Manipulation of Growth & Meat Quality by Vitamin D and its Analogues

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Abstract

Recent published work indicates a role for pre-slaughter dietary vitamin D supplementation to promote post mortem meat tenderization in cattle (Montgomery *et al.*, 2000; Foote *et al.*, 2004; Montogmery *et al.*, 2004), pigs (Wilborn *et al.*, 2004) and sheep (Wiegand *et al.*, 2001; Boleman *et al.*, 2004). The hypothesis being that vitamin D supplementation at supra-nutritional levels is able to cause increases in the calcium status of the animals, increasing the activity of the calcium-dependant proteolytic enzymes, the calpains, which are responsible for meat tenderization (Koohmaraie & Geesink, 2006). Muscle fibre type is a variable factor in muscle and is related to meat quality (Klont *et al.*, 1998). Vitamin D has been suggested to play a role in regulating skeletal muscle function through the creation of vitamin D receptor knockout mice models (Endo *et al.*, 2003) and observations that muscle weakness and falling risk in vitamin D deficient patients is linked to a loss of fast muscle fibres (Aniansson *et al.*, 1986; Larsson *et al.*, 1979; Sorenson *et al.*, 1979; Sato *et al.*, 2005).

This thesis investigated two vitamin D pre-slaughter diet regimes on their effects on meat quality of the most economically important cut of the carcass, the *longissimus dorsi* (LD) muscle (Molina *et al.*, 2005) and the expression of the calpain system. Trial 1 fed sheep vitamin D at 2.0 X 10^6 IU/day for four days prior to slaughter and found that this had no effect on shear force of LD chops. Calcium levels were unchanged but mRNA levels of calpain I and II were increased 3.7 and 10% respectively ($P=0.099$ and $P=0.014$) but there was no effect on calpastatin mRNA nor changes in the calpain system at the protein level. Trial 2 fed sheep the same dose of vitamin D for 7 days with an additional calcium bolus, resulting in a 10% increase in calcium concentrations of both serum and LD. Toughness of LD chops was increased ($P<0.01$), there was no effect on mRNA of the calpain system but there was an increase in the protein levels of calpain II and calpastatin by 16 and 17% respectively ($P=0.05$ and $P=0.087$).

A microarray study of rat primary myoblasts treated with $1,25(OH)_2D_3$ for 24 hours highlighted a number of responsive genes significantly up and down regulated 1.5 fold or more ($P<0.05$). Pathway analysis identified novel targets of $1,25(OH)_2D_3$ with a possible relationship to muscle growth and function; these included C/EBPβ, metallothionein 2A and the MAPK, ERK. Three muscle cell strains, the rat primary muscle cells, L6 Aston and C2C12, were assessed for myosin heavy chain (MHC) gene expression using semi-quantitative PCR and western blotting analysis. The muscle cell line demonstrating the broadest range of MHC genes relevant to mature muscle tissue was used for the

final experiments; this was the C2C12 cell line demonstrating expression of the slow MHC I/β, an isoform which was absent or showed much lower expression in the other cells.

C2C12 cells treated with $1\alpha(OH)D_3$ for 48 hours at varying stages of development responded in changes in myogenic regulatory factors (MRFs), MHCs and novel target gene expression. Real time PCR analysis of C2C12 cells treated with the active vitamin D metabolite $1\alpha(OH)D_3$ affirmed C/EBPβ mRNA expression to be upregulated ($P<0.001$) and MAPK ERK 1/2 phosphorylation to be down regulated ($P<0.001$) by $1\alpha(OH)D_3$ in muscle cells. The effect of $1\alpha(OH)D_3$ in myoblasts was reduce proliferation and promote differentiation, as myotubes formed the effect of $1\alpha(OH)D_3$ was to promote MHC gene expression of an intermediate oxidative fibre type, increasing expression of MHC 1/β and 2A, decreasing MHC 2B.

In conclusion, there is no apparent benefit of a pre-slaughter dietary vitamin D feeding regime on meat quality, but the active metabolites of vitamin D, $1\alpha(OH)D_3$ and $1,25(OH)_2D_3$, exert changes in gene expression and MAPK signalling which are likely to affect muscle growth and fibre type, and is of relevance in terms of both meat quality and muscle function in the elderly.

List of Abbreviations

(-/-)	homozygous gene knockout
°C	degrees Celsius
7DHC	7-dehydrocholesterol
$1\alpha(OH)D_3$	1α-hydroxyvitamin D_3, alphacalcidiol
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
$25(OH)D_3$	25-hydroxyvitamin D_3
AAS	atomic absorption spectroscopy
ADG	average daily gain
ADP	adenosine diphosphate
AEBSF	2-(4-aminoethyl)-benzenesulphonyl Flouride
ATP	adenosine triphosphate
BAA	β-adrenergic agonist
BF	*biceps femoris*
bHLH	basic helix-loop-helix
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
cDNA	copy-deoxyribonucleic acid
CO_2	carbon dioxide
$CuSO_4$	copper sulphate
CREB	cAMP- response element binding protein
DAG	diacylglycerol
DBP	vitamin D binding protein
DFD	dark firm dry
DHS	donor horse serum
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic acid triphosphates
dp	decimal places
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELC	essential light chain
FCR	feed conversion ratio
FCS	fetal calf serum
FG	fast glycolytic
FRET	flourescence resonance energy transfer
FOG	fast oxidative glycolytic
HBSS	Hank's balanced salt solution
HPLC	high performance liquid chromatography
HRP	horse radish peroxidise
IGg	Immunoglobulin G
IMS	industrial methylated spirit
IP_3	inositol triphosphate
IU	international units
kDa	kilo Daltons

KNa Tartrate	potassium sodium tartrate
HGF	hepatocyte growth factor
PC	phosphocreatine
PS	*psoas*
LD	*longissimus dorsi*
nm	nano metre
mA	milli Amps
mM	millimolar
M	molar
mRNA	messenger ribonucleic acid
MAPK	mitogen activated protein kinase
MADs	MCM1 agamous deficiens serum response factor
MEF	myocyte enhancer factor
MHC	myosin heavy chain
MMP	matrix metallopeptidases
MRF	myogenic regulatory factor
MW	molecular weight
NaOH	sodium hydroxide
Na_2CO_3	sodium carbonate
NFAT	nuclear factor- activated T cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGC-1α	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
pHu	ultimate pH
PIP_2	phosphatidyl inositol 4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PPAR	peroxisome prolferator-activated receptor
PTH	parathyroid hormone
RNA	ribonucleic acid
RAR	retinoic acid receptor
RLH	regulatory light chain
RXR	retinoid X receptor
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium doecyl sulphate polyacrylamide gel electrophoresis
Shh	sonic hedgehog
SO	slow oxidative
SR	sarcoplasmic reticulum
ST	*semitendinosus*
TAE	tris base, acetic acid, ethylenediaminetetraacetic acid buffer
TBS	tris buffered saline
TBS-T	tris buffered saline- Tween 20®
TCF	T-cell Factor
TR	thyroid hormone receptor
UVB	ultraviolet B
V	volts
VDR	vitamin D receptor

VDRE	vitamin D response element
VDRmem	membrane vitamin D receptor
VDRnuc	nuclear vitamin D receptor
WBSF	Warner-Bratzler Shear Force
Wnt	Wingless & Int related protein

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Introduction

The overall aim of the work reported in this thesis was to examine the potential for vitamin D to influence muscle growth and function from an animal production perspective, investigating the influence of dietary vitamin D on the quality of meat as a product and the biologically active forms' effects on gene expression relating to the muscle's capacity to grow. Meat quality is a general term used to describe a wide variety of factors which contribute to and define the value of meat as a product. These factors can include the composition and conformation of the carcass, the health issues associated with meat products e.g. *Escherischa Coli* 0157 contamination and bovine spongiform encaphalopathy (BSE), production methods related to the animal's welfare and environmental impact as well as the actual eating experience of the end meat product (Maltin *et al.*, 2003). All of these are factors which affect consumer's decisions when choosing meat products but the most influential factor affecting their decision to re-purchase a chosen product is the tenderness of the meat, with 94.6% of consumers choosing more expensive meat as long as it had lower shear force scores (more tender meat) (Boleman *et al.*, 1997). Therefore a major focus of the meat industry is to gain an understanding of the mechanism of tenderization and thereby improve production methods to consistently produce tender meat (Bindon & Jones, 2001).

Tenderisation is a result of proteolytic activity in the muscle post mortem which acts to degrade the protein ultrastructure within the muscles and so reduces the amount of force required to cut the meat. It is widely acknowledged that the majority of the tenderisation process is performed by the calpain system (Koohmaraie & Geesink, 2006) which is regulated by intracellular levels of calcium (Goll *et al.*, 2003). In order to manipulate the activity of the calpain system during post mortem storage, a number of studies demonstrated that infusing carcasses (Koohmaraie *et al.*, 1988) or marinating steaks (Whipple & Koohmaraie, 1993) in calcium chloride solutions is proficient in reducing shear force of meat rapidly during the first 24 hours of storage. It has been shown that this rapid increase in tenderness with calcium chloride infusion is a result of increased calpain activity (Koohmaraie *et al.*, 1989). Although this treatment of meat post mortem was demonstrated to vastly improve the speed and extent of post mortem tenderisation, it required additional procedures to be performed in the abattoir and the use of additives in meat products could lead to products which are rejected by consumers (Hoover *et al.*, 1995). In light of these observations, the research work described in this thesis set out to examine whether supplementing animal feed with vitamin D, a compound which is metabolised into a hormone responsible for activating calcium absorption from

the diet, could produce elevated calcium levels in the muscle and thereby activate the calpains during post mortem aging.

A second variable trait which is reported to influence meat quality is the relative quantities of different fibre-types within the muscle (Schiaffino & Reggianni, 1996). These intrinsically differ in size, proteolytic and metabolic activity all of which influence tenderisation post mortem (Klont *et al.*, 1998). In relationship to manipulation of fibre type, multiple studies in human patients have reported a relationship between vitamin D deficiency in elderly patients and the number of falls as a result of impaired muscle function, findings which have led several researchers to the suggestion that vitamin D could affect transitions in muscle fibre type (Bischoff *et al.*, 2003; Bischoff-Ferrari *et al.*, 2006, Snijder *et al.*, 2006). Reduced vitamin D status is associated with a reduction in fast fibres and this leads to a higher incidence of falling in older patients (Aniansson *et al.*, 1986; Larsson *et al.*, 1979; Sorenson *et al.*, 1979; Sato *et al.*, 2005). In addition to its responsibility for calcium homeostasis, acting through the classical nuclear receptor (Norman *et al.*, 2002), the vitamin D receptor has been found to modulate intracellular signalling pathways which are not predominantly associated with calcium homeostasis in muscle cells (Buitrago *et al.*, 2006; Buitrago *et al.*, 2001). Some of these signalling pathways are associated with effects on proliferation and differentiation of cells, with biologically active vitamin D reducing proliferation (De Haes *et al.*, 2004) and enhancing terminal differentiation in non-muscle cell types (Berry & Meckling-Gill, 1999). A fundamental aspect of animal production is the enhancement of lean tissue mass which is predominantly associated with an increase in fast type muscles. Therefore these general observations suggest that vitamin D may have a role as an agent which can modulate both growth and the phenotype of muscle tissue in ways which could have relevance to the meat production industry.

To this end, the latter experiments of this study were designed to investigate the effects of vitamin D signalling in muscle cells, particularly changes in fibre type and growth of muscle cells. Overall, these findings will improve the understanding of vitamin D as an agent to enhance meat quality both in terms of activating proteolysis during post mortem storage, presumably via the calpain system, and in changes in fibre type. In addition the studies will help to improve the general knowledge of vitamin D's role in muscle growth both in immature muscle through to manipulating muscle function in aged muscle tissue.

Literature Review

1.1 Introduction to Literature Review

Vitamin D has been commonly known to prevent rickets and osteomalacia for almost a century, and its role in calcium homeostasis to prevent these bone disorders is considered to be the compound's primary function (Holick, 1999). Recently, novel roles have been considered for vitamin D in tissues unrelated to calcium homeostasis, and this has led to research to further understand its mechanisms in "non-classical" tissues. The studies reported in this thesis investigate the effects of vitamin D in muscle tissue in both the classical sense of calcium homeostasis and in the more recent role of vitamin D's ability to signal using pathways related to growth and differentiation. Therefore this literature review aims to provide a background of vitamin D's actions in both calcium and non-calcium mechanisms, before the process of muscle development and growth is described and finally the aspects of muscle tissue which affect the quality of meat as a product are considered.

1.2 Vitamin D

Vitamin D is the common term used to describe a group of cholesterol derived compounds which act as pro-hormones in the calcium homeostasis mechanism. There are two major forms of vitamin D, vitamin D_2 and vitamin D_3, which are derived from the dietary plant and animals sources respectively and are known as the calciferols or simply as vitamin D (Norman & Collins, 2001). Vitamin D_2, or ergocalciferol, is produced in plants and yeasts from the plant sterol ergosterol and similarly vitamin D_3, or cholecalfierol, is produced in animals from the vertebrate sterol cholesterol. It is thought that both forms can have biological actions in vertebrate animals; however the efficiency of conversion of vitamin D_2 in the liver is thought to be much less than that of vitamin D_3 (Pfeifer *et al.*; 2002). Both vitamin D_2 and D_3 are seco-steroids, where the second carbon ring in the sterol structure is broken open, a process which requires energy from UV light and in most vertebrate species takes place in the skin. The actual vitamin D compounds are considered pro-hormones as the molecules need to be hydroxylated before they become biologically active, in animals these hydroxylation steps are catalyzed by enzymes, firstly in the liver producing 25-hydroxyvitamin D, or calcidiol, before the kidney produces the active vitamin D molecule 1,25 dihydroxyvitamin D ($1,25(OH)_2D$), or calcitriol; the structures of the products of these reactions are shown in figure 1.2-1. The biological function of vitamin D is traditionally considered to be associated with calcium homeostasis and the formation of healthy bones, however in the last decade research has looked towards understanding this

hormone's effects in recently discovered targets in non-calceamic tissues including the thyroid cells of the immune system, brain and nervous system, liver, lung and many others (Pfeifer *et al.*; 2002). It is known that vitamin D exerts its effects by binding to its receptor (VDR) and in doing so activates the classical calcium mobilisation responses, but more recently the discovery of the VDR in many other tissues has led to the realisation that this hormone has a much larger role in tissue development and maintenance. The VDR can be located in the membrane where it exerts immediate biological effects within minutes of the active vitamin D binding, the major result being increased calcium absorption in cells lining the intestine. However, the VDR-vitamin D complex can also translocate to the nucleus where transcription of specific genes is regulated, such as the calcium-binding protein, calbindin D-28K (Norman & Collins, 2001).

1.2.1 Sources, synthesis and deficiency of vitamin D

Vitamin D is a fat soluble substance required for normal calcium homeostasis and has few natural dietary sources, mainly oily fish and their liver oil and less so the livers of meat animals such as cattle, pigs and chickens (Holick, 1999). The largest source of vitamin D for most organisms is in fact the exposure to sunlight, where vitamin D can be photosynthesised in the skin using the energy from the UVB sunrays to convert the precursor, pro-vitamin D or 7-dehydrocholesterol (7DHC) to the subsequent pre-vitamin D and vitamin D compounds. However the ability of animals to produce vitamin D this way is variable depending on geographical location, seasonal variation in sunlight, skin pigmentation, age, as well fleece shearing and housing practices in animals (Hidiroglou *et al.*, 1979; Hidiroglou & Karpinski, 1989). In terms of farming practices where animals are increasingly being raised indoors it is a possibility that routine supplementation of vitamin D may be required to prevent deficiency (Montgomery *et al.*, 2004).

The natural vertebrate form of vitamin D_3 is derived from cholesterol (Voet *et al.*, 2008). The major source of vitamin D to vertebrate species is the photosynthesis of vitamin D during sun exposure where vitamin D is metabolised from its steroid precursor 7DHC to pre-vitamin D by action of UVB light (wavelength in the region of 295-315nm) which breaks open the second carbon ring of the steroid structure (figure 1.2-1). Pre-vitamin D is then converted to vitamin D by a process of isomerisation which does not require any enzymatic control. The endogenous vitamin D as well as that absorbed from the diet is transported in the circulation by binding to its specific vitamin D binding protein (DBP) (Walters, 1992). At the liver the enzyme 25-hydroxylase adds a hydroxyl group to the carbon at the C25 position to form 25-hydroxyvitamin D (25(OH)D), before this product is again transported in the blood to the kidney tubular cells where the enzyme 1α-hydroxylase adds

another hydroxyl group to the carbon at C1 position, generating 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the biologically active form of vitamin D.

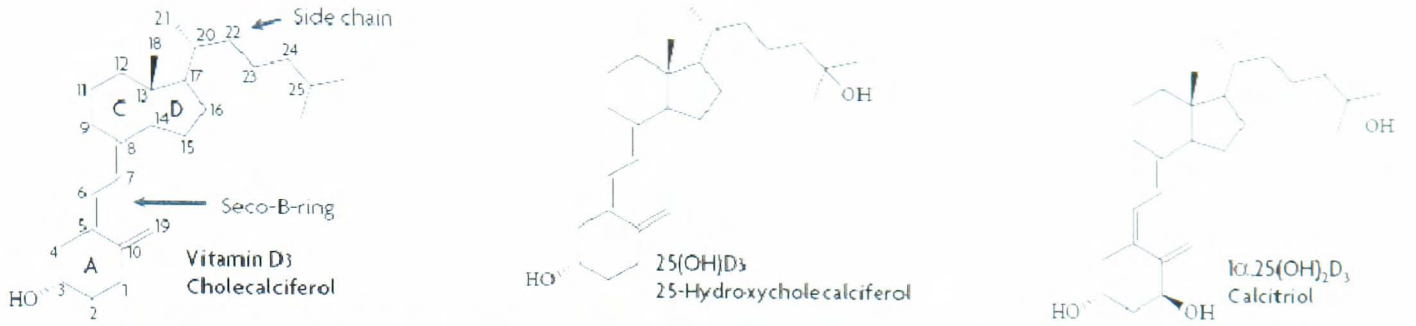


Figure 1.2-1. Structure of vitamin D and its metabolites, beginning with vitamin D, cholecalciferol, which is synthesised in the skin before the addition of a hydroxyl group by the liver converts it to 25-hydroxycalciferol or calcidiol, and finally the kidney produces the active form 1α,25-dihydroxycholecalciferol or calcitriol. From Deeb *et al.*, 2007.

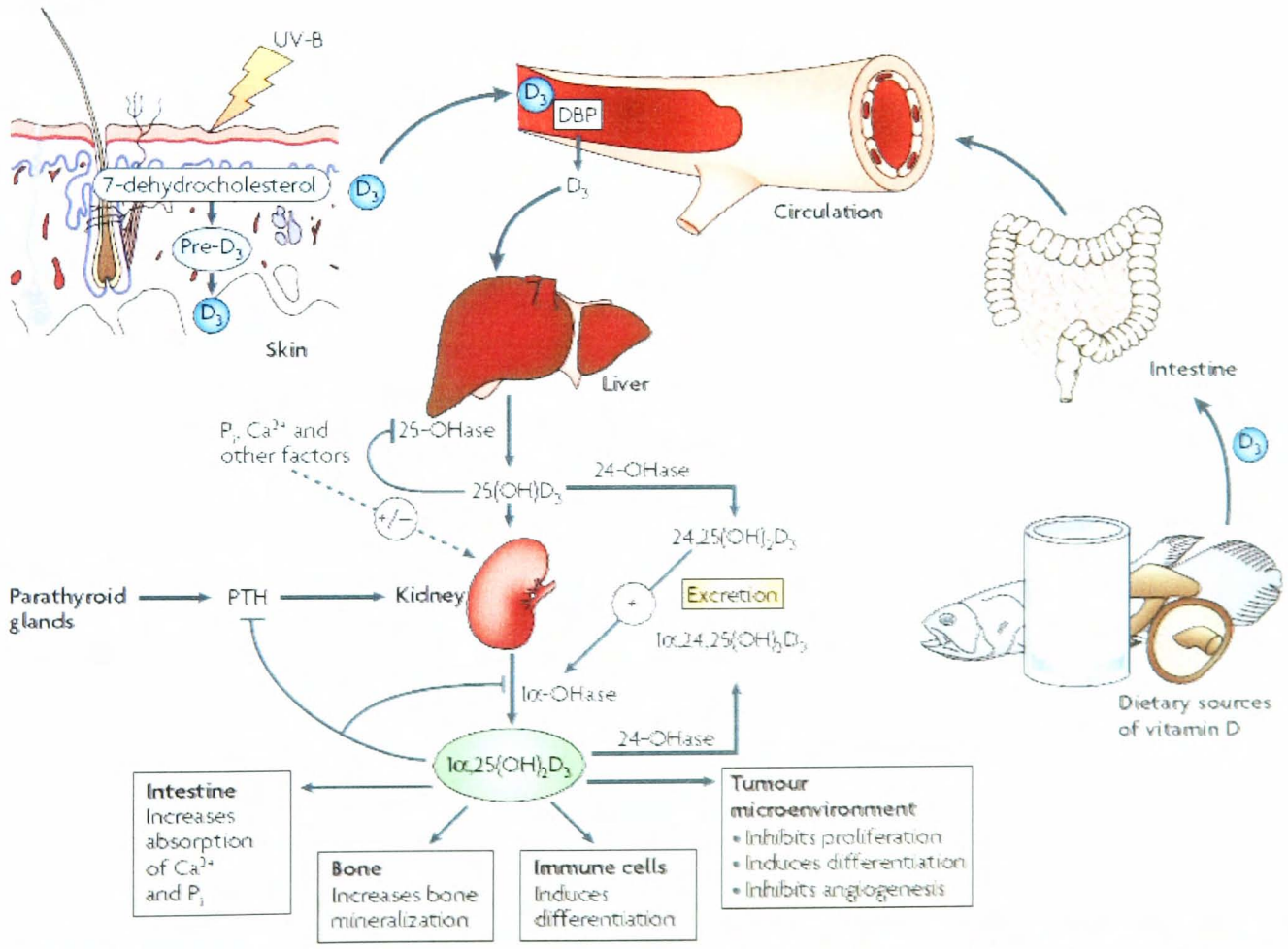


Figure 1.2-2. Summary of overall metabolism, activation and regulation of vitamin D within the body. Image from Deeb *et al.*, 2007.

The circulating forms of vitamin D, 25(OH)D and $1,25(OH)_2D$, are not equal in concentration as circulating levels of the active form $1,25(OH)_2D$ are under tight control at the activation step in the kidney (Brown *et al.*, 1999). In light of this, the circulating levels of the 25(OH)D are representative of bodily stores of vitamin D and this metabolite is routinely used to evaluate vitamin D status of animals (Hidiroglou *et al.*, 1979). Synthesis of the active form is controlled at the kidney hydroxylation step by parathyroid hormone in response to fluctuating calcium and phosphorus levels as well as a negative feedback of $1,25(OH)_2D$ on the 1α-hydroxylase. The active metabolite, $1,25(OH)_2D$, is produced in response to these stimuli and is transported bound to DBP in the blood to its primary target tissues; the small intestine and bone as well as the parathyroid gland (Deeb *et al.*, 2007), depicted in figure 1.2-2.

1.2.2 Action and Regulation of vitamin D production

The active form of vitamin D is produced in response to a drop in the circulating levels of calcium and phosphorus ions in the blood. A drop in calcium levels is detected by the parathyroid gland and this stimulates the glands secretion of parathyroid hormone (PTH) into the bloodstream. PTH then increases the synthesis of $1,25(OH)_2D$ in the kidney by up-regulating the activity of 1α-hydroxylase. This causes a rise in the circulating levels of $1,25(OH)_2D$ which then works to normalise the calcium and phosphorus levels in the blood by three mechanisms. Firstly the hormone prevents further loss of ions by increasing calcium retention from the urine produced in the kidney (Yamamoto *et al.*, 1984) and by reducing the amount of phosphorus lost to the urine indirectly by decreasing PTH secretion at the parathyroid gland (Bonjour *et al.*, 1977). Secondly the hormone increases dietary absorption by activating calcium uptake in the epithelial cells of the small intestine. Lastly, when dietary supply is insufficient $1,25(OH)_2D$ can release calcium and phosphorus from their stores in bone, stimulating bone resorptive cells, known as osteoclasts, to release calcium and phosphorus back into the blood stream. This response is in turn auto-regulated by action of $1,25\text{-}(OH)_2D$ which downregulates the production of PTH at the parathyroid gland as well as inhibiting 1α-hydroxylase in the kidney. Further, the circulating levels of $1,25\text{-}(OH)_2D$ are diminished by action of 24-hydroxylase , an enzyme which is most abundant in the kidney but is also present in the target tissues. This enzyme catalyzes the catabolism of $1,25\text{-}(OH)_2D$, converting it to the inactive form $1,24,24(OH)_3D$ (Holick, 1999). The overall metabolism, activation and regulation of vitamin D within the body is summarised in figure 1.2-2. This is the first step in the catabolism of vitamin D and eventually results in the formation of calcitroic acid (1α, (OH)-24,25,26,27-tetranor-23-COOH-D_3) which is excreted from the body in the bile (Holick, 1999).

1.2.3 Vitamin D deficiency

The symptoms of vitamin D deficiency indicate the hormone's vital role in calcium and phosphorus homeostasis and its direct effect on bone formation (Brown *et al.*, 1999). The appearance of the symptoms of vitamin D deficiency is a result of a lifestyle lacking two factors; firstly when exposure to sunlight is reduced thereby reducing production in the skin and secondly when the diet is itself is lacking in vitamin D (Holick, 1999). The most notable signs of vitamin D deficiency are rickets in young animals where the long bones do not form properly, causing stunted growth, in conjunction with the classic bone deformities such as knock-knees or bowed legs (Holick, 1999). In adults vitamin D deficiency presents with more subtle symptoms since the bones have already formed, and so vitamin D deficiency is characterised by osteomalacia (unmineralized bone matrix), usually causing pain and weakness in the bones and muscles, and associated osteoporosis (porous bones from calcium resorption) where bones are weak and susceptible to fracture (Holick, 1999).

1.2.4 Vitamin D and muscle function

Vitamin D deficiency has been frequently reported to present with muscle myopathy in addition to the well understood bone disorders of osteomalacia in adults and rickets in children (Schott & Willis, 1976). Eastwood *et al.*, (1977) reported that patients with long term kidney failure suffering with osteomalacia could be treated with supplementation of 25-hydroxyvitamin D and found, besides the effects on bone mineralization; patients also exhibited an increase in the muscle strength with vitamin D treatment. The relationship between vitamin D deficiency and loss of muscle function was further supported by studies describing an association between vitamin D deficiency and muscle pain, weakness and loss of type II fibres ("fast twitch" muscle fibres) in human patients (Russel, 1994; Ziambaras & Dagogo-Jack; 1999). Direct effects of vitamin D on muscle contractile function were reported by Curry *et al.* (1974) whose study demonstrated that the sarcoplasmic reticulum (SR) of muscle in vitamin D deficient rabbits had a slower uptake of calcium ions compared to the SR of muscle from rabbits fed a diet sufficient in vitamin D. This discovery was further investigated by Rodman & Baker (1978) who reported that muscles in vitamin D deficient rats had a slower relaxation time in comparison to normal animals, a response which is mediated by calcium uptake by the SR and in this case the vitamin D effect was found to be independent of calcium and phosphorus levels. Further to the evidence for vitamin D's effects on muscle contractile properties, Wassner *et al.* (1983) reported that vitamin D deficient rats showed reduced muscle growth rates with a higher rate of myofibrillar protein degradation. A further study into the effects of 25(OH)D on rat diaphragm muscle reported an increase in ATP levels and leucine incorporation into protein, again

demonstrating the possibility for vitamin D metabolites to regulate muscle metabolism and growth (Birge & Haddad, 1975). Although these studies provided the insight for the connection between vitamin D and muscle function, it was the discovery of the expression of the vitamin D receptor in muscle which affirmed the association between vitamin D and a direct effect on muscle tissue. Simpson *et al.,* (1985) first reported the presence of the $1,25(OH)_2D$ receptor (vitamin D receptor, VDR) in muscle cell lines and found that treatment of cell lines with the active form of vitamin D reduced DNA synthesis and inhibited proliferation of muscle cells; thus indicating skeletal muscle as a direct target organ of vitamin D and demonstrating that the hormone plays a role in regulating the terminal differentiation of muscle cells. Another piece of the puzzle into the hormone's role in muscle cell growth and differentiation was supplied by Endo *et al.*'s study into VDR (-/-) mice (2003) whereby the group observed reduced muscle fibre size in muscles of the hind limb and abnormally high expression of the muscle growth regulatory genes; Myf5, myogenin and E2A, as well as abnormal expression of the embryonic forms of the contractile protein myosin heavy chain (MHC). The group performed an additional experiment treating the C2C12 muscle cell-line with vitamin D and found treatment with the active hormone metabolite incurred downregulatory changes of these genes, suggesting a novel role for the hormone in controlling normal muscle cell growth and development.

1.2.5 Vitamin D signalling

Activated $1,25(OH)_2D$ exerts its actions on target tissues by binding to its specific receptor, the VDR, a member of the steroid hormone nuclear receptor family, whose general structure contains domains for ligand binding, DNA binding and activation of transcription (Cooper & Haussman, 2007). The understanding of the mechanism by which steroid receptors signal begins with the diffusion of the steroid hormone across the cell membrane where it binds to the receptor in the cytoplasm. Upon binding the ligand-receptor complex dimerizes with another nuclear receptor, the retinoid X receptor (RXR), and this $1,25(OH)_2D$-VDR-RXR complex translocates to the nucleus where it directly binds DNA and regulates expression of specific genes, depicted in figure 1.2-3. The RXR is required for a number of nuclear receptors to exert actions on gene transcription, including the thyroid hormone receptor (TR) and retinoic acid receptor (RAR), shown in figure 1.2-4. Vitamin D is able to affect transcription of its target genes by the action of the VDR-RXR complex binding to response elements (VDREs) in the promoters of its target sequences which subsequently causes activation or inhibition of the expression of these genes. Genes which are known to be upregulated by vitamin D and its receptor are calcium binding proteins such as calbindin D-9k and -28k, and the 24-hydroxylase enzyme which catalyzes breakdown of active vitamin D (Minghetti & Norman, 1988). In

contrast, genes which are down regulated by vitamin D-VDR-RXR binding are the 1α-hydroxylase enzyme in the kidney and the VDR itself. Response elements for the VDR are typical of steroid receptors which recognise their target genes by a code of short sequences of "half-sites" which are usually repeated within a short distance of each other (Lewin, 2008). The nucleotide sequences of the VDREs were elucidated by Carlberg *et al.* (1993) who reported that there were two mechanisms for vitamin D to activate gene transcription; firstly they described response elements which were responsive to VDRnuc alone, possibly as a homodimer, and finally those which were activated by the VDR-RXR heterodimer. The sequences, or half sites, recognised by VDRnuc alone were GGGTGA arranged in three specific patterns, either as a direct repeat with a space of 6 nucleotides, as a palindrome with no space or as an inverted palindrome with 12 nucleotides between. The half-sites required for activated gene transcription by the VDR-RXR heterodimer were direct repeats of pairs of 6-nucleotide motifs; GGTCCA, AGGTCA and GGGTGA with a space of 3 nucleotides between the repeated motifs e.g. 5'-**GGTCCA**NNN**GGTCCA-3'**, where, for activation of gene transcription, the RXR binds the 5' most motif and the VDR binds the 3' motif (Kurokawa *et al.*, 1993), shown in figure 1.2-4.

1.2.5.1 Rapid-responses of VDR

More recently, the discovery of the VDR's presence in non-calcaemic tissues has opened the door to the elucidation of vitamin D's roles beyond calcium homeostasis, including processes such as cell proliferation, differentiation, regulating the immune response and apoptosis (Walters, 2002). The realisation that vitamin D was able to stimulate intracellular signalling pathways usually associated with membrane receptors was a result of multiple studies reporting vitamin D's ability to increase levels of second messenger molecules. Morelli *et al.*, (1993) were first to report that treatment of muscle cells with $1,25(OH)_2D$ resulted in a rapid increase (seconds after vitamin D administration) in inositol 1,4,5-trisphosphate (IP_3) and diacylgycerol (DAG); two second messenger molecules produced from the hydrolysis of phosphatidylinositol 4,5- bisphosphate (PIP_2), a reaction catalyzed by activation of the enzyme phospholipase C (PLC). The authors of that study reported the major responses of the DAG and PIP_2 signal in muscle cells to be the release of calcium from intracellular stores, the sarcoplasmic reticulum (SR) by ligand gated Ca^{2+} channels, and the activation of the enzyme protein kinase C (PKC).

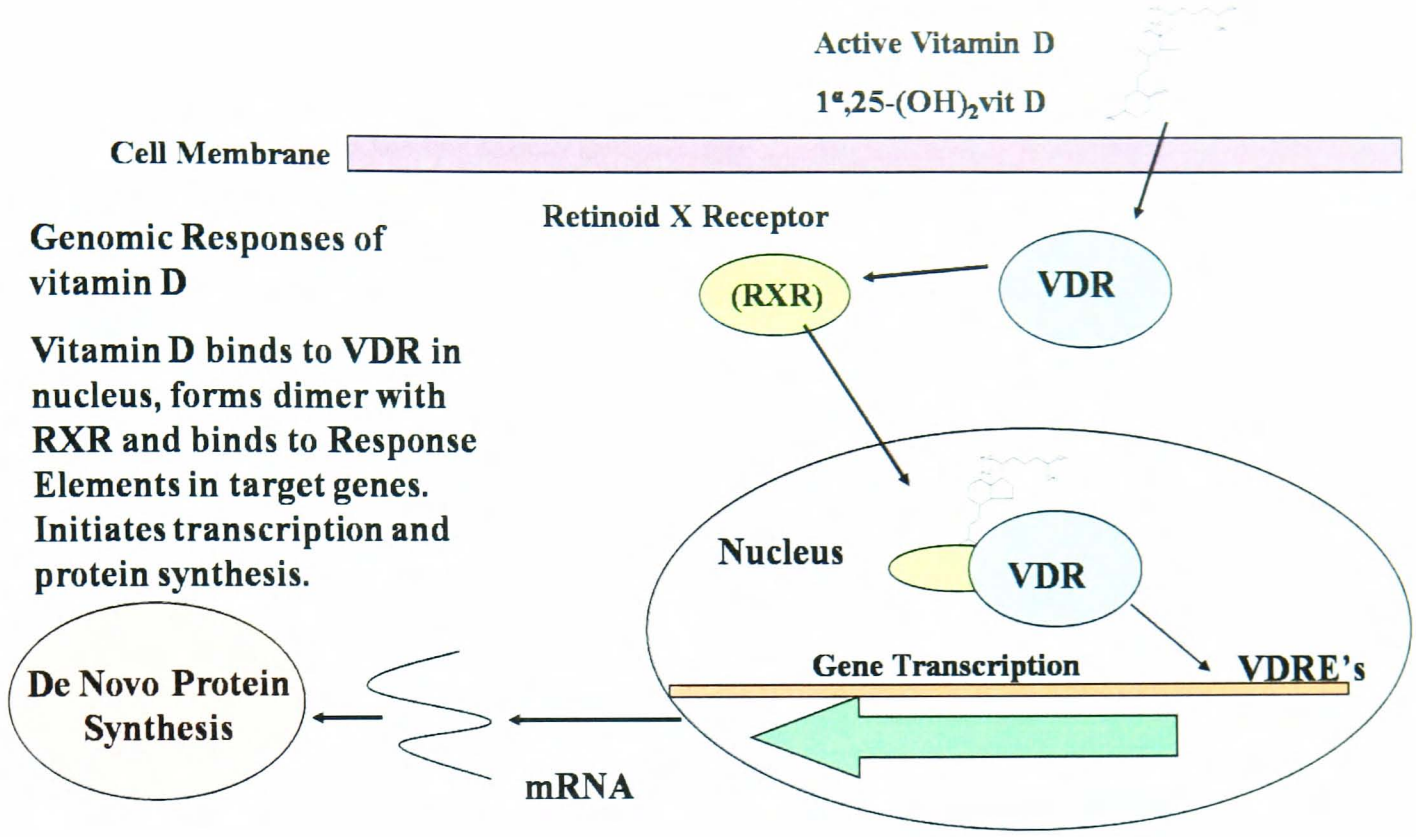


Figure 1.2-3. Mechanism for vitamin D signalling via the nuclear vitamin D receptor (VDRnuc) to facilitate changes in gene expression via binding of the VDRnuc to the vitamin D response elements (VDREs) in promoter regions of genes.

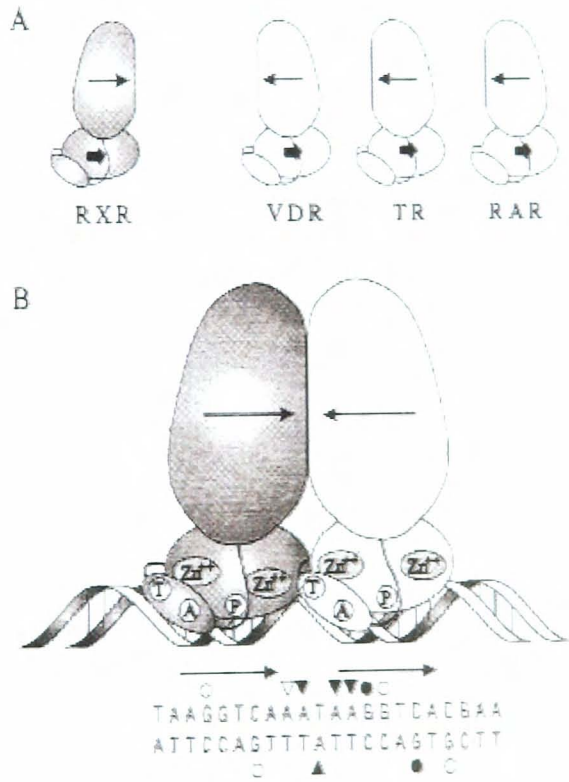


Figure 1.2-4. RXR forms heterodimers with a number of steroid hormone receptors including the vitamin D receptor (VDR), thyroid hormone receptor (TR) and retinoic acid receptor (RAR). Following the formation of the heterodimers, the complex binds to repeats of 6 nucleotides in the promoter region of genes, spaced 3 nucleotides apart, oriented so that VDR is downstream of RXR. From Kurokawa *et al.* 1993.

Despite these secondary signalling molecules having well known roles in the signalling of other membrane receptors, it was unknown at the time of this study whether the VDR itself was present at the membrane of cells. A further report of the rapid signalling effects of vitamin D in muscle was produced by Vazquez *et al.* (1995) who observed changes in intracelullar calcium levels mediated by the adenylyl cyclase and cyclic AMP (cAMP) mechanism of second messenger signal transduction, again usually activated by membrane receptors. Both these studies provided substantial evidence for the presence of a membrane receptor for vitamin D which was capable of inducing rapid responses to ligand binding, unrelated to the classical VDR signalling via gene transcription, but had not yet elucidated the receptor responsible. The final missing piece of the jigsaw came from Nemere *et al.* (1998) who performed immunohistochemical staining for the VDR in the membrane of chondrocytes (bone forming cells) and provided the evidence that was required to show the VDR could be present at the cell membrane and was responsible for the activation of PKC by vitamin D. Whether or not the VDR that is present at the cell membrane is the same VDR responsible for induction of gene transcription is still unclear. Brown *et al.* (1999) made a clear argument for two distinct VDR in their review of literature, however, Capiati *et al.* (2002) reported that the VDR was able to translocate from the cytosol to the plasma membrane within 10 minutes following binding of the ligand to the receptor.

Despite the lack of understanding over the exact form of receptor required for membrane signalling, it is now clear that vitamin D is able to mediate responses via a number of intracellular signalling pathways beginning with second messenger systems such as cAMP and PIP_2 hydrolysis, which lead to the activation of intracellular signal transduction pathways such as the mitogen-activated protein kinase (MAPK) cascade (Buitrago *et al.*, 2001) and the protein-tyrosine kinase, c-Src (Gniadecki, 1998; Buitrago *et al.*, 2001) in muscle, schematic diagram presented in figure 1.2-5. There are many enzymes which constitute the MAPK system and they respond to physical and chemical stimuli in order to coordinate cellular responses in survival and adaptation (Chang & Karin, 2001). Briefly there are three tiers to the MAPK pathway, the MAPkinase (MAPK) itself, which is activated by a MAPK kinase (MAPKK) which again is activated by a MAPKKkinase (MAPKKK), forming a multi-level cascade demonstrated in diagram 1.2-6. Taken together this body of research provides evidence for vitamin D's ability to activate intracellular signals which are now understood to be responsible for the control of cell growth, differentiation, proliferation and survival (Cooper & Hausman, 2007).

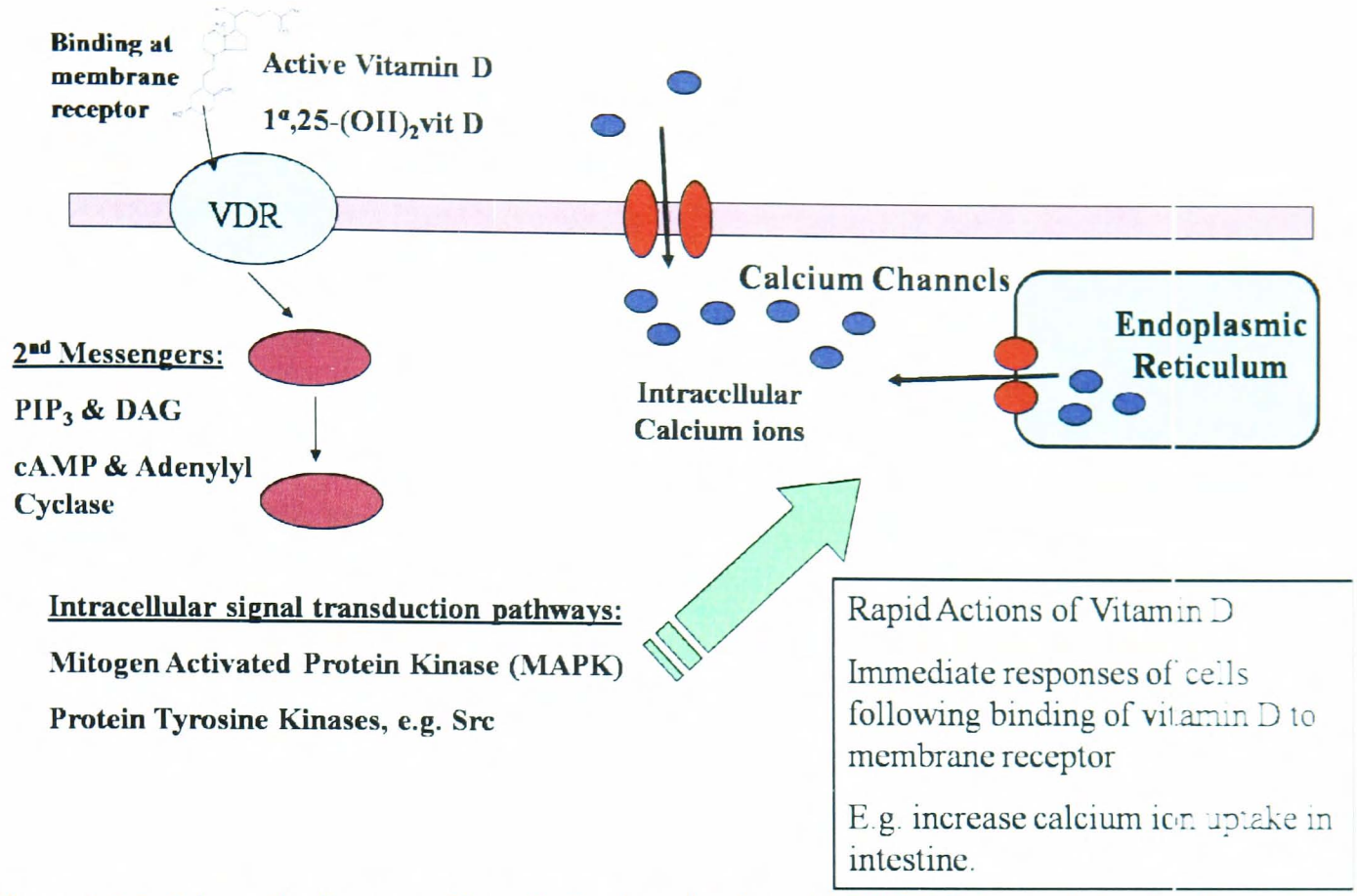


Figure 1.2-5. Schematic diagram of hypothetical mechanism of $1,25(OH)_2D$ intracellular signal transduction in rapid responses to vitamin D.

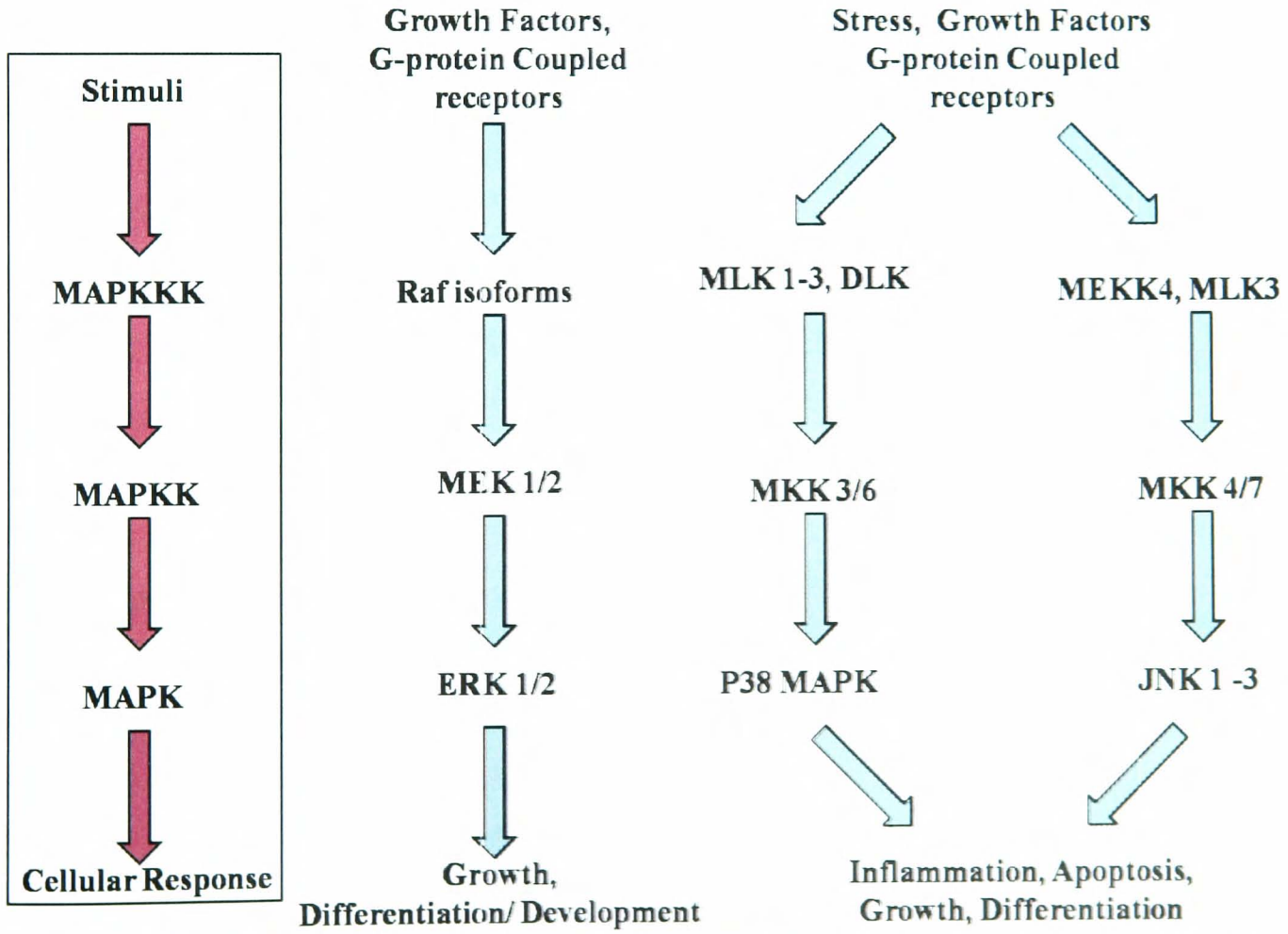


Figure 1.2-6. Schematic diagram of the MAPK signalling cascades from extracellular stimuli to cellular response (adapted from Voet *et al.*, 2008)

1.2.6 Vitamin D and Muscle: Summary

In light of the studies reported so far, there is a clear role of vitamin D to maintain muscle function and strength, as shown by reports of myopathy in vitamin D deficient individuals which can be rectified with re-alimentation of vitamin D (Schott & Willis, 1976; Eastwood *et al.,* 1977;. Russel, 1994; Ziambaras & Dagogo-Jack, 1999). In addition, the fact that muscle tissue has been shown to express the vitamin D specific receptor, VDR, suggests the effect of vitamin D on muscle to be hormone dependant and not only a secondary effect of calcium and phosphorus homeostasis (Simpson *et al.,* 1985). Finally, in view of the muscle deformities and abnormal expression of the myogenic regulatory factors (MRFs) in VDR (-/-) mice there is substantial justification for investigations into vitamin D's possible role in this tissue as a regulator of growth and development (Simpson *et al.,* 1985; Endo *et al.*, 2003).

1.3 Introduction to muscle metabolism, development and growth and its relevance to meat quality

Understanding the growth and development of muscle tissue is important in terms of meat production for 3 reasons; firstly, the factors which affect the growth of muscle during early muscle cell proliferation ultimately affect the yield of meat; secondly, factors controlling the fibre type in terms of metabolism and contractile speed (twitch) of the muscle will also affect meat quality and finally the mechanisms for muscle growth in adult muscle tissue affects both yield and meat quality (Maltin & Plastow, 2004). All these factors will be discussed within this literature review along with the definition of meat quality and how it can be influenced, before the hypothesis behind vitamin D's possible role in influencing meat quality and overall production are discussed.

1.3.1 Myogenesis

Skeletal muscle formation is a highly organised procedure that is controlled by a number of transcription factors working together to cause pluripotent stem cells of the embryo to specialise and differentiate into the highly organised skeletal muscle tissue described in section 1.3.2. This process, myogenesis has three main phases; the first is cell line determination, where cells are programmed to become muscle cells, myoblasts, followed by proliferation, which increases the population of these muscle destined cells (myoblasts), and lastly differentiation, where the cells begin to specialise into the phenotype characteristic of muscle cells (Buckingham M., 1992). This programme of cell specialisation is regulated by four transcription factors, known as the muscle regulatory factors (MRFs), whose presence at specific times of myogenesis control the expression of muscle specific genes (Edmondson & Olson, 1993).

1.3.1.1 Initiation and Regulation of Myogenesis

Muscle tissue is derived from the neural tube of the embryo where the somites form. The somites compartmentalize to create the scleratome and the dermamyotome, which eventually give rise to bone and cartilage and appendicular muscle respectively. The dermamyotome then divides into the myotome and the dermatome; the dermatome leading to the dermis and the myotome producing skeletal muscle, schematic diagram of these events is shown in figure 1.3-1 (Buckingham M., 1992, Cossu *et al.*, 1996). Initiation for the signal to differentiate into muscle cell lines comes from other regions of the embryo via signalling molecules Wnt and Sonic hedgehog (Shh). The dorsal neurotube and surface ectoderm release Wnts, which is a positive initiator of MRF expression, in addition to Shh released from the notochord and floor plate neural tube (Buckingham M., 2001). Wnts acts via its receptor, Frizzled1, and activates its signalling pathway through β-catenin and T-cell Factor TCF (a transcription factor) which, with Shh, induces expression of the early MRFs Myf5 and MyoD. Negative regulators of the muscle determination pathways also exist to assist in the control of embryo development. Notch, a transmembrane signalling receptor between cells (Cooper & Hausman, 2008), has been proposed as an antagonist for Wnt signalling (Buckingham M., 2001) and similarly Twist and the Id proteins have been shown to be negative regulators of the basic helix-loop-helix (bHLH) transcription factors involved in myogenesis (Yun & Wold, 1996). There are many other small signalling molecules released within the embryonic tissues which have been investigated for their role in muscle cell line determination and formation, including Pax3, c-met, Lbx1, Dach2, Eya2 and Six1. Knockouts of these signalling molecules with and without knockouts of MRFs have all shown abnormal muscle development ranging from no muscle in the embryos to specific and regional abnormalities (Birchmeier & Brohmann, 2000, Buckingham M., 2001). Some of these signalling proteins are required extremely early on in skeletal muscle development, where it has been shown that mice lacking Pax3, a transcription factor responsible for activating expression of c-met, do not have muscles in their limbs (Tajbakhsh *et al.*, 1997). Without Pax3 there is no transcription of c-met, which is a kinase enzyme required to regulate the migration of muscle pre-cursor cells from the somite to the limb buds of the developing embryo, hence animals lacking Pax3, c-met or HGF, a ligand of c-met, (hepatocyte growth factor, also known as scatter factor) do not develop muscle in the limbs (Buckingham *et al.*, 2003).

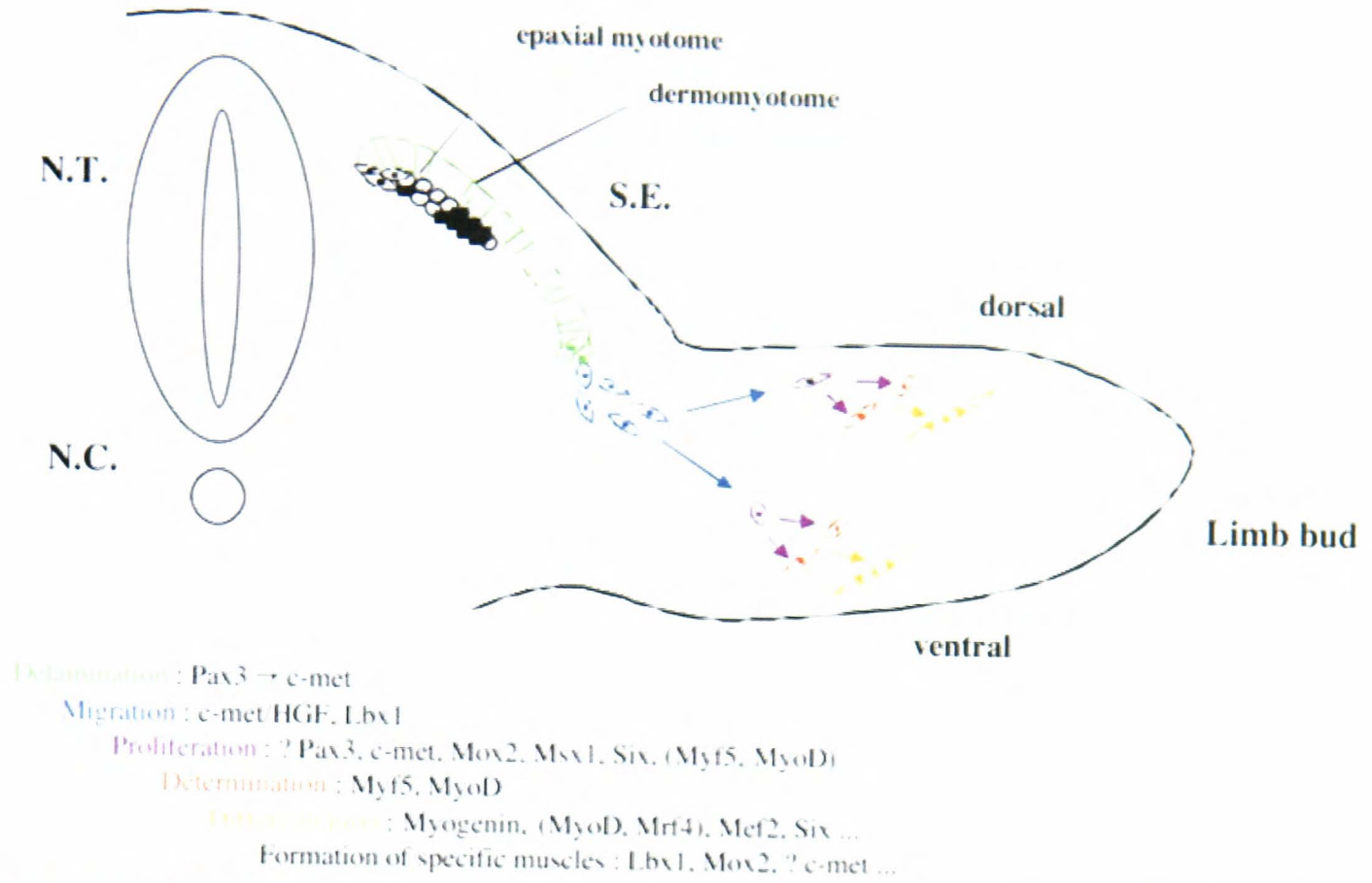


Figure 1.3-1. Schematic diagram of the early stages of myogenesis and the signalling factors responsible. Initial signals for the onset of the myogenic development programme are released as Wnts from the neural tube (NT) and Sonic Hedgehog (Shh) from the notochord (NC). Migration of muscle cell precursors from the somite to the limb bud is regulated by a number of signalling molecules in addition to the MRFs. In green font, delamination from the dermomyotome allows muscle cell precursors to migrate to the limb buds; this process is activated by the Pax3 transcription factor which induces c-met expression. The migration of the muscle cell pre-cursors to the limb buds, in blue font, requires the presence of both c-met and its ligand HGF (hepatocyte growth factor, or scatter). Once cells have migrated, they undergo proliferation to increase their population, in purple font, classically regulated by the MRFs Myf5 and MyoD, however it is known that other signalling proteins are required, including Mox2, Msx1 and Six as well as Pax3 and c-met. Pre-cursor cells are then determined into the muscle-cell line by Myf5 and Myod, in red, and begin to express specific muscle cell genes, before they differentiate into multinucleated myotubes under the influence of Myogenin, in orange font. Final specifications of specific muscles and fibre type attributes are regulated by further signalling molecules. From Buckingham *et al.* (2003).

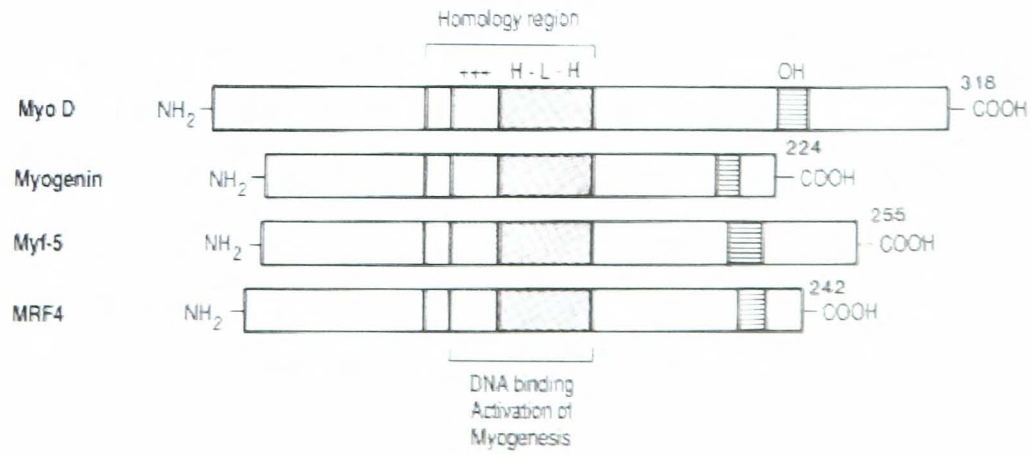


Figure 1.3-2. Schematic diagram of the structure of the four myogenic regulatory factors (MRFs), demonstrating their similar basic-helix-loop-helix (bHLH) DNA binding regions. OH denotes a serine-threonine rich region which is a site of phosphorylation. Protein sizes denoted above carboxyl terminal end (COOH) refer to mouse and human peptides. Image from Olson 1990.

1.3.1.2 *The Myoregulatory Factors*

The MRF family are members of the basic-helix-loop-helix (bHLH) family of transcription factors with similar structures shown in figure 1.3-2, all of which are responsible for the control of cell-type-specific gene expression in their varying tissues (Edmondson & Olson, 1993). The MRFS act to alter gene expression by forming homo- and hetero-dimers which bind to DNA at specific nucleotide sequences containing the motif **CA**NN**TG** (where N is any nucleotide), known as E-boxes (Ludolph & Konieczny, 1995), present in the control region of most muscle-specific genes as well as other cell-type specific genes in other tissues (Edmondson & Olson, 1993). All of the four MRFs are required throughout the process of myogenesis and experiments investigating the development of MRF knockout models in mice have elucidated each MRF's role in myogenesis. As mentioned above, the specification of the early cells in the mesoderm into the muscle cell line begins with early signals from other regions of the embryo before expression of the MRF family begins within the early muscle cells. The first MRFs to be expressed are Myf5 and myoD which act to promote proliferation and switch on the muscle-cell specific genes. Myogenin is expressed later in gestation and controls the process of muscle cell differentiation, whereby single nucleate cells leave the cell cycle and elongate before fusing and forming multinucleate myotubes. MRF4 is the last MRF to be expressed in the myogenic process and remains the most predominantly expressed following birth. A schematic representation of the MRF's involvement in the myogenic programme is shown in figure 1.3-3.

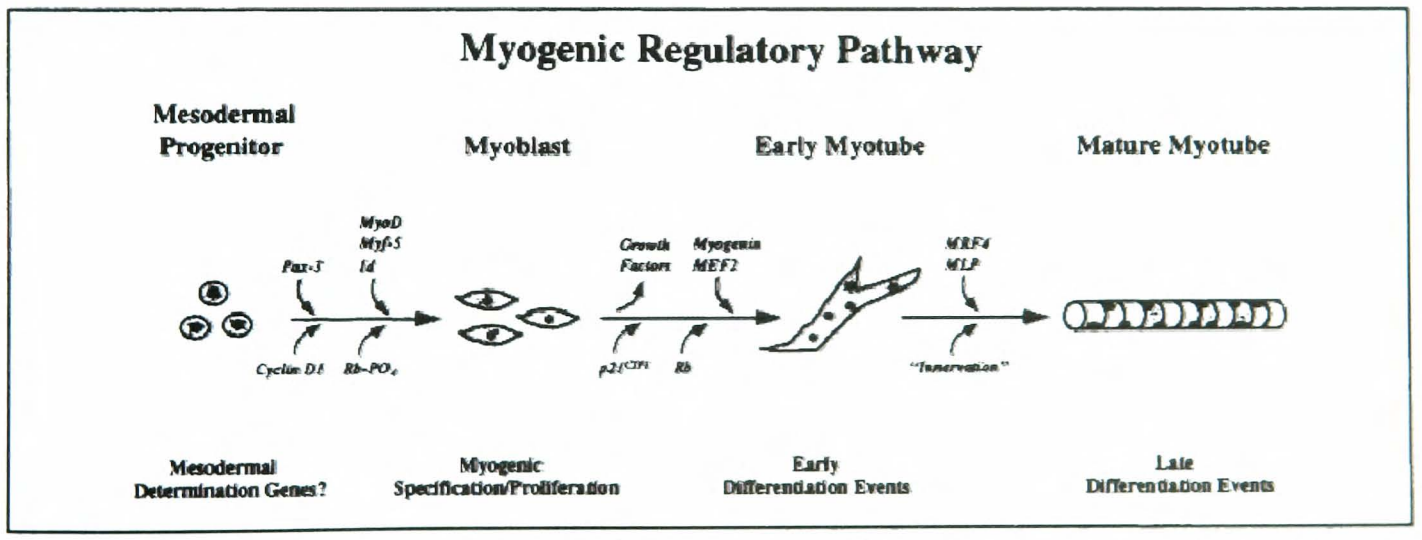


Figure 1.3-3. Schematic diagram of the control of muscle cell differentiation by MRFs (Ludolph & Koniecny, 1995). Specification into the muscle cell line is initiated by early signals from Pax -3 as well as MyoD and Myf5. Muscle cell precursors, or myoblasts, proliferate under the influence of MyoD and Myf5 until differentiation is induced by both extracellular and intracellular signals. Myogenin is the MRF responsible for regulating differentiation into fused and multinucleated myotubes until MRF4 becomes the most predominant MRF to be expressed in maturing muscle.

Table 1.3-1. Muscle phenotypes observed in mice lacking expression of MRFs.

MRF (-/-)	Normal Myogenesis?	Deformities?	other MRF expression	Reference
MyoD	Yes	No	Myf5 ↑ X 3	Rudnicki *et al* ., 1992
Myf5	Yes	defects in rib region	none reported	Braun *et al* ., 1992
MyoD + Myf5	No myoblasts present			Rudnicki et al., 1993
Myogenin	Myoblasts are present but no terminal differentiation, no functional muscle			Hasty *et al* ., 1993, Nabeshima *et al* ., 1993
MRF4	Yes	mild rib deformities	Myogenin ↑ X 3	Zhang *et al* ., 1995

MRF knockout studies have contributed immensely to the understanding of each MRF's role in regulating skeletal muscle formation, with all this data combined the roles of the MRF family can be identified, summarised in table 1.3-1. The first MRF to be investigated in this way was MyoD (Rudnicki *et al.*, 1992) where it was observed that mice embryos developed normally without abnormalities, however there was an increased expression of Myf5 by three times that in wildtype siblings. This was closely followed by a Myf5 knockout model where muscle tissue again appeared to progress normally although there were some deformities in the ribs of embryos (Braun *et al.*, 1992). These two studies demonstrated the ability for Myf5 and MyoD to compensate for the other's absence, but when both Myf5 and MyoD expression was removed there was no muscle cell formation at all, demonstrating the requirement for at least one to be present for fetal survival (Rudnicki *et al.*, 1993). This information leads to the conclusion that MyoD and Myf5 are required for determination of pluripotent cells to the muscle cell lines and without these MRFs there are no myoblasts present in the foetus. Following these studies the role of myogenin was investigated by the knockout model where mice embryos were observed to survive gestation before dying shortly after birth (Hasty *et al.*, 1993, Nabeshima *et al.*, 1993). These myogenin (-/-) mice did have normal myoblast populations but showed no terminal differentiation into muscle cells and as a result had no functional muscle tissue. This model highlighted the vital role of myogenin to induce terminal differentiation of myoblasts into functioning muscle cells. Finally a knockout mouse model for MRF4 was investigated and found there was no affect on the determination and differentiation of muscle and all animals appeared healthy with the exception of some mild rib deformities (Zhang *et al.*, 1995). These MRF4 (-/-) mice did have increased expression of myogenin in comparison to wildtype siblings which gave way to the notion that lack of MRF4 expression is compensated by myogenin and that MRF4's role was of importance of after muscle cell development.

Upon expression, the MRFs up-regulate expression of a number of muscle specific genes, for example all MRFs are capable of up-regulating the expression of desmin (Li & Capetanaki, 1994) and each MRF is capable of activating its own array of genes, for example MyoD upregulates creatine-kinase, tropnin I, α-actin and α-acetylinecholine receptor; whereas MRF4 only activates α-actin and α-acetylinecholine receptor (Ludoplph & Konieczny, 1995).

1.3.1.3 Other factors regulating myogenesis

As mentioned previously, the MRFs form both homo- and hetero-dimers prior to binding to the E – box of target genes; however, this process is further regulated by the presence of many other HLH proteins which are capable of binding with the MRFs and modifying their actions. Of these the MEFs and the E proteins are thought to be positive regulators of muscle differentiation whereas the Id protein is a negative regulator of muscle differentiation.

1.3.1.4 Myocyte enhancer factors (MEFs)

The myoctyte enhancer factor 2 family (MEF2) are a family of transcription factors with similar function to the MRFs and are expressed in a broad range of cell types including brain, cardiac and visceral tissue where differential gene splicing produces tissue specific isoforms (Ludolph & Koneiczny, 1995). The MEF family of transcription factors are members of a larger superfamily of MADS transcription factors (MCM1 agamous deficiens serum response factors) which all have the characteristic MADS box, a 56 amino acid motif responsible for binding to DNA and dimerization with other proteins. In addition, the MEF2 family contain another conserved 19 amino acid sequence, known as the MEF2 site responsible for binding AT-rich DNA sequences in the regulatory regions of many muscle specific genes (Ludolph & Koneiczny, 1995). There are four members of the MEF2 family, MEF2A, MEF2B, MEF2C and MEF2D, all of which function by forming homo- and hetero-dimers with the MRFS plus other transcription factors, and activate the expression of muscle specific genes such as desmin (Li & Capetanaki, 1994), creatine kinase, myosin heavy chain, and other MRFs (Ludolph & Konieczny, 1995).

1.3.1.5 E proteins

The MRF family of transcription factors are members of the class II of basic helix-loop-helix transcription factors and are able to form dimers with members of the ubiquitously expressed E proteins which are members of the class I bHLH factor family (Berkes & Tapscott, 2005). The E proteins function in a similar way to the MRFs and bind DNA sequences at the E-box due to their bHLH domain. The E2A gene is able to encode three different E proteins due to differential splicing of mRNA leading to the production of E12, E47 and E2-5, in addition, other similar related proteins E2-2 and HEB are encoded by distinct genes (Shirakata 1993). These proteins are able to form heterodimers with the MRFs and in doing so, change their affinity for DNA binding. For example, when MyoD forms a hetero-dimer with E12, its affinity for binding to the E-box in the creatine kinase gene enhancer sequence is much greater than would be for a homo-dimer of MyoD alone (Benezra

et al., 1990). The exact role of the E-proteins in myogenesis is still under debate, and knockout studies of specific E-proteins show no effect on the formation of skeletal muscle, demonstrating some redundancy in the family (Berkes & Tapscott, 2005).

1.3.1.6 Id protein

The Id family of proteins contains three members, Id1, Id2 and Id3, all of which contain the helix-loop-helix domain but lack the basic region required for DNA binding as seen in the bHLH transcription factors MRFs. However, the Id proteins are still able to form dimers and complexes with bHLH proteins but in doing so restrict their ability to bind DNA at the E-box, thus inhibiting muscle gene expression. In their study in the C2C12 mouse-derived muscle cell line, Jen *et al.* (1992) demonstrated that overexpression of Id mRNA was capable of inhibiting muscle cell differentiation.

1.3.1.7 Myogenesis Summary

The development of muscle cells from the embryo to fully formed specialised muscle cells is a highly regulated process and requires the presence of a multitude of signalling factors. The MRFs are muscle specific transcription factors which can induce expression of muscle genes in non muscle cells and myogenesis does not occur in their absence. Some MRFs are vital such as Myf5 and Myogenin and can act to oversee myogenesis in the absence of other MRFs in a way that MRF4 and MyoD cannot. In addition to the MRFs a large array of proteins are also required and play their part from the very beginning of muscle-cell migration to the limb buds through to the maturation of fully differentiated, mulitnucleated mature myotubes.

1.3.2 Skeletal Muscle Architecture

Skeletal muscle is responsible for allowing the voluntary movement of animals and is a highly adapted tissue to perform this function. Skeletal muscle is structured in a hierarchy of components, beginning with the overall muscle which is made up of bundles of fibres. Each fibre within the bundles, known as a myofibre, is a single muscle cell containing multiple nuclei as a result of many cells forming together to create one extremely large muscle cell, which is around 50μm in diameter and several centimetres long (Cooper & Hausman, 2007). Each individual cell is further made up of many smaller fibres, known as myofibrils, and these each have a distinct sub-structure of proteins organised in repeating units along their length. Skeletal muscle is also referred to as striated muscle owing to its appearance under the microscope; this striped appearance is a result of many repeating protein units known as the sarcomere, which are made of multiple proteins organised into a distinct

arrangement, demonstrated in figure 1.3-4. The proteins within the sarcomere are described in figure 1.3-5 along with the sub-structures of each region of the sarcomere.

1.3.2.1 Structure of the Sarcomere

The thick filaments are comprised of many molecules of myosin gathering together to form a long chain with the reactive large heads of each protein orientated to face outwards. Each molecule of myosin is formed from a complex of 6 smaller myosin proteins, consisting of two heavy chains (myosin heavy chain, MHC) and two pairs of light chains (regulatory light chain, RLC and essential light chain, ELC). These form the much larger myosin molecule which has a long chain which associates with other myosin molecules to bind the thick filament and a large globular head where the protein can bind to actin in contraction. Actin is a cytoskeletal protein, present in many tissues of the body and exists in three forms, α-, β- and γ. β- and γ-actin are expressed by most cell types as part of the cytoskeleton whereas α-actin is muscle specific (Au, 2004). Actin in the thin filament is a globular protein, noted as G-actin, which polymerises to form a long molecule known as filamentous actin or F-actin. The F-actin is formed around 2 molecules of the filamentous cytoskeletal protein nebulin, which acts as a "molecule ruler" to regulate the length of the thin filament, and around these is another long-chain molecule, tropomyosin, a coiled-coil protein which wraps around the thin filament and prevents the myosin heads from the thick filament binding (Au, 2004). In addition to tropomyosin, the troponin complex is also associated along the thin filament and it is this complex that removes the inhibition of tropomyosin in response to calcium. At rest the tropomyosin is positioned over the active sites of the actin proteins, but during muscle innervation calcium ions are released which bind to troponin and alter its tertiary structure and in doing so moves tropomysoin, revealing the active site for myosin binding and allowing contraction. The interaction between actin and myosin requires energy from ATP and the head of the myosin protein is an ATPase able to hydrolyse ATP to ADP + P, providing the energy for the interaction between the two filaments.

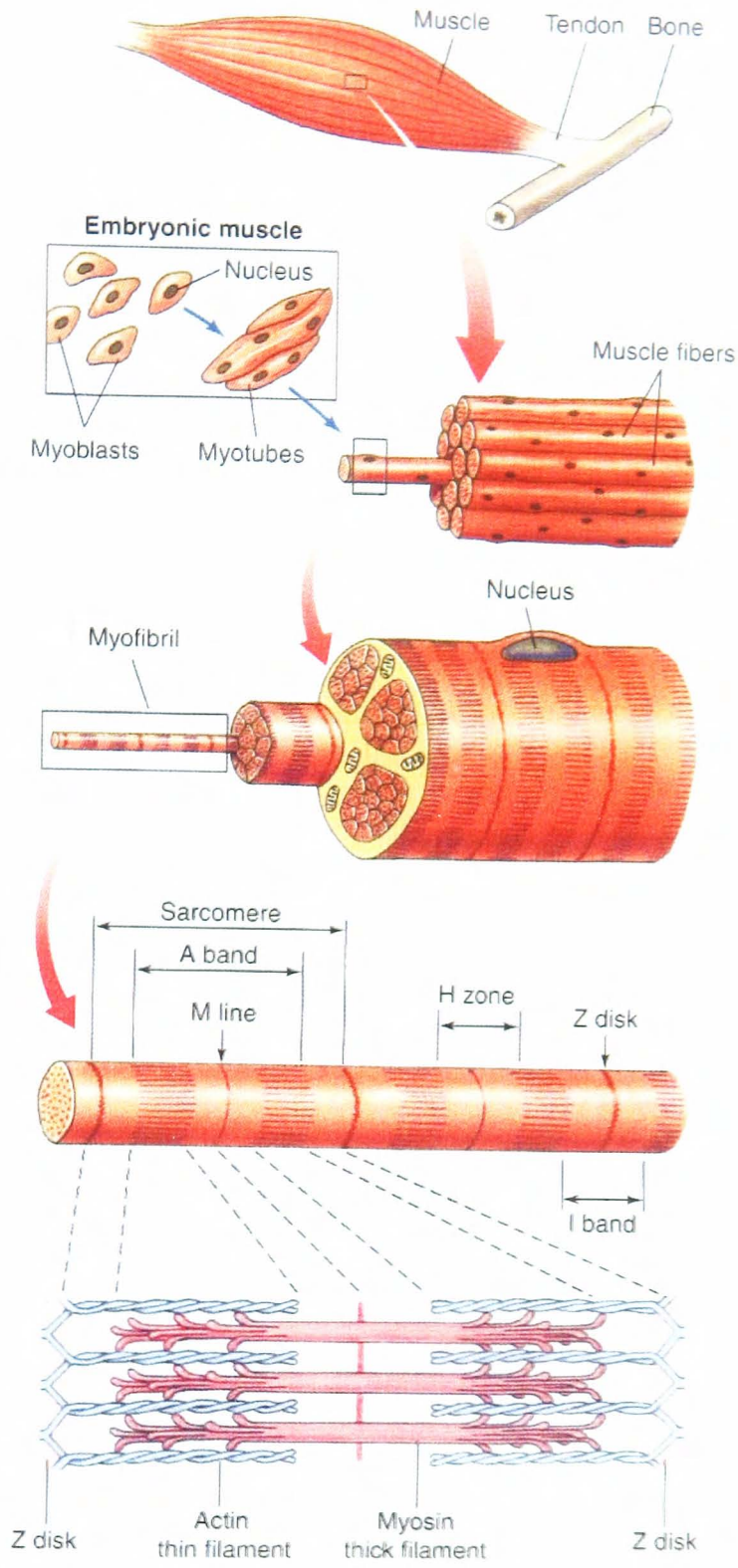


Figure 1.3-4 Structure of muscle from tissue to individual myofribril strand. Each muscle is attached to a bone of the skeleton at either end and is used to coordinate animal movement. The muscle itself is made of bundles of fibres which are effectively muscle cells, formed when multiple muscle cell walls break down to form larger massive cells with multiple nuclei. Each muscle cell, or myofibre, contains further smaller fibres known as myofibrils and within this structure the characteristic protein ultrastructure of the contractile apparatus is seen. Taken from Randall *et al.* (2002).

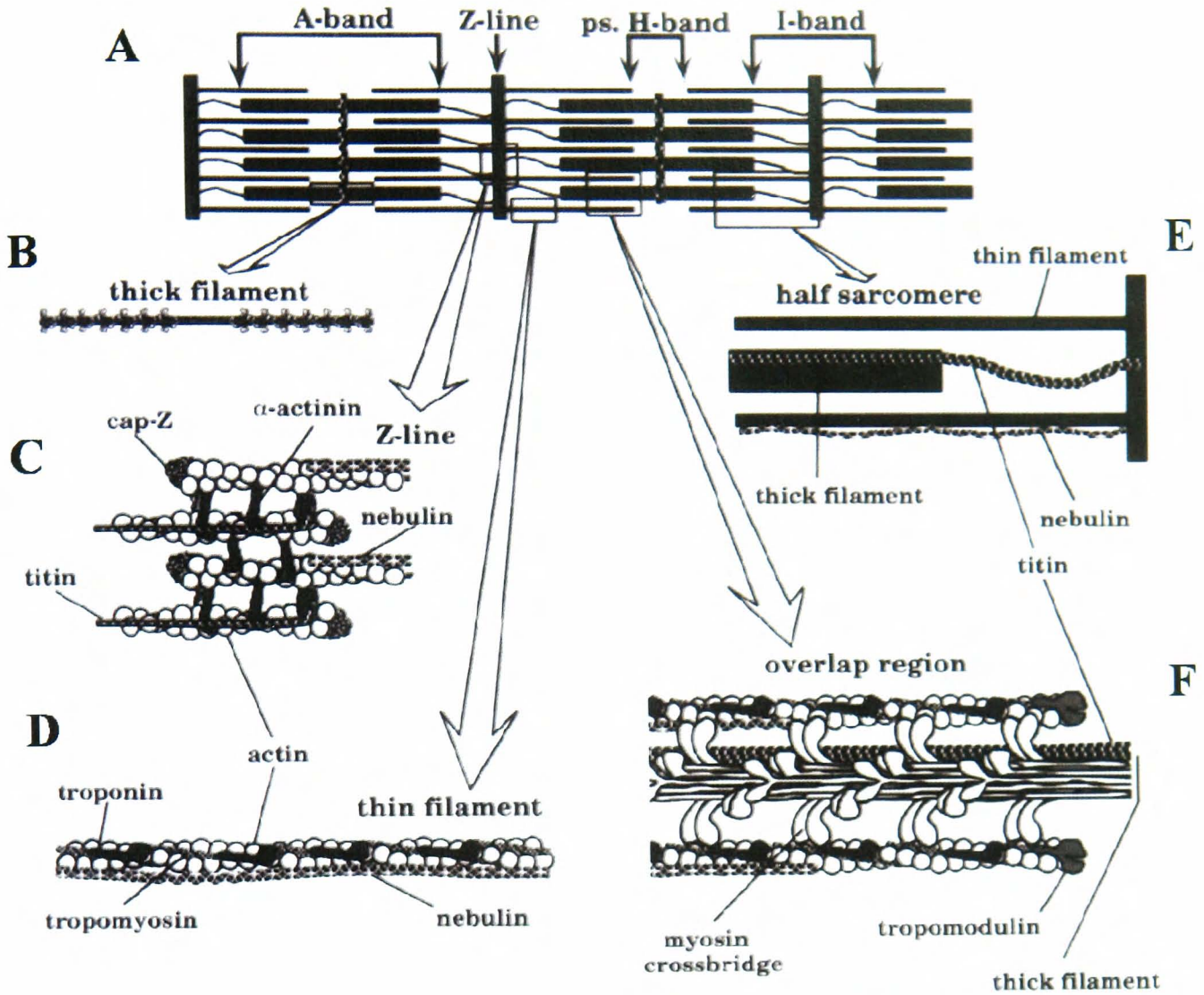


Figure 1.3-5. Proteins within the sarcomere, adapted from Swartz *et al.* (2000). A) The characteristic bands of the sarcomere defined by the overlap of the thick and thin filaments and the space between. B) The thick filament is composed of myosin molecules, bundled together in a formation that leaves the reactive globular head facing outwards from the filament. C) The thin filament is anchored at the Z-line by CapZ to α-actinin. D) The thin filament is comprised of many globular actin proteins polymerising to form a long chain, constructed around two molecules of another long filamentous protein, nebulin. E) Titin is the largest protein known and spans the length of the sarcomere, preventing over-stretching of muscle fibres. F) The actin proteins provide many binding sites along their chain to which myosin heads from the thick filament can bind (myosin crossbridge) and facilitate sliding of the filaments past each other. This reaction is regulated by the presence of tropomyosin which is associated with troponin complexes along the thin filament (D). Together tropomyosin and troponin regulate the binding of myosin to the active sites on the thick filament.

1.3.3 Muscle Function and Fibre type

There is great variation in the roles skeletal muscles are required to perform, ranging from maintaining posture to powering short, powerful sprints (Bottellini & Reggiani, 2000) and in order to perform these roles skeletal muscle can be highly heterogeneous in terms of mechanical and metabolic properties (Buckingham, 1992; Schiaffino & Reggiani, 1996). There are two major components to muscle protein, the myofibrillar protein fraction involved in muscle contraction and movement, and the sarcoplasmic proteins which include enzymes and myoglobin (Peterson *et al.*, 1991). After skeletal muscle cells differentiate and fuse into adult myotubes, they can begin to express different isoforms of muscle specific genes allowing muscle cells to be able to specialise further into fibres with varying degrees of physical properties, such as differing contraction time, maximal power, shortening velocity and resistance to fatigue (Bottellini & Reggiani, 2000), and metabolic properties; with differing levels of glycogen and lipid in the cells, fibre diameter, colour and their oxidative and glycolytic capacities (Chang, 2007). The speed with which the actin and myosin crossbridges interact differs as a result of different myosin heavy chain isoforms. The muscle fibres can react differently to neural stimuli resulting in slow gradual contraction lasting minutes or with a quick contraction which is over in milliseconds (Randall *et al.*, 2002). The number of calcium-ATPase enzyme pumps present on the membranes of the SR can affect the speed with which a muscle recovers from a contraction (Rodman & Baker, 1978) and the mitochondrial population density affects the energy supply required for aerobic respiration.

Muscle contraction requires energy in the form of ATP and, in order to generate ATP, muscle is able to metabolize fuels such as glucose, fatty acids and ketone bodies (Voet *et al.*, 2008). Choice of fuel consumption and energy production varies depending on the duration of work being done. For extremely short, rapid contraction muscle produces ATP from the reaction between phosphocreatine and ADP, producing creatine and ATP, a reaction which can generate enough ATP to fuel around 10 seconds of muscle "work". Following exhaustion of phosphocreatine supplies, muscle can utilise glycogen stores in the process of glycolysis, which involves the anaerobic respiration of glycogen, resulting in the production of lactate. Human muscle can use glycolysis as an energy store for around 2 minutes of exercise before build up of the by-product lactate begins to lower pH of muscle and causes muscle fatigue. For longer, sustained muscle use, oxidative phosphorylation of fatty acids must be used, a process which produces ATP most efficiently although more slowly. Training of athletes can alter the metabolic profiles of muscles so that sprinters rely on glycolysis as their major fuel supply whereas long distance runners rely on oxidative metabolism (Voet *et al.*, 2008). Overall, these differences in muscle metabolism allow muscle fibres to be

classified depending on two factors, whether they utilize predominantly glycolysis or oxidative phosphorylation as their energy source, and secondly, the speed of contraction, i.e. slow or fast twitch (Reggiani & Mascarello, 2004). These two factors are the predominant properties used to define the three major classes of muscle fibre type.

Peter *et al.* (1972) suggested fibres be classified under three terms based on their twitch speed and their metabolism; 1) slow oxidative (SO), 2) fast twitch glycolytic (FG) and 3) fast twitch oxidative glycolytic (FOG) which are intermediate fibres using a combination of both oxidative and metabolic metabolism. Generally, slow oxidative (SO) fibres have small fibre diameter, low force per cross-sectional area, slow rate of contraction and low myosin ATPase activity. SO fibres have high resistance to fatigue with a large population of mitochondria, along with little capacity for energy generation anaerobically by glycolysis. In contrast, fast glycolytic (FG) fibres have a large diameter and force per cross-sectional area and have high myosin ATPase activity. FG fibres fatigue quickly and have few mitochondria for oxidative respiration due to their high capacity for anaerobic glycolysis. Fast oxidative glycolytic (FOG) fibres have properties between SO and FG, where FOG are intermediate in size, myosin ATPase activity, resistance to fatigue and enzymes in glycolytic energy production. Similar to FG fibres, FOG fibres have a large force per cross-sectional area and a fast rate of contraction, but are similar to SO fibres in that they have a large capacity for oxidative metabolism due to their large mitochondrial population, summarised in table 1.3-2 (adapted from Randall *et al.*, 2002).

Table 1.3-2. Physiological properties in each muscle fibre type, adapted from Randall *et al.* (2002).

Muscle Characteristic	Slow Oxidative (SO) Type I	Fast Oxidative Glycolytic (FOG) Type IIa	Fast Glycolytic (FG) Type IIx
Fibre diammeter	Small	↔	Large
Force per cross-sectional area	Small	High	Large
Rate of contraction (Vmax)	Slow	Fast	Fast
Myosin ATPase activity	Low	↔	High
Fatigue resistance	High	↔	Low
Mitochondria/ capacity for oxidative metabolism	High	High	Low
Enzymes for anaerobic glycolysis	Low	↔	High

Key: ↔ = intermediate

A study in rats (Armstrong & Phelps, 1984) found that within the hind-limb muscle mass of rats the collection of fibres was predominantly fast glycolytic (76%) with less fast twitch oxidative (19%) and very few slow oxidative fibres (5%). In comparison, a study in the muscle population of sheep (Suzuki & Tamate, 1988) found that type I, SO, fibres were much more abundant in sheep muscles, where SO fibres accounted for between 50 and 75% in some leg muscles, an observation they linked to the species requirement to stand for long periods of time during grazing.

1.3.4 MHC Genes

There are several MHC protein isoforms expressed in mammals as a result of multiple MHC genes in the genome and their expression is tissue related, such as the cardiac isoforms in the heart, encoded by gene MYH6, and the extraocular in the eye, encoded by gene MYH13. Of these multiple gene variants, six isoforms are predominant in skeletal muscle, as summarised in table 1.3-3 (Maccatrozzo *et al.*, 2004). All the MHC genes are highly homologous and there is little variation between their nucleotide sequences in the highly conserved regions of the protein structure, but in the globular head region where the ATP binding region is associated there are differences, culminating in varying speeds with which the proteins catalyze ATP to produce energy for actin binding and contraction (Schiaffino & Reggiani, 1996, Pette & Staron, 2000). During mammalian embryonic development, the primary MHC genes to be expressed in skeletal muscle are the embryonic, neonatal and the I/β slow isoforms until birth, after which the expression of the embryonic and neonatal isoforms decreases and they are replaced by the adult fast isoforms IIA, IIX and IIB which are expressed through adulthood, along with the I/ β slow isoform (Chang, 2007). In small mammals such as dogs and rodents(Schiaffino & Reggiani, 1996) there are four major adult MHC isoforms present in varying abundances in skeletal muscle, whereas in larger species such as cattle and horses there are only three adult isoforms present, I/ β slow isoforms, IIA and IIX (Chikuni *et al.*, 2004; Maccatrozzo *et al.*, 2004). The organization of the MHC within the chromosome is not related to the spatial expression pattern and the 6 skeletal muscle MHC genes are split between two chromosomes in several species including human and mice (Weiss *et al.*, 1999). In humans the distribution of the isoforms is the same aside from locations in chromosomes 17 and 14, demonstrated in figure 1.3-6.

Table 1.3-3. Myosin protein isoforms, gene name and associated tissue types.

MHC Isoform	Gene Name	Tissue
MHC Iα / Cardiac	MYH6	Heart
MHC Extraocular	MYH13	Eye
MHC I β/Slow	MYH7	Heart/ Skeletal Muscle
MHC IIa	MYH2	Skeletal Muscle
MHC IIx	MYH1	Skeletal Muscle
MHC IIb	MYH4	Skeletal Muscle
MHC Embryonic	MYH3	Developing Skeletal Muscle
MHC Neonatal	MYH8	Developing Skeletal Muscle

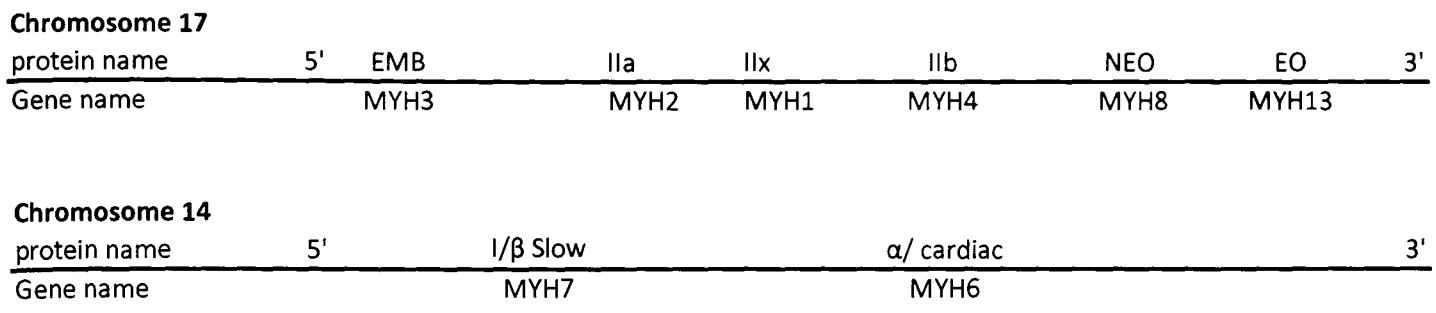


Figure 1.3-6 Schematic representation of position of MHC encoding genes on human chromosome. Location of skeletal MHC genes is conserved between some mammalian species with the I/β slow isoform localized with the α-cardiac isoform, in this example of human organization these two genes are situated on chromosome 14. The two developmental MHC are located either side of the adult fast isoforms located in chromosome 17 in human with the extraocular isoform. Common name for each MHC protein encoded is above, gene name is annotated below. Adapted from Maccatrozzo *et al.* (2007).

The variation in each isoform's activity in the ATPase reaction leads to variation in the protein's contraction speed and hence types of isoforms expressed in skeletal muscle sarcomeres determines their contraction speeds and thereby force. This leads to the classification of muscles in terms of their contractile speed; fast or slow. The slow isoform is MHC I, also known as MHC β or slow, and is associated with a slower muscle contraction in comparison to its faster relatives; MHC IIA, IIX and IIB. Chang (2007) further defined muscle fibre types in light of the recent discovery of MHC gene properties as follows; 1) slow oxidative express slow MHC isoforms, are red in colour due to high levels of myoglobin and large numbers of mitochondria which support oxidative respiration, have the highest lipid contents and have the most dense capillary networks; 2) fast glycolytic fibres predominantly express the fast MHC IIB isoforms, have the largest diameter of the fibre types, have least myoglobin and mitochondria content, contain a large store of glycogen to fuel their predominant metabolism and have low levels of lipid, 3) intermediate fast fibres have a fast twitch but use a combination of oxidative and glycolytic metabolism and express the MHC IIA and IIX isoforms.

This variation of fibre type between species and the distribution of these fibre types within an animal is related to the requirement of muscles to perform a variety of roles. Slow moving animals require more slow oxidative fibres whereas fast moving such as rodents tend to have more fast glycolytic. This is reflected in the MHC gene expression profile of animal species whereby large animals such as cattle and horses MHC IIB are almost exclusively absent, despite being abundant in smaller species such as pigs, dogs and rodents (Chang, 2007). Similarly fibre types vary between muscles as those involved in posture tend to contain predominantly SO fibres where as those muscles involved in movement can contain a variety of fibre types (Suzuki & Tamate, 1988; Ariano *et al.*, 1973). Distribution of fibres is also varied within a muscle with studies into a number of species

finding agreement that SO fibres tend to aggregate in the deepest regions of the muscle and the faster twitch fibres found towards the outer regions of the muscles (Armstrong *et al.*, 1982; Armstrong *et al.*, 1984; Suzuki & Tamate, 1988, Klont *et al.*, 1998). The arrangement of the slow fibres within the depths of the muscle surrounded by faster fibres is a result of the development of the fibres pre-natally. The first fibres to form in the embryo, known as primary fibres, are slow and the fast fibres form around them, known as secondary fibres, at the same time as the neural tissue is forming (Buckingham *et* al., 2003).

1.3.5 Muscle growth and fibre type

The development of the differential fibre types begins with the onset of innveration during foetal development (Buckingham, 2001) and the control of muscle fibre type beyond birth can be manipulated by a number of methods and is of interest to the meat industry as it is known to affect meat quality (Klont *et al.*, 1998). It has been known for some time that muscle contraction properties can be altered by altered electrical stimulation applied directly to the muscles, causing fast to slow fibre type transitions supported by related expression changes in MHC genes (Pette, 1998; Jaschinski *et al.*, 1998). In addition studies have investigated the fibre type changes during animals growth and reported that from birth to mature liveweight, fibre type of muscle progresses from fast to slow, observed in pigs (Handel & Stickland, 1987), sheep (de Macado *et al.*, 2000) and cattle (Wegner *et al.*, 2000) and have attributed these changes to the rate of growth of the animals whereby increased growth rate leads to increase in slow/fatigue resistant fibres probably due to an increased load effected by increased liveweight (Handel & Stickland, 1987). Importantly, these studies reported that these changes in properties of muscle fibre types were not due to growth of new muscle cells but due to changes within existing fibres. The number of muscle cells present in the adult tissue is defined during the early stages of muscle development (myogenesis) and post-natal muscle growth involves an increase in the size of those muscle cells (hypertrophy), not an increase in number (hyperplasia) (Rehfeldt *et al.*, 2004).

An example of agents that have been investigated for their effects in promoting muscle growth in the meat industry are β-adrenergic agonists (BAA). BAAs are potent promoters of lean muscle growth and studies have reported their effects in cattle (Beerman D.H., 2002; Yang & McElligot, 1989; Vestergard *et* al., 1994) and sheep (Koohmariae & Geesink, 1991). The results of these studies indicated that the majority of muscle hypertrophy as a result of BAA treatment was specific to fast glycolytic fibres, in the case of Verstergard *et al.*'s study the ratio of FG fibres in the LD of BAA treated animals was 71.1% compared to 51.7% in controls. The BAA studies show that the fibre type changes seen with BAA treatment are specific to fast glycolytic fibres, and the overall action in

muscle is to increase glycolytic potential and reduce oxidative capacity, in addition to increased protein synthesis and reduced protein breakdown. While the phenomenon of muscle hypertrophy observed with BAA treatment is of great interest to the meat industry in terms of increased production, it provides a clear link between changes in fibre type of muscle and the resulting meat quality, as there are reported adverse affects of BAA treatment on the tenderisation process post mortem (Koohmaraie & Geesink, 1991) and thus the gains of muscle growth are offset with losses in product quality. Although the mechanism of fibre type specific hypertrophy observed with BAA treatment is of interest to the studies reported in this thesis, the primary aim of this investigation is to improve meat quality in terms of tenderness. Thus the following literature review aims to outline some of the mechanisms responsible for fibre type switching and the relevance of muscle fibre type to meat quality.

1.3.5.1 Muscle fibre Type Transitions

It has been known for some time that muscle contraction properties can be altered by altered electrical stimulation, causing fast to slow fibre type transitions supported by related expression changes in MHC genes (Jaschinski *et al.*, 1998). In their experiments, Jaschinski *et al.* (1998) demonstrated that over a long period of chronic electrical stimulation, a fibre transition could be seen in muscles from rat expressing predominantly MHC IIB to switch to predominantly expressing MHC IIX. This was in agreement with another experiment where fibres from rabbit hind limbs switched MHC gene expression from predominantly MHC IIX, to MHC IIA and eventually to MHC I/β (Leeuw & Pette, 1993). These experiments demonstrate muscle's ability to change gene expression and to alter its phenotype in order to meet the demands more efficiently.

These transitions in MHC genes follow a sequential order, where MHC proteins of the fibre are converted from one form to another, in the sequence set out below (Jaschinski *et al.*, 1998, Pette & Staron, 2000), where the change in isoform expression is sequential. For example MHC IIA expression must lead to MHC IIX before the final transition to MHC IIB, with hybrid fibres in between.

$$MHC\ I\ (\beta) \leftrightarrow MHC\ IIA \leftrightarrow MHC\ IIX \leftrightarrow MHC\ IIB$$

The transformation from one fibre type to another allows muscle to adapt to altered requirements and demonstrates plasticity in muscle fibre populations of muscle. The mechanisms for these transitions are complex and are regulated in a variety of ways including neuromuscular acitivity, hormones and aging (Pette & Staron, 2000). In particular relevance to this study, it has been shown that thyroid hormone has been demonstrated to change MHC gene expression, whereby low levels

of thyroid hormones (hypothyroidism) results in muscle fibre type changes in a fast to slow transition (Nwoye & Mommaerts, 1981), and in contrast, high levels of thyroid hormones (hyperthyroidism) the transition is slow to fast (Li *et al.*, 1996). Aging has been related to a loss of type II fibres and a subsequent study verified that there is a transition from MHC IIX to MHC I at both mRNA and protein levels of expression levels in both men and women during aging (Short *et al.*, 2005). The signalling mechanisms that regulate fibre type transitions are complex and require a change in gene expression of a large variety of proteins, ranging from enzymes involved in energy metabolism to the MHC proteins required for contraction. Although still not fully elucidated, a number of mechanisms for fibre type changes are considered below.

1.3.5.2 Mechanisms controlling Fibre type transitions

1.3.5.3 Calcineurin and NFAT

Calcineurin, a calcium-calmodulin-regulated protein phosphatase was demonstrated to up-regulate the expression of MHC I slow in C2C12 muscle cells and promote muscle cell differentiation (Delling *et al.*, 2000). Calcineurin is an excellent candidate to be mediator of fibre type switching as it is responsive to fluctuating levels of calcium and therefore its activity is a reflection of the contractile activity of the muscle (Alzuherri & Chang, 2003). Calcineurin is known to be up-regulated by increased intracellular calcium levels, as seen in prolonged nerve contraction, and the enzyme functions in removing phosphate groups from the nuclear factor of activated T cells (NFAT) family of transcription factors, thus activating them to translocate to the nucleus, shown in figure 1.3-7. Once in the nucleus the activated NFAT protein can bind DNA and affect gene transcription. It has been previously shown that this mechanism is involved in the slow fibre regulation, but that the NFAT transcription factors require other muscle specific transcription factors such as the MEF2 family, and together these proteins are able to promote slow fibre formation (Chin *et al.*, 1998). In their study Delling *et al.* (2000) could demonstrate a role for one of the NFAT protein family members, NFATc3, in promoting cell differentiation but not in slow fibre type conversion, however they did not investigate the role of other members of the NFAT family and postulated NFATc1 and NFATc2 could be involved. A further study by da Costa *et al.* (2007) demonstrated that calcineurin signalling via NFAT was able to upregulate the oxidative fibre phenotype in C2C12 cultures by upregulating the expression of the fast but oxidative MHC IIA and downregulating MHCs IIX and IIB.

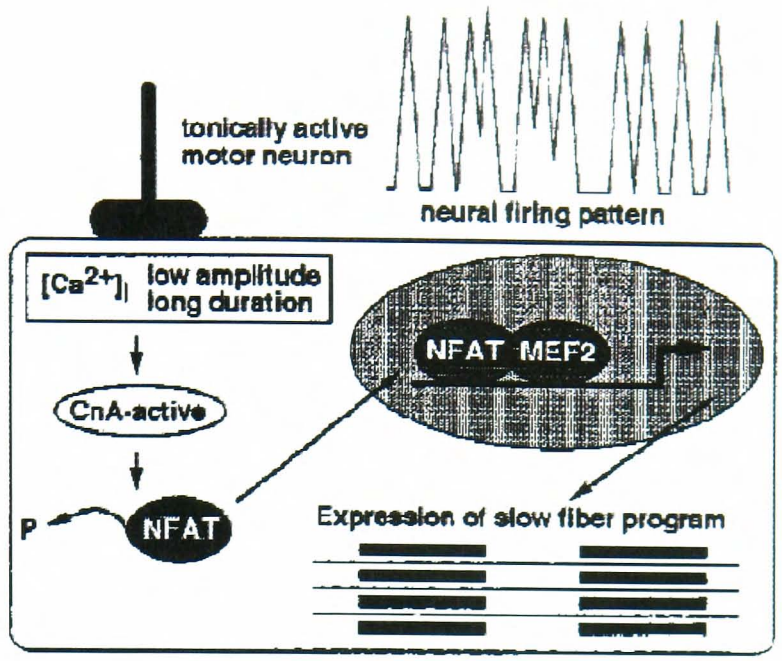


Figure 1.3-7. Proposed mechanism for calcineurin in activation of the slow muscle fibre program of gene expression leading to increased expression of the MHC I/β slow isoform (from Chin *et al.*, 1998). Neuron firing patterns cause fluctuations in intracellular calcium ions. Calcium ions activate the calcium regulated calcineurin A (CnA) enzyme which de-phosphorylates the nuclear factor of activated T-cells (NFAT) family of transcription factors. NFAT then dimerizes with the MEF2 family of transcription factors and promotes transcription of the slow muscle fibre related gene programme.

1.3.5.4 Protein Kinases

The explanation for the modulation of slow fibre phenotype by calcium fluctuations is further supported by the actions of the other calcium regulated signalling molecules within muscle cells, the Ca2+/calmodulin-dependant kinases, known as calmodulin kinases, and protein kinase C (PKC) . Calmodulin-kinases are a family of phosphorylating enzymes which are activated by increased levels of free calcium in the cell as seen in frequent contraction of muscle. Calmodulin kinases are known to up-regulate genes involved in oxidative metabolism such as mitochondrial enzymes involved in fatty acid metabolism and electron transport, as well as the transcription factors NFAT and PGC-1α, which are important regulators of slow muscle fibre phenotypes (Chin, 2005). Meissner *et al.*, (2007) investigated the relationship of calcium and the MAPK signalling pathways in C2C12 muscle cells and found the p38/MAPK was associated with MHC IIX expression via recruitment of the transcription co-activator CREB-binding protein and MEF2 proteins C and D. Finally, Akt is a member of the serine/threonine protein kinases and is activated via the phosphatidylinositol-3 kinase (PI3K) pathway, and in an experiment causing over-expression of Akt, type IIB fibres were formed and glycolytic metabolism was increased (Izumiya *et al.*, 2008).

1.3.5.5 Peroxisome Proliferator-Activated Receptors (PPARs)

In addition to the role of calcineurin in upregulating the transcription of the MHC I/β slow gene, a study by Lin *et al.* (2002) demonstrated that calcineurin activates the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) co-activator 1-α (PGC-1α). Upregulated PGC-1α was shown to act with the other transcription factors of the MEF2 family and caused fibre type changes from the fast to slow isoforms and increased expression of mitochondrial metabolism. Schuler *et al.* (2006) demonstrated that PGC-1α expression was under the control of another member of the PPAR family, PPARβ, which is implicated in activating PGC-1α expression via a PPAR response gene in the promoter region of the PGC-1α gene. Together these two transcription factors were shown to promote slow fibre formation (Schuler *et al.*, 2006). In addition to PGC-1α, other members of the peroxisome proliferator-activator receptors (PPARs) family are related to muscle fibre type with PPARδ overexpression leading an increased proportion of type I fibres in mice (Wang *et al.*, 2004), PPARα overexpression in mice causing changes in muscle oxidative metabolism (Finck *et al.*, 2005) and mice overexpressing PGC-1α have an increase in the proportion of type I and IIA fibres (Lin *et al.*, 2002). In contrast to the PGC-1α promotion of slow oxidative phenotype, Arany *et al.* (2007) demonstrated that the related protein PGC-1β was involved in transcriptional upregulation of the MHC IIX gene, via co-activation with PPARα, and so induced intermediate fast twitch fibres with oxidative metabolism.

1.3.6 Muscle fibre type control: Summary

Muscle is a heterogenous tissue which is capable of performing a large range of functions. The ability of muscle to perform these functions stems from the presence of different fibre types as a result of the differential expression of specific isoforms of muscle genes. Mechanisms to improve meat yield by increasing muscle growth have been investigated and in particular β-adrenergic agonists (BAAs) are potent in increasing muscle growth, but adversely affect the quality of the meat produced. There is now increasing understanding of the mechanisms involved in the manipulation of fibre type and the possibility of a growth promoting agent which could increase muscle mass via hypertrophy of the oxidative fibre types known to be associated with tender meat could be of significant advantage to the meat industry.

Sorenson *et al.* (1979) performed an investigation into vitamin D supplementation and its effects in fibre type changes. In their study a vitamin D analogue, 1α-hydroxycholecalciferol or alfacalcidiol, plus calcium was administered to elderly female patients with low bone density and found oxidative capacity of the muscle as measured by succinate dehydrogenase activity, was low before the

supplementation regime and was subsequently increased with treatment. In addition they observed an increase in the number of fast oxidative fibres and a decrease in fast glycolytic fibres, providing valuable evidence not just for vitamin D and calcium supplementation in improving oxidative capacity (resistance to fatigue) as well as inducing fibre type transitions. A final study into vitamin D supplementation in muscle fibre type was performed by Sato *et al.* (2005) who gave elderly women ergocalciferol at 1000 IU/day for 2 years and found falling risk decreased by 59%, muscle strength increased and the number and size of type II fibres increased. In addition to vitamin D's effects on aged muscle, it has been shown that the hormone could play a role in normal muscle growth and development. In a study using knockout VDR (VDR -/-) mice Endo *et al.* (2003) found mice born with the VDR -/- genotype exhibited abnormal muscle development, with elevated levels of the myogenic regulatory factors (MRFs) Myf5 and myogenin, along with the MRF-coactivator E2A. In addition these mice expressed higher levels of embryonic and neonatal isoforms of the myosin heavy chain genes. The muscle fibres of the VDR -/- mice were 20% smaller than in VDR +/+ control mice. The same study followed up these observations using the mouse derived C2C12 skeletal muscle cell-line and found the expression of Myf5, myogenin and neonatal MHC were down-regulated in response to activated vitamin D treatment and provides evidence for the importance of vitamin D in regulating the process of muscle tissue development.

These studies provide evidence for vitamin D to exert direct effects on muscle tissue and fibre type, but have not investigated the molecular mechanisms through which vitamin D could potentially signal, and thus the objective of the work performed in this thesis aims to determine the potential pathways through which vitamin D might regulate MHC gene expression and muscle fibre type, which is of economic relevance to the meat industry through possible improvements in meat quality. The final section of this literature review aims to define meat quality, outline what factors affect meat quality, with particular reference to the calpain system, and the subsequent hypothesis for vitamin D's role in modulating meat quality.

1.4 Meat Quality

There are three major contributors to the development of the quality of meat in terms of tenderness 1) the degree of background toughness, 2) the toughening phase prior to rigor and 3) the tenderization phase. Background toughness is a result of the extent of connective tissue, primarily collagen, and affects the ease with which unconditioned muscle can be cut (Koohmaraie & Geesink, 2006). Connective tissue increases with the age of animals and thus in the production of premium steak meat it is standard to slaughter animals after their first growth phase before background

toughness is significant; culling of elderly animals leads to meat with tougher tendencies. Of the remaining contributors to meat quality, the toughening phase is constant in all carcasses which are processed the same, however, it is the tenderization phase that is most variable (Koohmaraie & Geesink, 2006) and is of interest to meat scientists to manipulate in order to produce meat of guaranteed high quality. The major factor in the development of tenderization is the activity of proteolytic enzymes post mortem (Koohmaraie & Geesink, 2006) and the manipulation of these enzymes has been the focus of many studies in order to understand how production methods can optimise meat tenderness. In particular these enzymes are activated by intracellular calcium levels, the levels of which are expected to be regulated by vitamin D. It is likely that increases in calcium status of meat animals, as regulated by vitamin D, could affect the tenderisation process post-mortem through activation of the calpains.

1.5 Conversion of Muscle to Meat

In order to produce meat of high quality, it is important to understand the reactions that take place in muscle post mortem and how they contribute to the development of tender meat that is of acceptable quality to the consumer. The conversion of muscle to meat can be defined by three phases; the pre-rigor phase, rigor mortis and post rigor tenderization (Sentrandeu *et al.*, 2002).

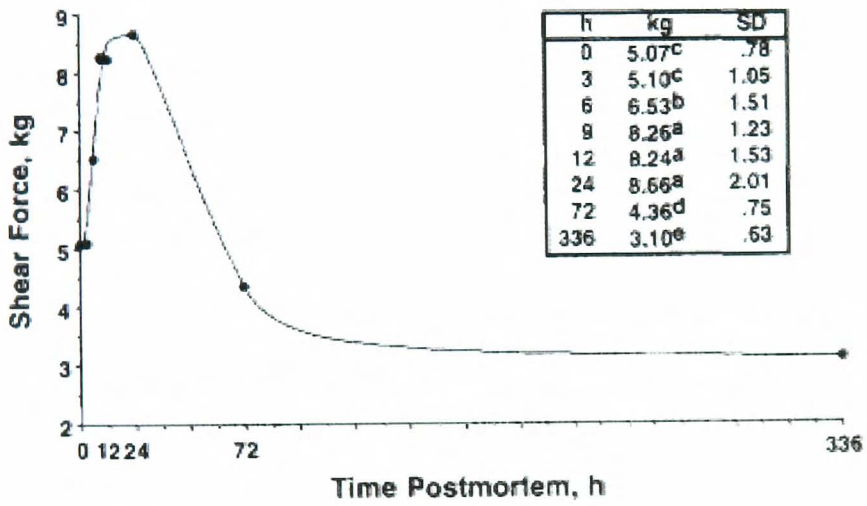
1.5.1 The Pre-rigor Phase

At death, blood circulation ceases and oxygen and nutrient supply to tissues is cut off causing the metabolic conditions in the muscle to change and the tissue begins to undergo a series of events that ultimately create the end product of meat. The pre-rigor phase in lamb muscle lasts around 12 hours and in this phase the muscle is still excitable and is still metabolizing available energy sources of ATP (Adenosine triphosphate), PC (phosphocreatine) and glycogen (Sentrendau *et al*; 2002). When the muscle is supplied with oxygen and nutrients i.e. during "life", these energy sources are primarily used to control the extent of muscle contraction and homeostatic conditions of the cells, predominatly keeping free calcium inside the sarcoplasma at low concentrations. There are two mechanisms to maintain the low free calcium ion concentration of the sarcoplasma. The first mechanism is that which sequesters free calcium ions within the muscle cell into the sacroplasmic reticulum (SR) via calcium ATPase, and requires energy in the form of ATP to perform calcium ion movement across the membrane. The second mechanism is to constantly remove calcium from the internal environment into the extracellular fluid, again a function performed by an ATP-dependant calcium pump on the cell membrane. As both these calcium pumps require ATP, it is the eventual decrease in this energy source in the muscle post mortem that allows the rise in calcium levels inside

the muscle cell and leads to the sustained uncontrolled muscle contraction that defines the second phase of muscle to meat conversion; the rigor phase (Jaecocke, 1993).

1.5.2 The Rigor phase

During rigor the free calcium levels in the sarcoplasma rise, thereby removing troponin inhibition of the actin binding sites and initiating muscle contraction, this phase begins in lamb meat around 12 hours post mortem. In normal muscle contraction, the release of calcium from the sarcolemma results in a change in the calcium concentration from the region on 10^{-7}M to 10^{-5}M, and this calcium is subsequently sequestered back into the sarcoplasmic reticulum to allow relaxation, but post mortem the energy supplies become exhausted and the contraction is maintained. Jeacocke (1993) found calcium levels at onset of rigor to be in the region of 10^{-4}M, allowing the much stronger and sustained contraction causing the characteristic increase in toughness of meat at this stage, shown in the shear force measurements demonstrated in figure 1.5-1.



h	kg	SD
0	5.07[c]	.78
3	5.10[c]	1.05
6	6.53[b]	1.51
9	8.26[a]	1.23
12	8.24[a]	1.53
24	8.66[a]	2.01
72	4.36[d]	.75
336	3.10[e]	.63

Figure 1.5-1. The post mortem changes in shear force (kg) of lamb *longissimus thoracic et lumborum* muscle (Wheeler & Koohmaraie, 1994). In lamb, the pre-rigor phase lasts approximately 12 hours before the onset of rigor, where meat reaches maximum toughness, in this case at 24 hours. The meat then begins the tenderization phase where shear force is slowly reduced; this process can take up to two weeks in lamb muscle.

A defining characteristic change of muscle tissue during the conversion of muscle to meat is that of the shear force of the meat. Shear force is the measurement of the degree of resistance with which meat shows to being cut when the fibres are sheared transversely along their length. Changes in shear force during the conversion of muscle to meat is shown in figure 1.5-1 (Wheeler & Koohmaraie, 1994), with the peak of rigor beginning at around 6 hours post mortem and lasting around 12 hours. Following the rigor phase the meat undergoes a slower, gradual decrease in toughness.

1.5.3 The Tenderisation phase

In lamb, the tenderization phase begins at around 24 hours post mortem and is a result of initiation of proteolysis (Wheeler & Koohmaraie, 1994). The meat slowly becomes more tender, taking around a week to reach ultimate tenderness, but the rate and extent of tenderness development is highly variable. It is this phase of meat production, known as conditioning or aging that is of concern in the present study. Improving the time taken to reach acceptable meat tenderness would help reduce the length of cold storage and reduce costs of meat, in addition to being able to guarantee a tender meat product.

The further development of tenderness during the aging period is catalyzed by the activity of proteolytic enzymes in the muscle which break down the muscle ultra-structure. The importance of the role of proteases in the tenderization process was demonstrated by Wheeler and Koohmaraie (1994) showing that between 24 and 72 hours post mortem the shear force of lamb muscle halved, but the length of the sarcomere (the extent of muscle contraction) was not significantly changed. This information leads to the conclusion that post-rigor, the development of tenderness is catalyzed by proteolytic enzymes and not by further changes in muscle contraction state, which just increases the "density" of the structure of the sarcomere causing the increased resistance to cutting i.e. toughness.

1.5.4 Properties of Meat changed during the conversion

There a number of properties of the meat which change during the conversion process and these are key identifiers of aspects which affect the quality of the meat.

1.5.4.1 Muscle pH

Tissues can continue to respire aerobically until available oxygen is used up following which they respire anaerobically, regenerating adenosine tri-phosphate (ATP) initially from phospho-creatine and then for a more prolonged period from glycolysis. When muscle glycogen is metabolised via glycolysis the products are pyruvate and ATP, although the amount of ATP produced is much less than is seen with aerobic respiration. In a final effort to produce energy anaerobically, pyruvate is metabolised into lactate, a by-product which is normally removed by the blood circulation. However, in post mortem muscle lactic acid accumulates and causes a decrease from neutral pH 7.5 down to pH 5.4-5.7 (Maltin *et al.* 2003), known as ultimate pH (pH_u). The amount of glycogen available prior to slaughter is a large factor in the normal development of post mortem pH. In animals with depleted glycogen stores the pH of the carcass does not decrease causing the meat to become dark

firm dry (DFD). Thus it is of high importance in the meat industry that animals are not stressed prior to slaughter and transport of animals to the abattoir, and conditions therein, must cause minimal upset to meat animals (Peterson *et al.*, 1997).

1.5.4.2 Temperature

As the muscle ceases to metabolize energy sources and the carcass is dressed, the muscle temperature begins to drop to that of the environment. The rate of temperature loss is affected by the fat content of the muscle (owing to fat's insulating properties), the size of the carcass and the reduction in metabolic activity of the tissues. The rate of temperature change is another contributing factor to the final tenderness of meat as rapid cooling prior to rigor can lead to cold- shortening of the meat and cause toughness, as a result of irreversible muscle contraction.

1.6 Proteolysis and Tenderisation

There are numerous enzymes present in muscle which can potentially contribute to the development of tenderization, these are the cathepsins (lysosomal peptidases), the proteasome (ubiquitin-marked proteolysis), caspases (apoptotic proteases), matrix metallopeptidases (MMP), muscle serine peptidases, and the calpain system (Ca^{2+}-dependant, cysteine proteases) (Sentrandeu *et al*, 2002). The major enzyme system reported to have effects on meat tenderness are the calcium-activated protease enzymes the calpains, and in particular the micro calpain (calpain I) isoform (Koohmariae & Geesink, 2006). It was the members of the calpain system which were investigated in this study. Although other protease enzymes have been attributed to playing some role in post-mortem proteolysis, the consensus is that the calpains play the major role. There is little activity of the ubiquitin-proteasome system in contributing to meat quality development, as the system is ATP-dependant and such energy stores are already depleted in the early stages post mortem. However there is some contribution towards degrading proteins such as troponin-C in the thin filament and myosin light chains -2 and -3 of the thick filament (Koohmaraie, 1992). In addition the lysosomal enzymes also do not appear to be highly active (Maltin *et al.*, 2003), although the cathepsins are capable of degrading α-actinin, actin and myosin (Okitani *et al.*, 1980) which are not substrates of the calpain system, but this proteolysis does not occur until late post mortem at 7 to 10 days (Matsukara *et al.*, 1981).

1.6.1 The Calpain System

The calpains are endogenous cysteine protease enzymes, with optimum activity in neutral pH (pH 7.2-8.2). There are two calpain isoforms responsible for proteolysis post mortem calpain I, or μ-calpain, and calpain II, m-calpain. Both enzymes have multiple calmodulin like EF-hand calcium motifs within their structure. The levels of calcium required to activate them has given rise to their suffixes; μ-calpain requiring calcium levels (Ca^{2+}) in the region of 3-50 μM and m-calpain requiring higher levels around 400-800 μM for half maximal activation (Goll *et al*, 2003). The enzymes exist as heterodimers, composed of two subunits; the larger subunit is around 80 to 75kda in size and the smaller around 30kDa. The 80kDa subunit structure can be divided into four major structural domains, domains I, II, III and IV, shown in figure 1.6-1. Domain I containing the N-terminal region is automatically removed in a process of autolysis upon activation of the enzyme. Domain II of the 80kDa subunit contains the active site, characteristic of other cysteine proteases, including the cathepsins, and domain III may be involved in binding phospholipids at the cell membrane and in contributing to folding of the protein. Domain IV contains four Ca^{2+} binding regions and is responsible for the activation of the enzyme, although there are an additional two other potential Ca^{2+} binding sequences in domain III. The 30kDa subunit has five Ca^{2+} binding regions in domain VI, similar to domain IV of the 80kDa subunit. The calpain enzyme subunits are encoded by distinct genes, with calpain I or μ-calpain and calpain II or m-calpain large subunit being encoded by the CAPN1 and CAPN2 genes respectively, however the smaller 30kDa subunit is encoded by a third gene, CAPN4 and this subunit is common to both calpain I and II.

The calpains are situated intracellularly, usually associated with organelles such as the myofibrils in skeletal muscle and the cytoskeleton in non-muscle cells. Upon activation by the binding of Ca^{2+} ions to the calmodulin-like domain IV, the enzyme translocates from the cytosol of the cell to the membranes. At the membrane the enzyme performs a further self-activation step whereby it removes the N-terminal region in domain I which results in the production of the active 75kDa form of the enzyme. This step is significant to the activity of the enzyme as the autolysed form requires a lower concentration of Ca^{2+} ions for activation, shown in table 1.6-1 (Melloni *et al.*, 1996). Following this autolysis step, the enzyme is then released back into the cytosol of the cell where it can degrade its substrates.

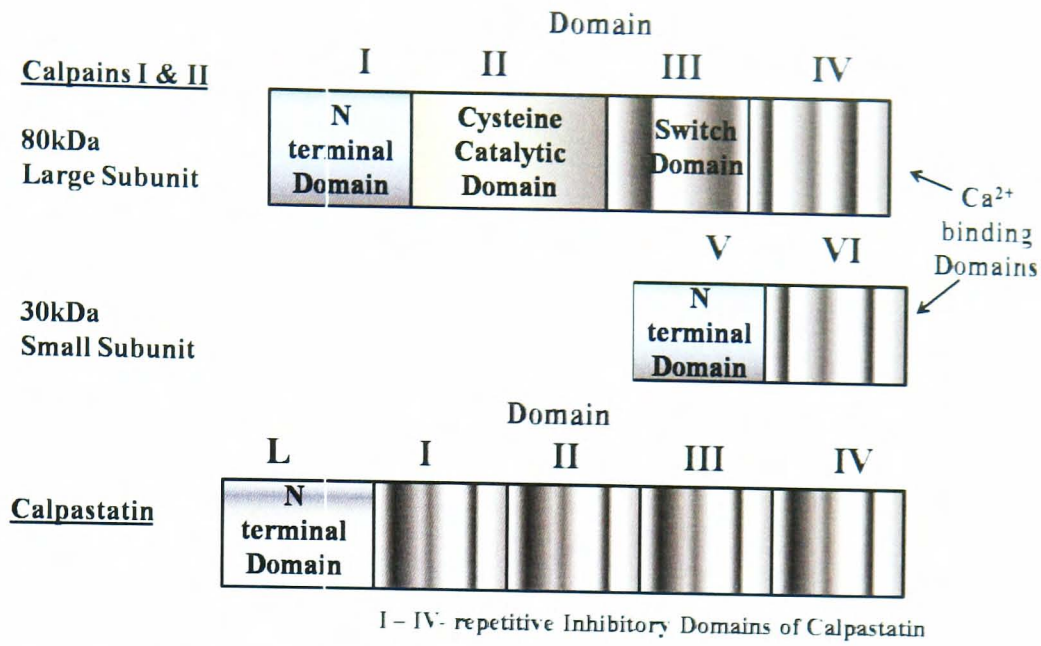


Figure 1.6-1. Schematic structure of the calpain enzymes and their endogenous inhibitor calpastatin, adapted from Sentrendau *et al.* (2003).

The activity of the calpain enzymes are regulated not only by the free calcium concentration in the muscle but by the presence of their endogenous inhibitor calpastatin, the structure of which is shown in figure 1.6-1. Calpastatin is a polymorphic protein and multiple isoforms exist as a result of different promoters in the gene and splicing events (Goll *et al.*, 2003), which gives rise to proteins ranging from 50 to 172kDa (Sentrandeu *et al.*, 2002), despite being encoded by a single gene, CAST. In each protein there are four domains responsible for calpain inhibition (domains I to IV) and the L-domain which is the N-terminal of the protein and is variable in size. The binding of calpain to calpastatin is calcium-dependant, although in a unique phenomenon the Ca^{2+} requirement for the binding of the inhibitor is less than that required for the enzyme to be activated (Wendt *et al.*, 2004), shown in table 1.6-1 and discussed further below.

Table 1.6-1. Physical properties of the calpain enzymes (adapted from Maltin *et al.*, 2003). Autolysis of the calpains reduces the concentration of calcium required for half-maximal activity but also decreases the calcium concentration required for binding of calpastatin.

Property of Calpain protease	μ-Calpain	Autolysed μ-calpain	m-Calpain	Autolysed m-calpain
Molecular mass	110	99	108	98
Large subunit	81	78	80	78
Small subunit	28	20	28	20
pH optimum	7·5	7·6	7·6	7·4
Ca^{2+} concentration for half-maximal activity (μm)	1–50	0·5–2·0	200–1000	30–150
Ca^{2+} concentration for binding calpastatin (μm)	42	0·042	400	24

1.6.2 Regulation of calpain activity by calpastatin

In their review in 2003, Goll *et al.* reinforced the significance of the regulation of the calpain enzyme activity with their estimation that there are enough calpain enzymes present within in a muscle cell to completely degrade all the z-discs within 5 minutes. Calpastatin acts as a reversible inhibitor of calpain activity and every calpastatin protein has four calpain binding regions, each capable of binding a calpain molecule. Within the four inhibitory domains of the calpastatin molecule there are three subdomains A, B and C; of which sub-domain A binds domain IV of the calpain 80kDa subunit and sub-domain C binds domain VI of the calpain 30kDa subunit (Wendt *et al*, 2004), however, sub-domain B is vital to inhibitory activity.

The binding of the calpains to calpastatin is calcium dependent and is reversible so that released calpain enzymes can continue to be proteolytically active (Imajoh & Suzuki, 1985), however the mechanisms of calpastatin and calpain binding were not clear until recently. Otsuka & Goll (1987) reported in their study that the calcium levels required for calpastatin to bind m-calpain was lower than that required for m-calpain to be catalytically active, when a calcium concentration of 0.53M was sufficient for half-maximum binding to calpastatin in comparison to a calcium concentration of 0.93M required for half-maximum activity of m-calpain in the absence of an calpastatin. This phenomenon was further investigated using both m- and μ-calpain and again found the same result (Kapprell & Goll, 1989). From these studies it was concluded that it was impossible for calpain enzymes to exist without being bound to calpastatin, until a study by Melloni *et al.* (2006) has shed more light on this phenomenon. In their study Melloni *et al.* (2006) report that phosphorylation of calpastatin by protein kinase C reduces the association between the inhibitor and enzymes. Protein kinase C is activated in response to calcium influxes within cells (Voet *et al.*, 2008) and therefore it seems likely that this is an additional step in calcium related control of calpain activity.

1.6.3 Function of the Calpains

The function of the calpains in living cells is not entirely defined and there has been some research into the calpain enzymes functions via knock-out models. A study performed by Azam *et al.* (2001) created mice null for the μ-calpain isoform and observed animals which survived but demonstrated altered blood platelet function. In another study Arthur *et al.* (2000) demonstrated an essential role of calpains in foetal survival in their study using knock-out mice lacking the gene for the small 30kDa subunit, where animals not expressing the small sub-unit did not survive beyond mid-gestation. These studies show the calpains are important in a large variety of functions and both Azam *et al.*

(2001) and Arthur *et al.* (2000) suggested the essential roles of cell division and migration, cell proliferation, apoptosis and cytoskeletal organization.

1.6.4 The calpain system and meat quality

The calpain enzymes are the major contributors to the development of tender meat; in particular it is μ-calpain that is largely responsible for the tenderization of muscle (Koohmaraie & Geesink, 2006). Wheeler & Koohmariae (1994) reported that as a result of the increased calcium levels during the rigor phase there is already detectable activity of the μ-calpain enzyme at 18 hours post mortem in lamb muscle. Lametch *et al.* (2004) reported that in *in vitro* post mortem conditions of pH = 5.5 and 4°C, μ-calpain retained 24-28% of its activity when compared to that in normal physiological conditions of pH=7.5 and 25°C. Further to support their major role in meat tenderisation, the calpains have been shown to be capable of degrading a large number of proteins *in vitro* and a study by Lametch *et al.* (2004) reported proteolytic digestion of a number of important structural proteins of the sarcomere including desmin, myosin light chain I, troponin T, tropomyosin α1, tropomyosin α4 and CapZ as well as myosin and actin of the sliding filaments, although not all of these are degraded during postmortem proteolysis (Koohmaraie & Geesink, 2006).

The discovery of the calpains and their activities in myofibrillar turnover was made in the 1970's (Dayton *et al.*, 1976) but investigations into their role in postmortem proteolysis began over ten years later. Bandman & Zdanis (1988) were some of the first to investigate specific proteins altered during post mortem tenderization and found titin was completely degraded within post mortem muscle but myosin was unaffected. Hwan & Bandman (1989) then investigated specific protein degradation in the Z-line in aging muscle and found desmin was degraded but not α-actinin, which is released from the Z-line intact. Taylor *et* al. (1995) investigated the major structural changes in the sarcomere during the early tenderization phase and found that the proteins linking the Z-lines together, known as N_2 lines, and proteins of the structures known as the costameres, which anchor the myofibrils to the membrane, were the major substrates of proteolysis. Members of the sarcomere which are degraded by the calpain enzymes include the key regulatory proteins troponin-T and –I along the thin filament and titin, the largest protein present, being responsible for regulating sarcomere length. Huff-Lonergan *et al.*, (1996) demonstrated μ-calpain was responsible for the digestion of five proteins, nebulin and filamin in addition to desmin, titin and troponin-T, and further, their study showed that the rate of the extent of the degradation of these proteins was correlated with improved tenderness of bovine muscle.

1.6.5 Evidence for the μ-calpain as major contributing enzyme to post mortem tenderization

The large majority of tenderisation develops early post mortem with 65-80% of tenderisation taking place between 3 and 4 days of storage at 4°C (Taylor *et al.*, 1995). During this period there is no degradation of actin or α-actinin and no large structural changes in the Z-line despite proteins of the Z-line being susceptible to degradation by the calpains. In fact this study showed that the major substances degraded during this early stage of tenderization are two cytoskeletal structures. Firstly, the costamere, which binds the contractile myofibril structures to the muscle cell membrane (sarcolemma), and secondly the structures which form a link between the Z-line and the thin filament known as the N_2 lines, shown in figure 1.6-2. In fact the majority of this early calpain activity is focussed at the costamere, where the enzymes degrade the proteins desmin and vinculin, in addition to nebulin and titin of the N_2 lines. Association of the loss of the costamere and the N_2 lines with the large change in tenderness observed early post mortem is evidence to show that calpain degradation of these structures is largely responsible for the tenderization mechanism and there is little degradation of the actual contractile apparatus (Taylor et al 1995). Further disassembly of the sarcomere does take place as calpains are responsible for degrading proteins associated with the thin filament such as troponin T and tropomyosin although there is no degradation of the contractile proteins myosin and actin.

In mice over-expressing the calpastatin protein there was a lack of post mortem protein degradation as measured by degradation of desmin and troponin-T (Kent, 2004). There was little change in activity of m-calpain between control mice and those which over-express calpastatin, where as μ-calpain activity was significantly affected, table 1.6-2. This suggested that μ-calpain is the major protease responsible for post mortem proteolysis and tenderization of meat. Geesink *et al.* (2006) further strengthened the argument with the use of μ-calpain knock-out mice. Their study compared the amount of substrate degradation in μ-calpain -/- against that observed in wild type and found that there was little post mortem degradation of key protein substrates of μ-calpain, nebulin, dystrophin, metavinculin, vinculin, desmin and troponin-T, despite the presence of m-calpain. These observations suggested that μ-calpain is responsible for a vast proportion of the post mortem degradation contributing to tenderisation of meat. With this knowledge in mind, it has been the aim of a number of studies to promote overall calpain activity post mortem by increased activation via increasing free calcium.

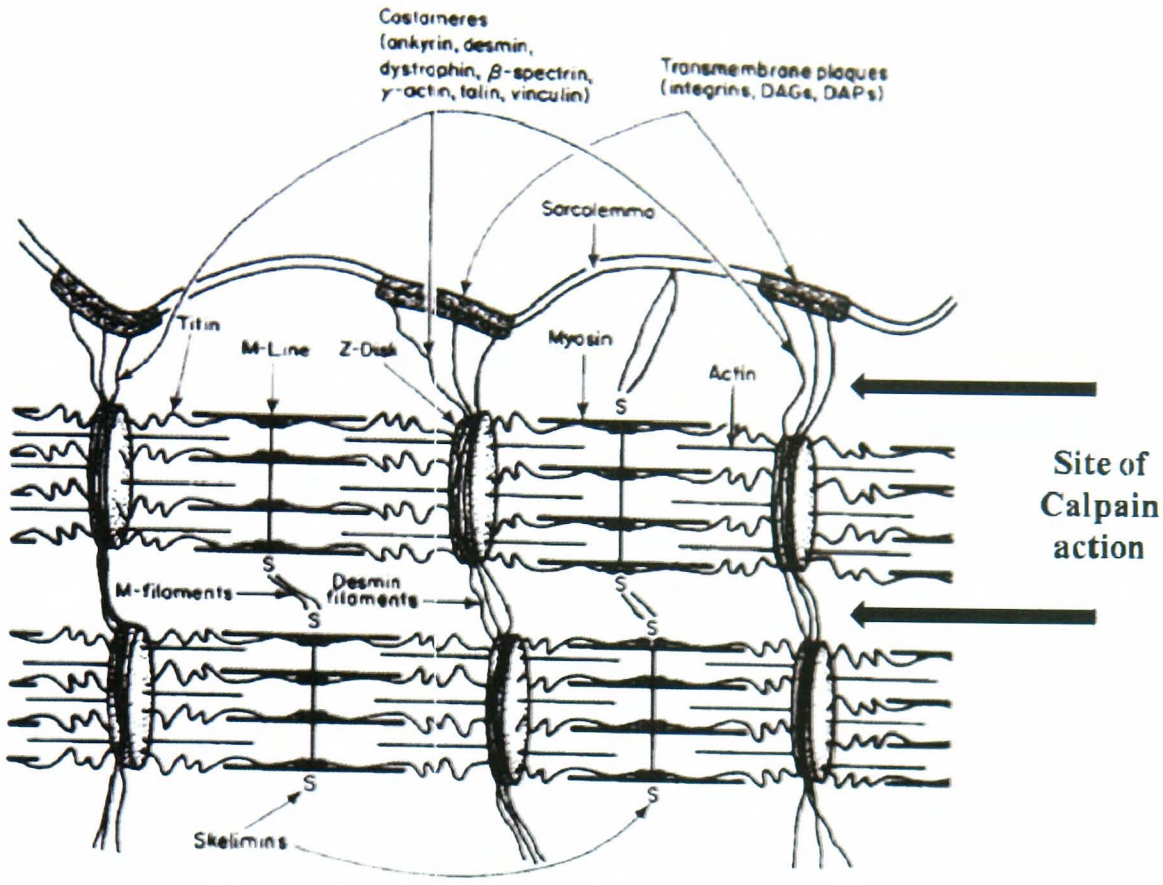


Figure 1.6-2. Schematic diagram of the proteins of the costamere which are responsible for anchoring the myofibrillar structures to the cell membrane. Further,three of these proteins, ankyrin, desmin, and vinculin, extend into the structure to anchor repeating myofibrils laterally at the Z disc. Image from Taylor *et al.* 1995.

Table 1.6-2. Changes in activity of m- and μ- calpain expressed as % values in control and over-expressed calpastatin transgenic mice, adapted from Kent *et al*, 2004.

		Percent Remaining Activity	
Enzyme	**Days P.M.**	**Control Mice**	**Transgenic CAST mice**
m-calpain	**1**	**94**	**96**
	3	**99**	**98**
	7	**93**	**91**
	sem	2.8	2.2
μ-calpain	**1**	**81**[b]	**97**[a]
	3	**29**[d]	**94**[a]
	7	**13**[e]	**67**[c]
	sem	4.4	3.5

Other evidence that further supports the role of calpain in meat tenderisation are the observations that treatment of animals with β-adrenergic agonists (BAA) results in an increase in muscle growth as a result of changes in the activity of the calpain system but with detrimental effects on meat quality (Koohmaraie *et al.*, 1991; Wheeler & Koohmaraie, 1992). These agents increase protein

synthesis and decrease protein degradation (Bardsley *et al.*, 1992) their predominant effect on the calpain system being an increase in mRNA of calpastatin (Parr *et al.*, 2001). The Callipyge breed is an example of an inherited trait for increased muscle growth (Rehfeldt *et al.*, 2004), the effect of which is a specific increase in the size of muscles associated with the hindlimbs, the muscles of the forelimbs showing little increase in muscle mass. Callipyge lambs yield more meat as a result of this increased hypertrophy, however, their meat is more tough compared to non-callipyge lambs (Ducket *et al.*, 2000). Although their increase in muscle mass is due to increased protein synthesis combined with decreased protein degradation, the predominant effect on proteolysis is an increase in calpastatin levels which adversely affects the tenderisation process through inhibition of calpain activity causing the characteristic toughness of the callipyge meat. Overall the BAAs and the callipyge genotype indicate further the calpain system's the role in increasing muscle growth and yield of meat, with unwanted subsequent adverse effects on meat tenderisation. If the meat industry wishes to improve muscle growth using such agents, then it must find ways to improve the post mortem tenderisation effects that are inhibited by over active calpastatin and reduced activity of calpain I.

1.6.6 Improvement of meat quality by calcium Infusion

There have been multiple studies investigating the effect of increasing calcium levels in carcasses post mortem by infusion of calcium solutions with the objective of increasing calpain activity and thereby improving meat quality. An experiment carried out by Koohmaraie *et al.* (1988) in lamb carcasses infused a 0.3M calcium chloride ($CaCl_2$) solution at 10% (w/w) of the liveweight into carcasses circulation system via the carotid artery. This provided a 40-fold increase in calcium levels in the *Longissimus Dorsi* (LD) of the infused carcasses and significantly reduced shear force in the calcium infused carcasses after 24 hours post-mortem (LD muscle chops shear force was 3.56Kg compared to 6.32kg in controls, $P<0.05$). This experiment did not evaluate directly the activity of the calpain system nor the effects of calcium infusion on eating quality at consumer level. In a second calcium chloride infusion study the activity of both m- and μ-calpain activities dropped significantly, with μ-calpain activity dropping to immeasurable levels in the 0.3M calcium chloride infusion (Koohmaraie *et al.,* 1989). The increased μ-calpain activity caused increased autolysis resulting in loss of activity early in the initial 24 hour post mortem period (Koohmaraie *et al.,* 1989). Koohmaraie & Shackelford (1991) then went on to investigate whether calcium chloride infusion of carcasses post mortem could overcome the reduced tenderisation that is seen in BAA treated animals. They found that calcium chloride ($CaCl_2$) was able to significantly reduce the shear force of BAA carcasses (which had a much higher shear force in comparison to control animals) when compared to BAA carcasses without $CaCl_2$ infusion, causing shear force to be reduced to levels usually observed in 14

day aged carcasses of animals fed a control diet, data shown in table 1.6-3. Koohmaraie & Shackelford (1991) attributed this much higher rate of tenderisation over 24 hours to increased activity of the calpain system, in particular μ-calpain, and demonstrated that this method could be applied to overcome known causes of tough meat.

Table 1.6-3. Efficacy of calcium chloride ($CaCl_2$) infusion in improving shear force in lambs fed β-adrenergic agonists (BAA). Adapted from Koohmaraie & Shackelford (1991).

		Shear force (kg)		
Diet Treatment	**$CaCl_2$ Infusion**	**day 1**	**day 7**	**day 14**
Control	**-**	**10.0**	**7.7**	**6.9**
Control	**+**	**3.0**	**3.1**	**2.7**
BAA	**-**	**11.4**	**9.6**	**9.1**
BAA	**+**	**6.0**	**5.2**	**5.2**
	pooled sem	0.7	0.7	0.9
Significance				
Diet Treatment		*	*	*
$CaCl_2$ Infusion		*	*	*
Interaction		N.S.	N.S.	N.S.

*** refers to significant effect (P<0.05)**

In 1993, Whipple & Koohmaraie published data showing that meat tenderness could be improved by 40% when beef steaks were marinated in a calcium containing solution. The steaks were marinated in a 150mM calcium solution for 24 and 48 hours, both of which gave significant decreases in toughness, although 48 hours gave optimal results. The activities of the calpain system were measured and it was found that the activity of both calpastatin and m-calpain were decreased. In their study Polidori *et al.* (2000) slaughtered meat-age wether lambs and infused the carcasses with 0.3M $CaCl_2$ at 10% ratio of the liveweight via the carotid artery immediately post mortem, and found the calcium level in the muscle tissue was increased 60-fold by $CaCl_2$ infusion, from 4.45μg/g tissue to 265.33μg/g. The shear force of treated chops was reduced although not as dramatically as was previously reported by previous studies, with control chops displaying shear force values of 5.99kg at 6 days post mortem in comparison to treated chops of 5.22kg. At 24 hours post mortem the treatment significantly reduced m-calpain activity ($P<0.01$) whilst the activity of μ-calpain had dropped to an undetectable level, suggesting its activity was exhausted through autolysis. Calpastatin activity was also significantly reduced in treated chops ($P<0.05$). This drop in activity was in agreement with Koohmaraie *et al.* (1989), where accelerated post mortem proteolysis had led to accelerated autolysis of the calpain enzymes and subsequent loss of activity.

The evidence presented suggests that meat can be treated with $CaCl_2$ solutions in order to produce a guaranteed tender steak, however, it was noted by Hoover *et al.* (1995) that there was resistance from the meat industry as consumers would not accept meat that had been injected with a

substance. In their study in 1995, the taste panel was subjected to restaurant conditions to assess the impact of calcium chloride on eating quality. Their study used the lower dose of 0.2M $CaCl_2$ solution at a 5% by weight injection rate, and found improvement in perceived tenderness and flavour of the treated steaks, although the evaluation was not performed by a trained taste panel.

1.6.7 Summary of the calpains and meat quality

The calpains and their endogenous inhibitor, calpastatin, have been shown to play a major role in the proteolytic events in the aging process of meat and are important factors in the development of tender meat that is acceptable to consumers. The use of growth promoters such as β-adrenergic agonists can increase growth of muscle partly by increasing calpastatin activity which reduces calpain-mediated protein degradation, which improves growth of muscle but inhibits tenderisation. As the calpain enzymes are calcium-activated, their activity post mortem can be increased by infusing, injecting or marinating carcasses with calcium chloride solutions. However, there is concern from the industry that this physical manipulation of the carcass or butchered meat has a low acceptability to consumers; therefore, if the manipulation of carcass calcium to improve meat quality could be achieved through dietary means, this may be more acceptable. As vitamin D is a dietary component and is activated into a hormone responsible for calcium homeostasis, this may present an opportunity to manipulate this feed component which would subsequently alter calcium levels and increase calpain activity post mortem.

1.7 Vitamin D_3 Supplementation and Meat Quality

There has been a practice in dairy farms to administer vitamin D_3 prior to onset of lactation as a method of increasing calcium levels in the serum in order to reduce the incidence or severity of hypocalcaemia associated with milk fever (Littledike & Goff, 1987). In light of the apparent meat quality benefits of increasing calcium concentration in meat post mortem, Swanek *et al.* (1999), investigated the possibility of increasing calcium status of meat animals pre-slaughter via supplementing feed with vitamin D_3. In the first experiment of this kind, Swanek *et al.* fed vitamin D_3 beef cattle in three experiments. The first experiment investigated the effects of the level of vitamin D_3 supplementation on calcium status of the animals, feeding vitamin D_3 at either 0.0 (control), 2.5, 5.0 and 7.5 $x10^6$ IU/day (n=5 per dose) for 10 days and monitoring calcium levels throughout and for 5 further days. These levels of vitamin D supplementation are noted as supra-nutritional levels as they are up to 1000 times higher than the standard recommended vitamin D intake of meat animals (210 IU/day for lambs and 2400 IU/day for cattle (McDonald *et al.*, 2002), where one IU is around 0.025μg vitamin D (Holick, 1999)). The results of Swanek *et al.*'s study found that serum calcium

levels increased linearly (P<0.04) between days 6 and 13 in Vitamin D_3 groups and found maximum serum calcium levels appeared at day 9 onwards, with increases from 20 - 40% compared to controls, figure 1.7-1.

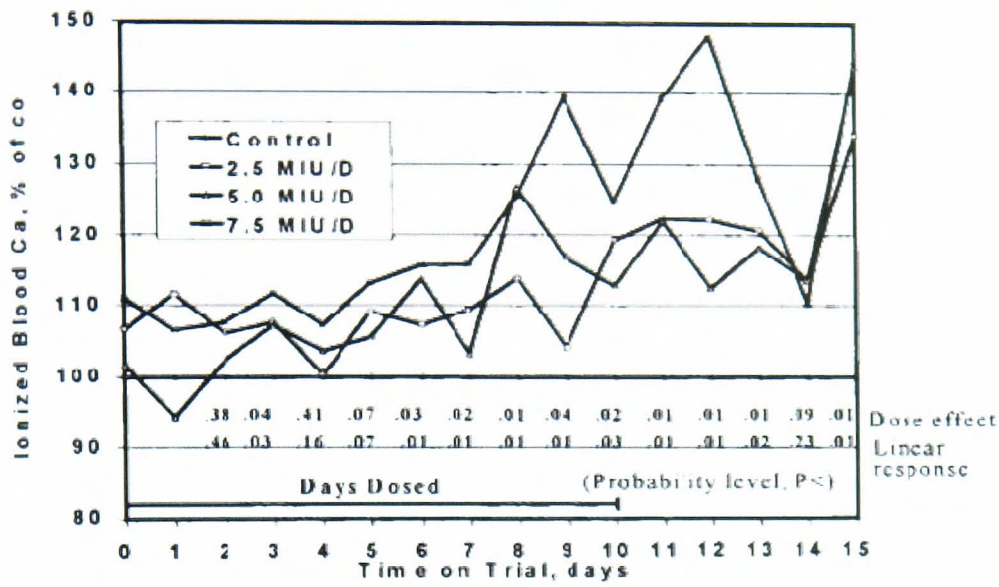


Figure 1.7-1. Changes in serum calcium in beef cattle fed vitamin D_3 prior to slaughter, Swanek *et al.*, 1999. Animals were supplemented with one of three levels of vitamin D; 2.5, 5.0 or 7.5 x10^6 IU/day (MIU/day) for 10 days and serum calcium compared to that of control animals, considered to be baseline. Serum calcium was highest in animals given 7.5 x10^6 IU/day and peaked after 12 days, whereas serum calcium in animals fed the intermediate dose of 5.0 x10^6 IU/day peaked after 8 days.

In their two successive experiments beef cattle were supplemented with the two highest doses of vitamin D_3, 5.0 and 7.5 x10^6 IU/day, for 7 and 10 days respectively. The lower dose of 5.0 x10^6 IU/day was sufficient to significantly increase both serum and in muscle calcium (20% increase, P<0.03 and 30% increase, P<0.05, respectively, table 1.7-2) and found that shear force was significantly reduced after 7 days of aging, compared to control animals, but no differences were seen with longer storage (table 1.7-2). With the higher dose of 7.5 x10^6 IU/day supplemented for 10 days, there was a larger increase in muscle calcium levels (50% increase, P<0.02, table 1.7-1) and this translated into improved shear force at both 7 and 14 days of conditioning (P<0.02 and P<0.07 respectively, table 1.7-2) along with significant decreases of both μ-calpain and calpastatin activity after 24 hours post-mortem (P<0.05 and P<0.04 respectively, table 1.7-2). This study showed that feeding vitamin D_3 to beef animals immediately prior to slaughter was sufficient to alter calcium status of animals and improve meat quality of the steaks, thus producing an alternative to manipulating carcasses with $CaCl_2$ solutions post mortem in the abattoir.

Table 1.7-1. Changes in calcium status in beef cattle fed vitamin D_3 prior to slaughter. Adapted from Swanek *et al.*, 1999.

Swanek *et al*, 1999	Dose Vit D_3 x10^6 IU	n per group	Time (days)	Plasma Ca (mg/dL)	Muscle Ca (µg/g)
Expt 2	0.0	59	7	9.23	13.9
	5.0			12.39*	19.9*
Expt 3	0.0	22	10	N.M.	14.2
	7.5				21.3†
				*P<0.03	* P<0.05
					†P<0.02

Table 1.7-2. Changes in shear force of *longissimus dorsi* steaks from beef cattle fed vitamin D_3 prior to slaughter, Swanek *et al.*, 1999.

Swanek *et al*, 1999	Dose Vit D_3 x10^6 IU	Shear Force (kg) Day 7	Day 14	Day 21	Calpain System Activity/g 24hr PM µ-calpain	m-calpain	calpastatin
Expt 2	0.0	4.70	4.03	3.58			
	5.0	4.12*	3.87	3.60			
Expt 3	0.0	5.13	4.40	4.04	0.56	0.99	4.83
	7.5	4.21†	3.81†	3.44	0.19†	0.62	4.11†
		*P< 0.01	P=0.25	P=0.90			
		†P< 0.02	†P<0.07	P=0.13	†P<0.05	P=0.0729	†P<0.04

Several further studies have been performed in cattle in light of this original experiment, investigating the possibility of improving meat tenderness using shorter vitamin D_3 supplemental regimes (5 and 7.5 x10^6 IU/day for 9 days, Montgomery *et al.*, 2000; 6 x10^6 IU/day for 4 and 6 days, Karges *et al.*, 2001), using additional calcium supplements (Scanga *et al.*, 2001), investigating the supplementation of vitamin D_3 at a variety of doses (0.5, 1.0, 2.5, 5.0 & 7.5 x10^6 IU/day for 9 days, Montgomery *et al.*, 2002), the efficacy of vitamin D_3 supplementation in reducing toughness from growth promoters (Reiling & Johnson, 2003), the use of 25-hydroxy vitamin D_3 in comparison to 1,25-dihydroxyvitamin D_3 (Foote *et al.*, 2004), efficacy of vitamin D_3 in reducing toughness in meat from cull cows (Rider-Sell *et al.*, 2004) and the differential effects of vitamin D_3 in differing cattle breeds (Montgomery *et al* ., 2004).

Not all of these studies produced shear force data or measured calpain activities, and results reported in many of the studies are inconclusive in terms of the effects on these experimental outcomes. The proposed mechanism for the benefits of vitamin D_3 supplementation prior to slaughter is that the calcium status of animals is increased leading to increased activity of the calpain system immediately post mortem, thus tenderizing steaks more quickly and reducing the time required for conditioning and the cost associated with cold storage. To provide conclusive evidence for benefits of vitamin D_3 in this role there must be an evaluation of each step associated with the mechanism; these are calcium status, calpain activity and the shear force of the chops.

At the time this study commenced, the majority of work performed into this mechanism was in cattle and only two studies had been carried out in sheep and another two in pigs. In 2002 Wiegand *et al.* investigated levels of 0.25 and 0.5 $x10^6$ IU/day doses of vitamin D_3 for their effects on serum calcium in pigs and found significant increases with the higher dose which gave a higher calcium rise more quickly. This higher dose of 0.5 $x10^6$ IU/day gave a 60% increase by day 4 compared with 0.25 $x10^6$ IU/day arising in a 40% increase by day 6. Following this initial experiment the group investigated effects on calcium status and LD tenderness of a short dose of 0.5 $x10^6$ IU/day for 3 days immediately prior to slaughter. They found serum calcium was increased by 20% in treated animals compared to controls ($P<0.01$) but there were no differences in shear force values. A second study performed in pigs (Wilborn *et al.*, 2004) used lower doses of vitamin D_3 of 0.04 and 0.08 $x10^6$ IU/day but fed for much longer than has previously been investigated, supplementing for 44-51 days prior to slaughter. This study did record changes in calcium levels in the blood after 7 weeks of supplementation (29% increase compared to controls, $P<0.05$) but there were no changes in calcium concentration of the LD muscle, nor effects on tenderness of the chops.

In 2001, Wiegand *et al.* performed a study investigating the possibility of vitamin D_3 supplementation in reducing the toughness of steaks from callipyge lambs, as noted earlier the callipyge breed exhibits hind limb hypertrophy and produces tough meat (Duckett *et al.*, 2000). The study performed a preliminary experiment using two doses of vitamin D_3, administering 1.0 and 2.0 $x10^6$ IU/day for 4 days to determine the optimum dose for calcium status changes. Their second experiment administered the higher dose of 2.0 $x10^6$ IU/day for 7 days prior to slaughter and recorded the changes in calcium status and shear force of LD chops. In the normal breed genotype of sheep, serum calcium increased 8.8% in the vitamin D group compared to controls ($P<0.05$, table 1.7-3), and in the callipyge genotype, a 6.1% increase was seen in vitamin D fed animals ($P<0.05$).

Table 1.7-3. Changes in calcium status in lambs of either callipyge or normal genotype fed vitamin D_3 for 7 days prior to slaughter; Wiegand *et al.*, 2001.

Wiegand *et al* 2001	Dose Vit D_3 $x10^6$ IU	Time (days)	n per group	Breed	Plasma Ca (mg/dL)	Muscle Ca (9mg/dL)
Expt 2	0	7	8	Normal	9.44	5.38
				Callipyge	9.29	4.24
	2			Normal	10.27*	7.15
				Callipyge	9.89*	4.14

* $P<0.05$

However, these changes in the calcium levels were not transferred to significant changes in the muscle calcium levels, nor was there any effect on shear force of LD chops. Callipyge lambs fed the control diet did present chops with higher shear force values than the normal genotype lambs

(P<0.05) and vitamin D_3 supplementation was not able to overcome this. Boleman *et al.* 2004 performed another study in sheep, investigating a number of doses of vitamin D_3 from 0.25 to 0.75 $x10^6$ IU/day for 4 days to assess the changes in serum calcium before administering the highest dose of 0.75 $x10^6$ IU/day for 14 days prior to slaughter. There was no effect of this vitamin D_3 supplementation regime on plasma calcium levels. Boleman *et al.* measured the shear force changes in four muscles; *longissimus dorsi* (LD), *biceps femoris* (BF), *semitendinosus* and *semimembranosus*, across an aging period from 5 to 15 days; however there was no effect of vitamin D_3 on the shear force of chops prepared from these muscles, with the exception of the LD at 15 days conditioning which was significantly higher (P<0.05) and the BF which was significantly lower at day 5. Neither of these studies performed in sheep were able to give conclusive evidence to support the hypothesis that pre-slaughter vitamin D_3 feeding regimes improve meat tenderness.

1.7.1 Summary of vitamin D_3 Sheep trials

Neither of the investigations by Wiegand *et al.* (2001) and Boleman *et al.* (2004) measured the effects of dietary vitamin D regimes on the activities of the calpain system in lamb and thus these studies do not provide clear answers to the vitamin D_3 and meat quality question. The changes in calcium status in cattle studies are considerable with serum calcium increases around 20 – 50% and did report positive changes in shear force of chops which were associated with effects on the calpain system (Swanek *et al.,* 1999). The work in sheep has failed to report such clear evidence, while Wiegand *et al.* reported a 14.9% increase in serum calcium when sheep were fed a high dose for 5 days, there was no clear effect on the shear force of the LD and no direct investigation into subsequent calpain system activity. Boleman *et al.* (2004) did not find any significant changes in circulating calcium levels and there was no effect on shear force and no investigation into the calpain system activity. It would be expected that the same responses observed in cattle could be replicated in sheep. In addition to ascertaining the influence of incorporating this agent in feed on meat quality, there is a need to investigate all the components of the hypothesised system, measuring calcium levels of the serum and muscle, shear force of the LD as a standard muscle to compare between investigations and investigate effects on the calpain enzymes which can be related to the shear force of the muscle.

1.8 Summary of Literature

The literature described in this chapter provides an insight into the available evidence indicating the potential of vitamin D to enhance meat quality by a variety of mechanisms. The recent work supplementing vitamin D to meat age animals in a short but high dose immediately prior to slaughter appears to provide a simple mechanism to promote tenderisation of meat post mortem. The model proposed in these studies is based on vitamin D's classical role in calcium homeostasis and the evidence of work performed in $CaCl_2$ marination studies. It is likely that such treatments will greatly influence the development of tenderness post mortem and will reduce the length of cold storage required to reach acceptable levels of tenderness through feed manipulation alone. The studies investigating these effects in cattle appear to provide evidence to support the functionality of the proposed model, however, the work in sheep is not as conclusive and there is a requirement for research to investigate optimum duration and level of vitamin D supplementation, and to confirm the mechanism affects both shear force and calpain system activity.

A second potential role for vitamin D in muscle tissue was implicated by the results of Endo *et al.*'s study, providing evidence for vitamin D to control the growth and development of muscle fibres from the earliest phase of myoblast development. The results of their study show that the absence of VDR results in smaller muscle fibres and abnormal MRF expression, which would be of relevance to animal production where muscle growth is a primary aim. In addition their study reported that treatment of muscle cells with active vitamin D, $1,25(OH)_2D_3$, could downregulate expression of some genes associated with muscle cell development and therefore further research into this mechanism could provide a novel role for vitamin D as an agent to promote muscle growth in animal production systems.

The potential for vitamin D to affect muscle growth has two-fold relevance, the initial gain to the industry being increased muscle growth and yield, but secondly the effects of the hormone of fibre type could also improve the quality of the meat. It is well known that the use of BAAs in meat animals significantly increases lean growth and therefore yield of muscle tissue, but with detrimental affects on the tenderness of the meat, as demonstrated in Koohmaraie & Shackelford's experiment (1991). This increase of growth observed in BAA treatments is associated with increases in the size and number of fast glycolytic fibres (Vestergard *et al.*, 1994) and it is likely that these fibres are responsible for the increased toughness of the meat. However, investigations of vitamin D supplementation in human patients reports that vitamin D can increase the size and number of fast oxidative fibres and reduce the number of fast glycolytic fibres simultaneously (Sorenson *et al.*,

1979; Sato *et al.*, 2005). Taken together these studies provide evidence to propose the use of vitamin D as an agent to promote muscle growth without the detriment to meat quality which is associated with BAA treatment.

Therefore it is the aim of the experiments in this thesis to investigate the use of vitamin D and active metabolites in their capacity to promote muscle growth and to promote the tenderisation of meat in the aging period. The results of these experiments will be relevant to the meat industry in terms of optimising production methods to produce a greater yield of meat which is likely to be tender, and could additionally provide benefit to elderly human patients at risk of falling through loss of fast fibres associated with vitamin D deficiency.

1.9 Hypothesis

The hypotheses of the work performed in this study are as follows:

- Supplementation of vitamin D to meat age lambs in the final week immediately prior to slaughter will promote tenderisation of meat via activation of the calpain system
- Administration of active vitamin D metabolites to growing muscle cells will promote muscle growth through effects in gene expression and subsequent translated protein, in particular those of the MRFs which control early muscle cell growth and development.
- Administration of active vitamin D metabolites to growing muscle cells will promote fibre type changes via mechanisms controlling MHC gene expression.

1.10 Aims

The aims of this thesis are as follows:

- To clarify and optimise the mechanism through which vitamin D supplementation in the final week immediately prior to slaughter will promote tenderisation of lamb meat by assessing the three major components of this system:
    - Increases in calcium status of the serum and LD
    - Increases in expression/activity of the calpain system in the LD
    - A reduction in shear force of the LD chops
- To assess targets of vitamin D in muscle which are of relevance to muscle growth and fibre type changes in rodent muscle cells, incorporating two experiments:
    - The first experiment will screen for novel gene targets of $1,25(OH)_2D_3$ in rat muscle cells via microarray analysis.
    - The second experiment, in a mouse derived muscle cell line, will assess the efficacy of an active vitamin D analogue, $1\alpha(OH)D_3$, to promote gene expression changes of:
        - Novel targets identified by the microarray screen
        - Expression of MRFs and effects on muscle cell growth and development
        - Expression of MHC genes and effects on muscle fibre type.

Materials and Methods

2.1 Materials used in this study

2.1.1 Equipment used at the University of Nottingham

- Bench top centrifuge; (volumes below 2ml) Microfuge 22R Centrifuge, Beckman Coulter
- Bench top centrifuge; (volumes over 2 ml and cell culture), Centaur 2, MSE
- Bench Top Shaker; Stuart See-Saw Rocker SSL4, Barloworld Scientific Ltd (Staffs UK)
- Polytron PT300 homogeniser, Kinematica (Litteu, Switzerland)
- Basic PCR machine; DNA Engine- Mastercycler, M.J. Research (MA, USA)
- Quantitative real time PCR machine; LightCycler® 480, Roche Diagnostics (Switzerland)
- Q-PCR Software, LightCycler® 480 SW V 1.5, Roche Diagnostics GmbH (Germany)
- SubGell GT tanks for agarose gel electrophoresis, BioRad (Hemel Hempstead, UK)
- Dri-Block Heat Block, Techne (Cambridge UK)
- Microplate Reader model 680XR, BioRad, Japan
- UV/Visible Spectrophotometer, Amersham Biosciences Ultrospec 2100pro, Biochrom Ltd (Cambridge, UK)
- SDS-PAGE 200mm x 100mm vertical dual plate unit, Fischer Scientific (Loughborough, UK)
- Trans-Blot with plate electrodes, BioRad, (Hemel Hempstead, UK)
- Texture Analyser Software, Texture Expert Exceed
- Spectrometer ND-1000 Nanodrop Products, Thermo Fischer Scientific (Wilmington, USA)
- DNA gel imager: Gel Doc 2000, BioRad Laboratories (CA, USA)
- Protein gel imager: Flour-S MAX2, BioRad Laboratories (CA, USA)
- Software to Analyse DNA and protein gels: Quantity One V4.1.1., BioRad Laboratories (CA, USA)

Laboratory reagents for work performed in North Laboratory, Sutton Bonington Campus were purchased from Fischer Scientific (Loughborough, UK), Sigma Aldrich (Poole, UK) and Promega (Southampton, UK). Water used in buffers and solutions was either glass distilled or column purified using MilliQ Elix®Purification system (Millipore, Watford, UK). Water for use in nucleic acid work was certified RNAse/DNAse free, purchased from Sigma Aldrich.

2.1.2 Equipment used in Pfizer Ltd. (Sandwich, Kent, UK)

- Bench top centrifuge; volumes below 2 ml; Centrifuge 5415R, Eppendorf
- Bench top centrifuge; cell culture, Centaur 2, MSE.
- Agarose Gel Tanks; Horizon 11.14 (Large) and Horizon 58 (Small) Horizontal Gel Electrophoresis Apparatus, Life technologies, GIBCO BRL
- Agarose Gel Imager; Universal Hood II, BioRad Laboratories, (Milan, Italy).
- Agarose Gel Imagine Software; Quantity One V4.6, BioRad Laboratories (CA, USA)
- Basic PCR machine; T3 Thermocycler, Biometra
- Quantitative real time PCR machine; 7900HT Fast Real-Time PCR System, Applied Biosystems (CA, USA)
- Quantitative real time PCR software; Sequence Detection Systems V 2.2.2.; 2004 Applied Biosystems (Foster City, CA, USA)
- Spectrometer ND-1000 Nanodrop Products, Thermo Fischer Scientific (Wilmington USA)
- Agilent 2100 Bioanalyzer, Agilent Technologies

Laboratory reagents for work performed in Pfizer, Sandwich, were purchased from Fischer Scientific (Loughborough, UK) and Sigma Aldrich (Poole, UK) unless otherwise stated. Water used in buffers and solutions was either glass distilled or column purified. Water for use in nucleic acid work was certified RNAse/DNAse free, purchased from Sigma Aldrich.

2.2 Experimental Design of Animal Trials

2.2.1 Animal housing layout

All animals were male Mule X Charolais lambs approximately 7 months old and were individually housed for the duration of the trials. Animals were weighed weekly to assess weight gain during the housing period and were arranged into 3 slaughter groups according to weight approximately 3 weeks prior to the supplementation week, shown in figure 2.2.1. Within each slaughter group, animals were paired with the animal housed in the pen behind, creating treatment-control pairs matched for age and weight, and during the supplementation period treatment diets were allocated to animals distributed equally within each slaughter group, demonstrated in figure 2.2.2. On the day of slaughter, animals were taken to the lairage in groups of four consisting of two treatment pairs, and were slaughtered in pair order beginning with vitamin D treated animals. The order of removal was sequential, for example in trial 1 slaughter began with the pair 373 and 208, where 373 was treated with vitamin D and therefore slaughtered first, followed by its control pair 208, shown in figure 2.2.2-A.

<u>Pen layout in the Metabolism 3 building</u>

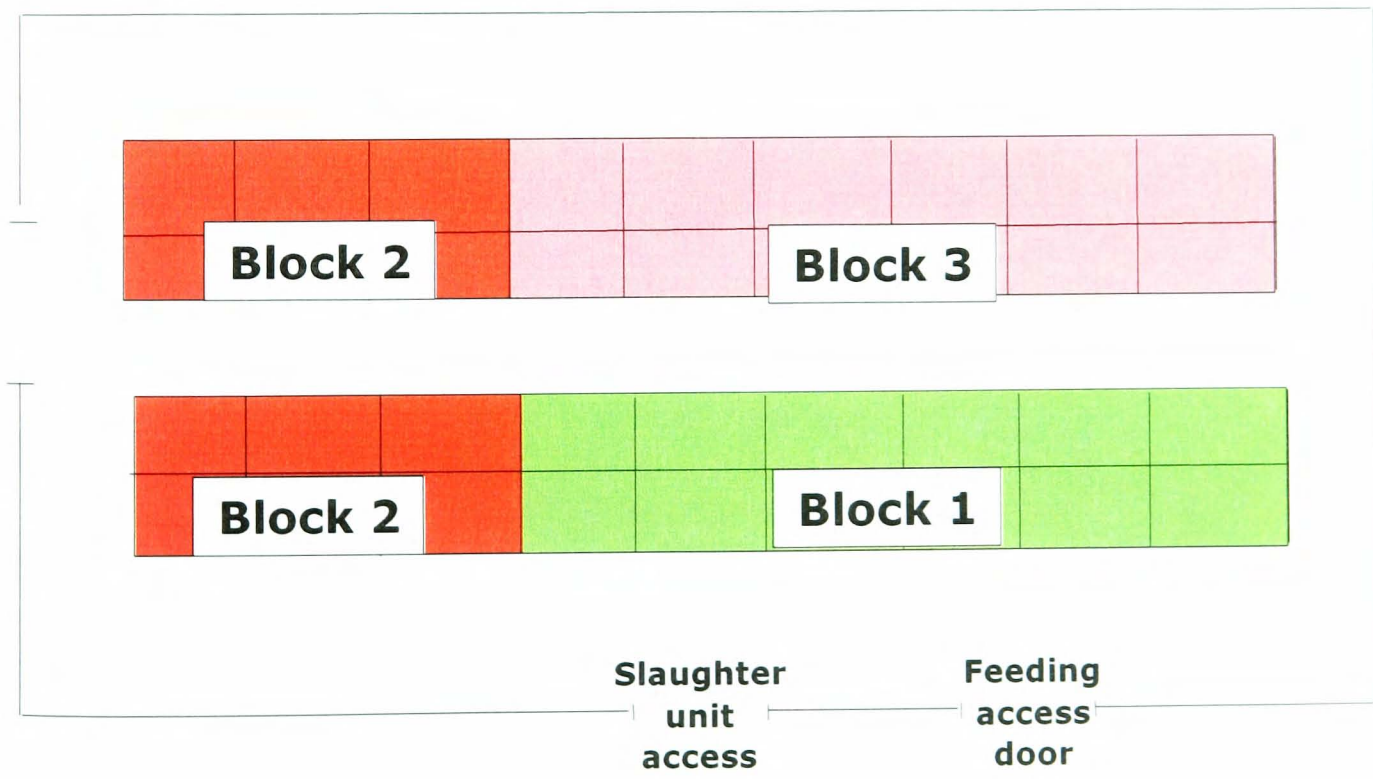


Figure 2.2-1. Pen Layout in the sheep housing. Each block possessed equal numbers of treated (n=6) and control (n=6) animals and animals were paired with the animal in the pen behind. Block 1 contained heaviest animals (n=12) randomly assigned to treatment group; Block 2 contained middle-weight animals (n=12) and Block 3 contained lightest animals (n=12).

A Allocation of treatment group

Group 3

263 D	169 C	152 D	462 C	276 D	182 D	258 C	171 D	380 C
310 C	332 D	377 C	464 D	475 C	421 C	474 D	168 C	386 D

Group 2

361 C	254 D	148 C	466 C	297 D	178 D	209 C	467 D	208 C
364 D	251 C	181 D	330 D	293 C	123 C	333 D	219 C	373 D

Group 1

DOOR

B Allocation of treatment group

Group 3

232 D	370 C	215 D	490 C	339 D	212 D	488 C	213 D	409 C
137 C	144 D	274 C	108 D	338 C	220 C	522 D	515 C	309 D

Group 2

101 C	228 D	180 C	378 C	455 D	281 D	234 C	308 D	246 C
141 D	450 C	296 D	145 D	478 C	535 C	158 D	518 C	511 D

Group 1

DOOR

Figure 2.2-2. A; Housing layout and diet treatment allocation to Trial 1 animals; B; Housing layout and diet treatment allocation to Trial 2 animals.

2.2.2 Diet Composition

Basal diet composition as fed to animals during housing prior to trial diets and fed as control diet to control group during trial periods is shown in table 2.2-1. The diet has no added vitamins or minerals. Vitamin D diet was based on this mix with vitamin D3-500 (Rovimix, DSM, Derbyshire UK) added prior to pelleting the diet.

Table 2.2-1. Basal diet composition as-fed basis.

Veg Oil	5 kg
Barley	240 kg
Oats	80 kg
Soya	25 kg
Grass Nuts	100 kg
Nutramol 30 (RUMENCO)	50 kg
Total	***500kg***

2.2.2.1 Vitamin D_3 diet formulation

The supplement D3-500 contains 500,000 IU per g, therefore to supply $2x10^6$ IU/day vitamin D, animals required 4g D3-500/day, given to them in 1.4Kg feed, hence feed supplemented with D3 500 required 2.86g D3-500 per Kg. For the second trial, basal calcium was calculated as 0.7% calcium by Frank Wright Ltd and calcium propionate was added to double the calcium in the vitamin D_3 + calcium diet. Basal diet supplied 0.7/100x 1Kg = 7g calcium/Kg. To provide an additional 7g calcium per Kg diet, calcium propionate (21% calcium) was added at (21/100*X = 7; X = (7x100)/21) = 33.3g calcium propionate per Kg diet. Final calcium concentrations were confirmed by AA spectrometry by Dave Bozon (Nutritional Sciences). Nutritional composition for each diet fed in trial 1 is summarized in table 2.2.2 and for trial 2 in table 2.2.3.

Table 2.2-2. Nutritional composition of diets fed during trial period in Trial 1.

Trial 1 Diets	Basal Diet/Control	Vitamin D
Energy (MJ/Kg)	16.41	16.46
Nitrogen (%N)	2.21	2.15
Calcium (mg/g)	15.88	15.82

Table 2.2-3. Nutritional composition of diets fed during trial period in Trial 2.

Trial 2 Diets	Basal Diet/Control	Vitamin D only	Vitamin D + Ca
Energy (MJ/Kg)	16.46	16.79	16.69
Nitrogen (%N)	1.87	1.78	1.58
Calcium (mg/g)	15.09	11.71	34.56

2.2.3 Standard slaughter procedure

All animals were slaughtered by electrical stunning to the head, followed by exsanguination, performed by a licensed slaughterman in the abattoir at Sutton Bonington Campus, University of Nottingham, under conditions designed to replicate those in a commercial unit. Procedures were performed as schedule 1 according to DEFRA guidelines and all animal experiments at University of Nottingham were first drafted then sent to Home Office inspector for approval to ensure they fell outside the Animals (Scientific Procedures) Act 1986 and therefore were not under UK Home Office Animal Licence.

2.2.4 Carcass handling

Immediately after death, carcasses were dressed and defleeced but not split, and their weight recorded, the time to prepare each carcass from stunning to final dressing and weighing was consistently 15 minutes. The pH was taken using a puncture electrode (Ingold, Mettler-Toldeo Ltd. Leicester, UK) from the left side of the carcass at 45 minutes post mortem and 24 hours after. Carcasses were placed in a chiller at 4°C four hours after slaughter and dressing to reduce the potential for cold shortening, and then hung overnight by Achilles tendon until sampling at 24 hours. At 24 hours post mortem the temperature and pH of the carcasses were measured at the same sample site again before the whole LD was removed for aging subsequent to Warner-Bratzler shear force (WBSF) analysis, see appendix A for diagram of snap LD, pH and temperature sample sites.

2.2.5 Sample Procedure

2.2.5.1 Blood Samples

Blood samples were collected at exsanguination, whole blood and plasma samples were collected in 15ml tubes containing ethylenediamine tetraacetic acid (EDTA) to prevent clotting; whole blood was stored at -20°C at the end of each slaughter day. Plasma was prepared at the end of the slaughter day by spinning tubes at 3000 rpm for 15 minutes at 4°C in a Beckman centrifuge. The straw coloured supernatant was removed into eppendorf tubes before storage at -20°C. Serum samples were prepared in 50ml tubes which were left to clot overnight in a chiller at 4°C. The following morning samples were spun at 3000 rpm for 15 minutes and the supernatant stored in eppendorf tubes as before.

2.2.5.2 Snap Tissue Samples

Snap frozen samples of live tissue were prepared by submersion in liquid nitrogen as quickly as possible after slaughter. The time of sampling was consistent for each animal and was within 2 minutes of stunning and exsanguination. Snap samples were taken in the following order; *Longissimus Dorsi* (LD) taken from both the left and right sides through the fleece immediately following stunning and exsanguination, between the 10^{th} and 11^{th} rib and *Semitendinosus* (ST) was sampled from the left leg (sample sites shown in Appendix A). Approximately 20g of the kidney tissues was then sampled, taken consistently from same side and included both cortices, as the carcass was dressed according to standard slaughter procedure. Finally the *Psoas* (PS) was sampled from the left side of the carcass and, once frozen, all samples were stored at -80°C. The carcasses were hung overnight in the chiller before snap samples were taken of the LD muscles, both left and right, caudal to the initial sample point, 24 hours after slaughter and again stored at -80°C. Snap samples were stored at -80°C until protein and total RNA extraction, approximately 6 months later.

2.2.5.3 LD Chop Samples for Shear Force Analysis

The remaining part of both sides of the *Longissimus Dorsi* was dissected 24 hours post mortem and prepared into chops 2.54cm thick before vacuum packing in pairs, 2 chops per sample point, left and right side chops packed separately. The chops were taken towards the caudal region of the muscle, beyond the sample site of the initial snap sample taken at around the 10^{th}-11^{th} rib. The chops were labelled according to their distance from the original snap site and numbered from 1 to 4 (1 being taken from the 11^{th} rib region down to 4 denoting from the dorsal region), see appendix A for diagram of LD chop preparation, numbering of chops and latin square arrangement for ageing period. These chops were then aged in a chiller at 4°C for up to 21 days before blast freezing at -80°C.

2.3 Analysis of Plasma Vitamin D Concentration

2.3.1 Trial 1 Plasma Samples

Plasma samples stored in eppendorfs were sent to Professor Bill Fraser of the Department of Clinical Biochemistry, University of Liverpool, for vitamin D analysis by Tandem Mass Spectrometry.

2.3.2 Trial 2 Plasma Samples

Plasma samples stored in eppendorfs were sent to Stuart Jones of the Nottingham University Hospitals NHS trust, city campus, Nottingham for vitamin D analysis by HPLC.

2.4 Fat Analysis of Animal Muscle

Fat content of LD was performed using the Soxtherm fat extraction method. Analysis of fat content on samples by the Soxtherm method is an industry standard procedure and is routinely used to measure fat content of meat and other food stuffs (Pérez-Palacios *et al.*, 2008).

2.4.1 Preparation of LD samples for fat analysis

Crushed LD samples to be used for analysis of fat content were first freeze dried to remove all moisture. Samples were weighed and placed in 50ml falcon tubes sealed with perforated parafilm and placed in freeze drying equipment (Freeze drying System, Edwards, UK). Samples were weighed every 24 hours until their weights became constant and were then deemed to have had all water removed.

2.4.2 Extraction of fat using Soxtherm method

Around 1.5g crushed and freeze dried LD muscle was weighed into a crucible, allowing two replicate crucibles per sample. The weight of the crucible plus freeze dried muscle tissue was accurately recorded before fat was extracted in petroleum ether using Soxtherm Fat Extraction Equipment (Gerhardt). The sample was heated with petroleum ether at 150°C for 35 minutes to encourage release of the fat component from the sample. The solvent and fat were then removed together into another thimble, where the fat extract was recovered from the solvent by drying in an oven at 103°C for one hour. The final weight of the extracted fat was then recorded and the fat content of the sample was calculated using the following equation:

Soxhlet Fat % = [Weight of Extract (g) / Sample Weight (g)] x100%

The average of the two replicates for each sample was calculated and the fat content expressed as a percentage value.

2.5 Calcium Ion Concentration Analysis: Atomic Absorption Spectroscopy

2.5.1 Principle of Atomic Absorption

Atoms consist of a nucleus comprised of protons and neutrons, orbited by electrons in a number of "shells" or "energy levels". The number of electrons orbiting the nucleus depends on the element and its chemical state. Atoms usually exist in a balanced energy state where the number of negative electrons orbiting the nucleus is equal to the number of positive protons on the nucleus, and the configuration of the electrons allows for stable orbits. The atom's stable energy state can be disturbed by the addition of energy from outside the atom, which can alter the configuration of the electrons. Atomic absorption occurs when light energy applied to the atom is absorbed and causes electrons to gain enough energy to "jump" from a lower energy level to another. This means the atom is in a higher energy state, known as the "excited state". The amount of light energy required for this to occur is unique to each element, and thus each element is known to absorb light of a specific wavelength (proportional to the energy of the light wave). Calcium absorbs light at wavelength 422.7 nm; hence by using a spectrometer to measure the amount of light absorbed at this wavelength the calcium ion concentration can be calculated.

This is the basis for AA spectroscopy; by measuring the amount of light absorbed at a specific wavelength, the quantity of an individual element in a sample can be calculated. The more of an element present in the sample, the more light energy is absorbed.

2.5.2 Preparing Calcium Standards

To calibrate the system, a set of known quantity calcium solutions were made. Calcium chloride was dissolved in deionised water at concentrations of 2mg/l, 4mg/l, 6mg/l, 8mg/l and 10mg/l. Measurements were made, example absorbance recordings for the standards are shown in table 2.5.1., and a calibration curve was obtained. The curve is linear across this range and samples were diluted to appear in the middle of the curve, shown in figure 2.5-1.

Table 2.5-1. Absorbance readings obtained for calcium standard solutions. Standard deviation (S.D.) and standard error of mean (S.E.M.) are shown.

Standard Concentration (mg/l)	Atomic Absorbance			Mean	S.D.	S.E.M.
2	2.210	2.196	2.197	**2.201**	**0.0078**	0.0045
4	4.023	4.042	4.005	**4.023**	**0.0185**	0.0107
6	6.101	6.158	6.093	**6.117**	**0.0354**	0.0205
8	8.347	8.234	8.324	**8.302**	**0.0597**	0.0345
10	10.455	10.457	10.393	**10.435**	**0.0364**	0.0210

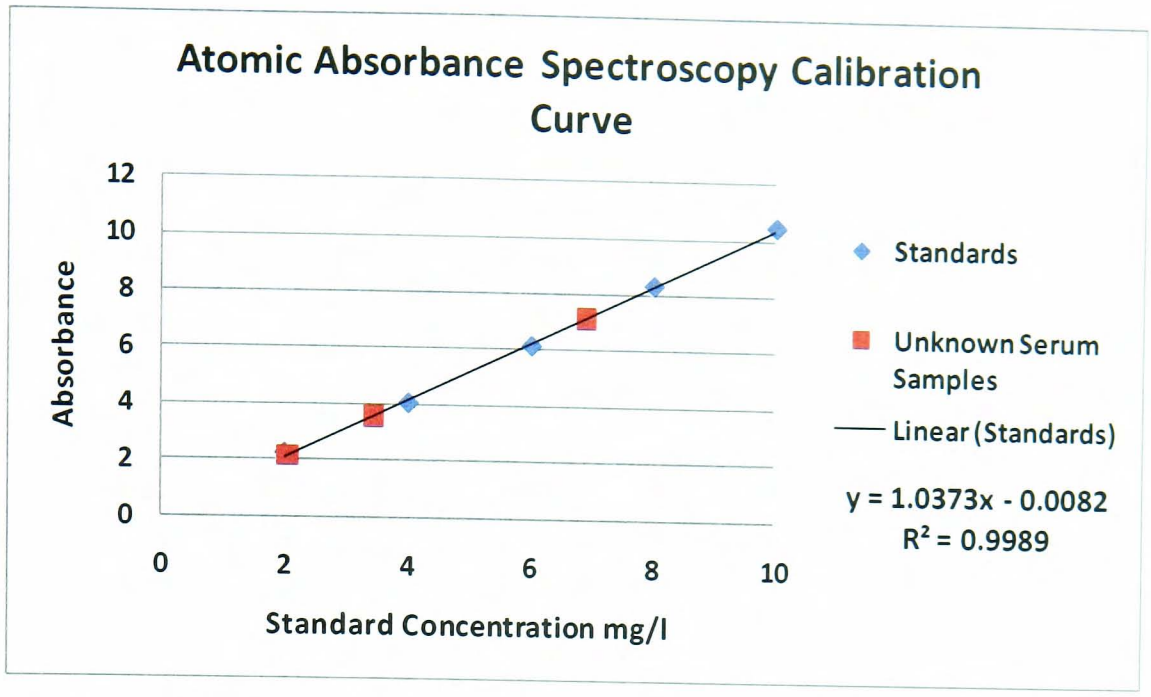


Figure 2.5-1. Calibration curve obtained with calcium standard solutions, showing position of example unknown serum samples on the curve.

2.5.3 Preparing Samples

2.5.3.1 Serum samples

Frozen serum samples were defrosted at room temperature for 45mins and mixed with a vortex mixer for approximately 15 seconds to ensure a completely homogenized sample before dilution and analysis.

2.5.3.2 Muscle samples

Muscle samples were crushed in liquid nitrogen using a pestle and mortar before approximately 2g of tissue was weighed into an acid (HCl) washed crucible and the weight recorded. Samples were then ashed overnight in a muffle furnace at 550°C (Muffle Furnace size 2, Gallenkamp). The following morning samples were allowed to cool to room temperature before preparation for analysis by Atomic Absorption Spectrometry. When cool, 1ml of 6M HCl was added to the crucible and used to rinse the sample into a clean 50ml conical flask. Another 1ml HCl was used to digest any remaining sample before the crucible was rinsed with lanthanum chloride solution (0.2% w/v) into the flask, then made to a final volume of 50ml (a 1:25 dilution). Once made up to 50ml, the solution was left for an hour to allow for precipitation of the lanthanum phosphate complex before analysed in the AA Spectrometer (AAnalyst 100, Atomic Absorption Spectrometer, Perkin Elmer Instruments, Norwalk, CT, USA).

2.5.3.3 Diluting Samples

0.2ml of sample was diluted with 9.8ml 0.1% w/v lanthanum chloride (1:50 dilution) to produce samples which could be measured in the linear range of the curve. The lanthanum chloride was used to react with any phosphate in the sample to reduce interference from phosphates.

2.5.4 Determining Calcium Concentration

The equipment measures absorption of light in triplicate and gives the average of the three measurements, with the standard deviation of each set. Any sample with a SD higher than 0.1 was repeated.

An example result

AA Concentration:

2.934; 2.932; 2.889

Mean: 2.919 mg/l SD = 0.0252

Original Sample Concentration: 2.919 x 50 = 145.95 mg/l

Standards were used randomly throughout sample determination to check variation of the method. The samples were analyzed in reverse slaughter order in Run 1, and then in actual slaughter order in Run 2. The results from both runs were then compared for repeatability of the method, and the average of the 6 measurements was taken as the final result.

2.6 Warner-Bratzler Shear Force Assay for Meat Tenderness

Once the LD had been aged at 4°C for the appropriate time (according to Latin square sample arrangement in Appendix A), the vacuum packed LD chops were stored at -70°C until required for tenderness analysis, when they were stacked, single layered on ventilated racks in a cold room at 4°C for 72 hours to allow thorough defrosting. The thickest chop was used for temperature monitoring, with an electrical temperature probe placed in the geometric centre. It was assumed that the temperature of all other chops would be reflected by temperature changes in this chop. Steaks were placed in an 80°C water bath and cooked until the largest chop reached a temperature of 78°C then an additional ten more minutes were given for cooking. All chops were removed and placed back on the racks at 4°C to cool overnight.

2.6.1 Preparation of samples-Core preparation

Samples were removed from the cold room in polystyrene boxes to prevent large temperature changes. Chops were then individually removed from the boxes at random and prepared for tenderness analysis. Warner-Braztler shear force analysis requires chops to be prepared into cores of certain diameter (1.27 cm) and length (2.5cm) achieved by use of a hand held corer. Each chop was removed from its vacuum pack and as many cores were prepared from the steak as possible, average core yield per chop was 4. Cores were cut in parallel to the alignment of the muscle fibres within the LD, using the back fat as a guide for the orientation of the chop within the muscle. The whole of the chop was sampled to reduce any variation in toughness between the outer and inner areas of the muscle. All chops were sampled in this way to cover all variation within chops. All cores were then analysed for shear force and the average of all cores was taken and assumed to be representative of the chop as a whole.

2.6.2 Warner-Bratzler Shear Force Assay

Each core was then placed at the centre of a guillotine fitted with a blade of thickness 1.016mm with a central v-shaped cutting blade of angle 60° as defined by Wheeler *et al.* (1997). The blade is part of the TA-XT2i Texture analyser (Stable Micro Systems, Surrey, UK) and was calibrated with a 25kg weight, set at a constant speed of 1.7 mm/s. This machine then measures the force required for the blade to shear the core whilst maintaining a constant speed using supplied software (Texture Expert Exceed). The result is expressed in kg.

2.7 Extraction and Measurement of total RNA

All water used in the extraction of RNA, reverse transcription into single standed DNA and further PCR reactions was RNAse/DNAse free water (Sigma) and all sample and reaction tubes, pipette tips and PCR plates were certified RNAse/DNAse free by manufacturers.

2.7.1 Extraction of total RNA from LD and Kidney

Total RNA for real time PCR analysis was extracted from sheep tissues using TRIzol® Reagent (Invitrogen, UK), whereby RNA is extracted by phase separation. Animal tissue samples were crushed in liquid nitrogen using a pestle and mortar prior to extraction and were stored at -80°C before TRIzol® extraction. 1ml TRIzol® reagent was added to 100mg frozen crushed animal tissue before homogenisation for two bursts of 30 seconds, and all samples were kept on ice until ready for phase separation. The prepared homogenates were left on the bench for 5 minutes to allow dissociation of nucleoprotein complexes. 200µl chloroform was then added and each sample shaken vigorously for 15 seconds. This mixture was then incubated for another 3 minutes at room temperature before

centrifugation at 12,000 rpm (Microfuge 22R Centrifuge, Beckman Coulter) for 15 minutes at 4°C. This separates the mixture into three phases, the top phase being a colourless aqueous solution containing the total RNA; it is this top phase that is carefully removed and transferred to a fresh tube. The total RNA was then precipitated from solution by addition of 0.5ml isopropanol, the tube contents were mixed using a vortex mixer and the solution incubated at room temperature for 10 minutes. This solution was then centrifuged at 12, 000 rpm for 10 minutes, allowing the total RNA to collect as a pellet at the base of the tube. The water/isopropanol supernatant was removed carefully and the pellet re-suspended in 1ml 75% v/v ethanol. Finally the total RNA pellet was cleaned of remaining traces of TRIzol® reagent constituents using 75% v/v ethanol; the pellet was vortexed briefly in 75% v/v ethanol before the pellet was collected again by centrifugation at 12,000 rpm for 15 minutes. The ethanol supernatant was removed and the pellet air-dried before dissolving in 40µl RNAse/DNAse free water and storing at -80°C.

2.7.2 DNAse treatment of total RNA

To remove possible contamination of genomic DNA in the extracted total RNA, the RNA was treated with a DNAse enzyme to digest any DNA present. 5µl DNAse enzyme (Promega, UK; Catalogue #M610A) was added with 5µl 10 x DNAse buffer to RNA dissolved in 40µl water. The contents of the tube were mixed by vortex mixer and then collected by brief centrifugation (up to 12,000 rpm for 30 seconds) using a bench top centrifuge (Microfuge 22R Centrifuge, Beckman Coulter). The reagents were then incubated at 37°C for one hour. The reaction was then stopped by addition of 150µl water with 200µl phenol/chloroform/isoamylalcohol (Ratio 25:24:1; Sigma Aldrich, UK) and the contents vortex mixed and spun for 5 minutes at 13,000 rpm at 4°C. This separated the enzyme and reagents from the RNA, leaving the total RNA dissolved in the aqueous upper phase. The top layer was removed to a clean tube and the RNA precipitated by addition of 375µl ethanol with 15µl 3M sodium acetate (pHed to 5.2 with HCl). The contents were vortex mixed and left to precipitate at -80°C overnight. Following precipitation, the samples were vortex mixed for approximately 15 seconds before the RNA pellet was collected by centrifugation at 13,000 rpm at 4°C for 15 minutes. The supernatant was removed and the total RNA washed in 1ml 75% v/v ethanol. The pellet was then re-collected by spinning at 13,000 rpm at 4°C for 5 minutes. Finally the ethanol supernatant was removed and the total RNA pellet air-dried before dissolving in 40µl water, ready for RNA quantification.

2.7.3 Total RNA Quantification

Quantity of extracted total RNA was determined using a Nanodrop spectrophotometer (ND-1000 Nanodrop Products, Thermo Fischer Scientific, USA). In addition, RNA purity was assessed by ratio of absorption at 260nm to 280 nm, thereby giving an indication of the level of protein contamination, where a 260/280nm ratio between 1.8 and 2.0 is optimal, where protein contamination reduces the ratio from the optimal 2.0; therefore a ratio below 1.8 indicates a sample containing unacceptable levels of protein. The average 260/280 ratio and the range of values for each RNA sample set used in this thesis is shown in table 2.7-1. Following RNA quantification the samples were all diluted to a concentration of 0.1µg/µl in water and stored until the reverse transcriptase step could be performed, approximately 3 months later. Total RNA samples were separated by agarose electrophoresis following the storage period to visually check that integrity was maintained.

Table 2.7-1 RNA 260/280 ratios from samples used in this thesis. Data for sheep tissues is from trial 1 of this thesis (trial 2 values were unavailable) n=36, C2C12 cell RNA n=48 and Rat Primary cells n=12.

Tissue	Mean 260/280 Ratio	SEM	Range		
			Minimum	Maximum	n
Sheep LD	2.10	0.005135	2.02	2.16	36
Sheep Kidney	2.03	0.011951	1.81	2.12	36
Rat Primary Muscle Cells	2.10	0.008657	2.06	2.13	12
C2C12 Cells	2.08	0.004803	1.96	2.11	48

2.7.4 Horizontal Agarose gel Electrophoresis of total RNA

RNA integrity and quality was visualised using non-denaturing agarose gel electrophoresis. 5µl RNA at a concentration of 0.1µg/µl was loaded with 1 µl 6 x loading dye (Promega, Cat #G190A) in a 1.5% w/v agarose gel made with 1x TAE (40mM Tris, 1mM ethylenediaminetetraacetic acid (EDTA), 0.12% (v/v) acetic acid), the gel was then electrophoresed for approximately 1 hour in 1 x TAE buffer. The gel was stained in 0.5µg/ml ethidium bromide in 1 x TAE buffer, and visualised using GelDoc system and Multi-analyst program (BioRad, Hemel Hempstead, UK). Intact total RNA from animal tissues should exhibit three clear bands, the larger 28S and 18S ribosomal RNA molecules, followed by a smaller transfer RNA band, and a slight smear of mRNA fragments, as observed in figure 2.7-1.

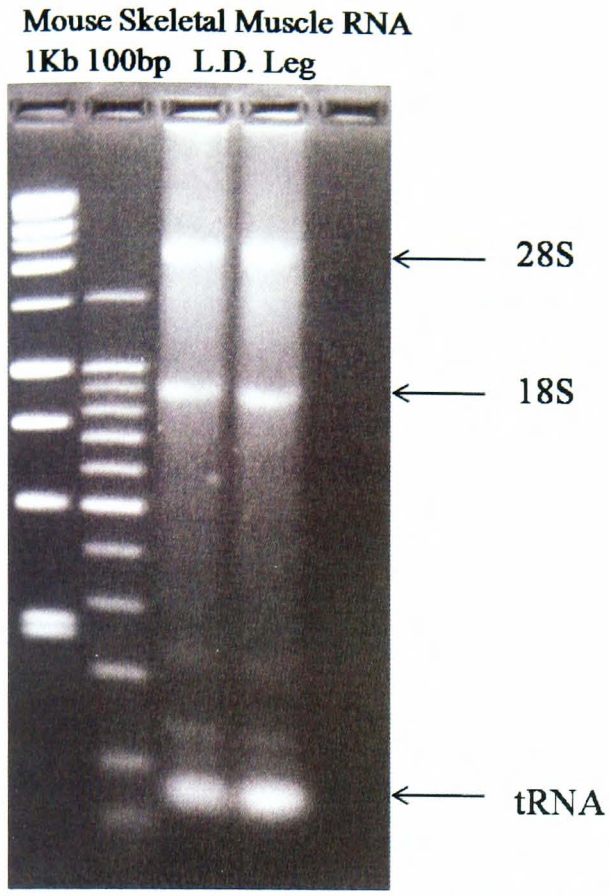


Figure 2.7-1 Horizontal agarose gel electrophoresis of total RNA extracted from mouse LD and leg muscle, three distinct bands are visible showing 28S, 18S and tRNA to be intact, markers are 1kb and 100bp DNA markers (Promega).

2.7.5 Reverse-Transcription (RT) of mRNA

To investigate changes in the expression of genes at the mRNA level, the extracted total RNA was reverse transcribed into copy DNA (cDNA), which was a template for use in Polymerase Chain Reactions (PCR). Extracted total RNA was reverse transcribed using reverse transcriptase (RT) enzymes to generate cDNA. All extracted RNA was diluted to 0.1µg/µl prior to RT step and the reverse transcriptase enzyme (Moloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT), Promega UK) was used to generate cDNA in the sheep studies. The RT reaction used the basic principles of heating the RNA to a high temperature to denature the tertiary structures, then cooling rapidly to allow annealing of short DNA hexamer primers (single stranded DNA molecules 6 bases in length) to the RNA. The hexamers were designed to anneal randomly to RNA and provide a start site for the reverse transcriptase enzyme, demonstrated in figure 2.7-2.

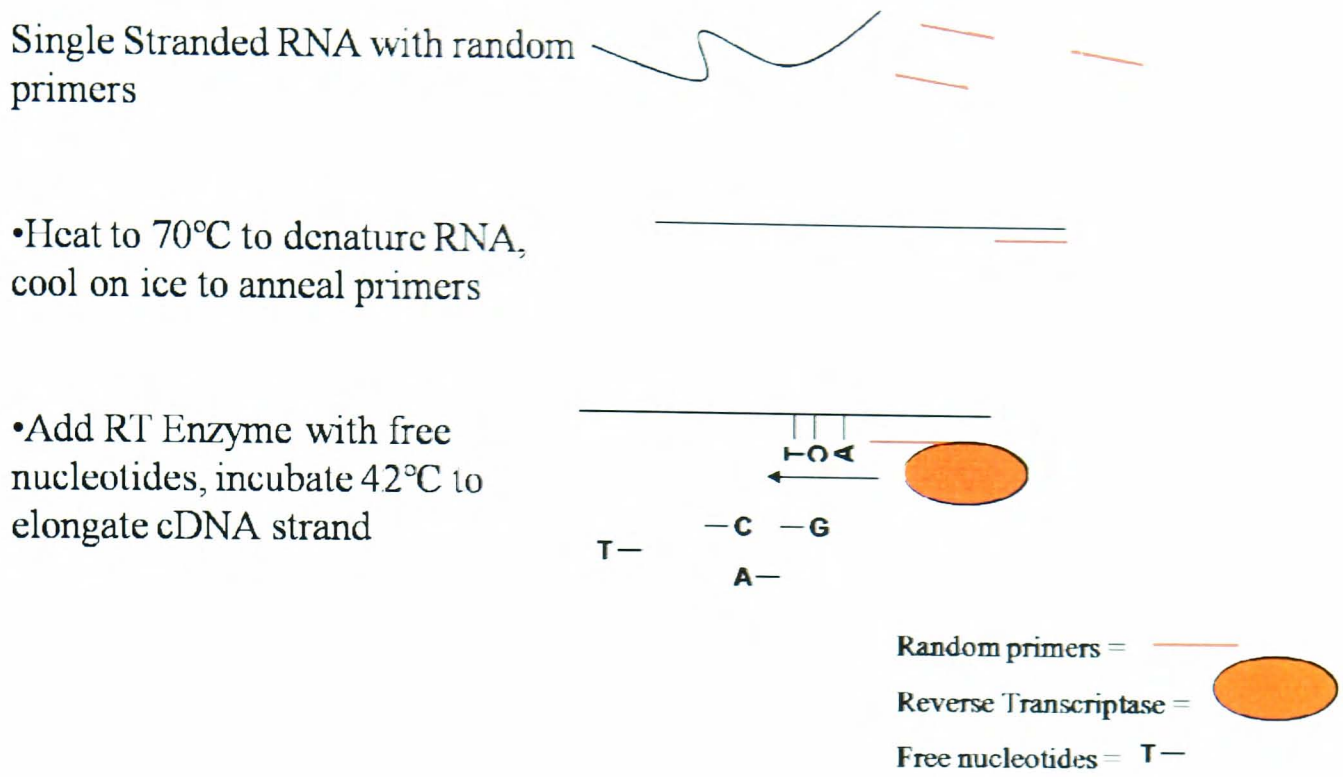


Figure 2.7-2 Schematic representation of Reverse Transcription of mRNA.

2.7.5.1 RT using MMLV-RT

1µl random primers (Promega, UK) at a concentration of 0.5µg/µl was added to 0.5µg total RNA in a thin-walled microfuge tube and the final volume made up to 15 µl with water, the reagents were then vortex mixed and collected by brief centrifugation (up to 12,000 rpm for 30 seconds). The tube was then heated to 70°C for 5 minutes on a PCR heat block, before transferring quickly to ice. This ensured the primers annealed while the RNA was denatured and prevents secondary structures forming. The sample was left on ice for at least one minute before addition of 5µl 5x MMLV-RT Buffer (250mM Tris-HCl, pH 8.3; 375mM KCl; 15mM $MgCl_2$; 50mM DTT; Promega UK), 1.25 µl nucleotides mix (dATP,dCTP, dGTP, dTTP, final concentrations 0.5mM), 0.5 µl Ribonuclease Inhibitor enzyme (25 units; Promega UK), 1 µl RT enzyme (200 units) and 2.25 µl water. The contents were mixed and collected briefly as before and incubated for 5 minutes at room temperature to allow the enzyme to extend the primers before transfer to the heat block of the PCR machine (DNA Engine-Mastercycler, M.J. Research; USA) for one hour at 42°C. The final 25µl of first strand cDNA mixture was diluted 1:4 to 100µl with water to be stored as stock cDNA at -20°C.

2.8 Development of sequences for primer and probe design in sheep

At the time of this study there were no available known mRNA sequences for the sheep calpain enzymes nor for the calbindin D-28K protein. To overcome this, calpain sequences needed to be created using available sheep partial length cDNA sequences in the EST (expressed sequence tag) database (National Center for Biotechnology Information (NCBI)). There had been previous work in this laboratory to develop and sequence a PCR product of calbindin D-28K in sheep (Miss C. Anike-Nkweze, University of Nottingham) and the present study used this sequence to develop a set of sheep specific primers. Primers and dual labelled fluorescent oligonucleotide probes for sheep mRNA measurement were purchased from Sigma Genosys (UK) and primers and probes for use in mouse and rat –derived cells and tissues were purchased from MWG (UK).

2.8.1 Development of calpain Sequences

There were no existing mRNA sequences for the ovine calpain enzymes I and II, so sequences needed to be generated using the EST database of sheep cDNA fragments. To do this the mRNA nucleotide sequence of the closest related species was used to search for related ovine sequences, which was in this case the bovine sequence.

Firstly the bovine peptide sequence was found using the "Swissprot" website [http://www.expasy.org/sprot/], where a search was performed using the common protein name (calpain I or calpain II). This then produced a link to EMBL (European Bioinformatics institute-EMBL) for the bovine mRNA nucleotide sequence [http://www.ebi.ac.uk/embl/]. This nucleotide sequence for the bovine mRNA of the translated protein was then used to search using the BLAST (basic local alignment tool) tool within the NCBI (National Center for Biotechnology Information) website for related ovine sequences using the EST-[non-human, non-mouse– others] database. This recovered a number of unidentified ovine partial length cDNA sequences with similarity to the initial bovine mRNA sequence.

Each of the collected ovine cDNA sequence fragments were then aligned with the relevant bovine calpain I or II sequences using the ClustalW alignment tool within the NCBI website, to ascertain the region of the mRNA sequence it was related to. The ovine sequence fragments were organised into their order relative to the bovine sequence, aligned and a contiguous cDNA sequence generated. The bovine mRNA sequence used to search the sheep EST database and the sequences obtained for each gene are shown in table 2.8-1. This final created sequence was then ClustalW aligned back to the original bovine and human sequences to assess variation, the results are shown in table 2.8-2.

Table 2.8-1 Bovine Sequence used to interrogate NCBI EST non-human, non mouse database and retrieved partial length cDNA sheep sequences.

Ovine Calpain primers		Ovine EST Sequence	
Gene	Bovine sequence	Accession No.	Length (bp)
Calpain I	NM_174259	EE820633	710
		DY507375	741
		EE814187	557
		DY491370	781
		created sequence	1404
Calpain II	NM_001103086	DY506459	722
		DY509248	658
		DY521444	568
		created sequence	990

Table 2.8-2 ClustalW alignment of created contiguous Ovine calpain cDNA sequences Vs human and bovine sequences indicating the similarity to ovine sequences. Length of created ovine calpain I sequence was 1404bp, created ovine calpain II was 990bp.

Ovine Calpain I alignment to	Accession #	Sequence Length (bp)	Similarity
Bovine Calpain I	U07849	286	97%
Human Calpain I	X04366	3007	82%

Ovine Calpain II alignment to	Accession #	Sequence Length (bp)	Similarity
Bovine Calpain II	U07850	623	96%
Human Calpain II	M23254	3213	89%

2.8.2 Design of Quantitative (Real -Time) PCR primers and probe

Intron and exon boundaries were identified using the exon boundaries for equivalent genes in bovine genomic DNA sequence as a guide, before primers and probes for real-time PCR were designed using Primer Express Software (V1.5, Applied Biosystems); the resulting sequences and the position of the primers and probes are shown in appendix B.

2.9 General and Quantitative Polymerase Chain Reaction

cDNA created from mRNA extracts was analysed for gene expression by either semi-quantitative or quantitative PCR. General PCR amplifies small quantities of cDNA into larger amounts that can be visualized under eithidium bromide staining. This method was used to validate primers and to verify expression of genes in different tissues. Quantitative (or real-time) PCR (Q-PCR) allows absolute or relative quantification of mRNA transcripts by determining the quantity of PCR amplicon produced after each cycle of the PCR reaction. Q-PCR was performed in all studies to quantify changes in gene expression at the mRNA level.

2.9.1 General (Semi-Quantitative) PCR

AmpliTaq Gold enzyme (Applied Biosystems) was used in general PCR reactions, in a thin-walled 200µl volume microcentrifuge tube; containing 0.25 µl AmpliTaq Gold Enzyme (1.5 units), 5 µl 10x PCR Gold Buffer, 3 µl 25mM MgCl2, 1 µl 10mM dNTP mix, 1.25 µl forward primer, 1.25 µl reverse primer (both primers at concentration of 10pmol/µl) and 5 µl template cDNA (equivalent to 0.25µg RNA), with the final volume adjusted to 50 µl with water. This reaction mixture was then vortex mixed and centrifuged briefly to collect at the base of the tube before incubation using a PCR thermocycler (DNA Engine- Mastercycler, M.J. Research; USA). The reaction was incubated at 95°C for 10 minutes to activate the enzyme before the amplification cycles began. Cycles contained three steps; 94°C denaturation step for 30 seconds, 55 to 65°C annealing temperature (primer set dependant) for 30 seconds and 72°C elongation for 30 seconds to 1 minute (dependant on length of amplicon), summarized in figure 2.9-1. These three steps were repeated 35 times before the final elongation step of 72°C for 5 minutes followed by cooling to 4°C.

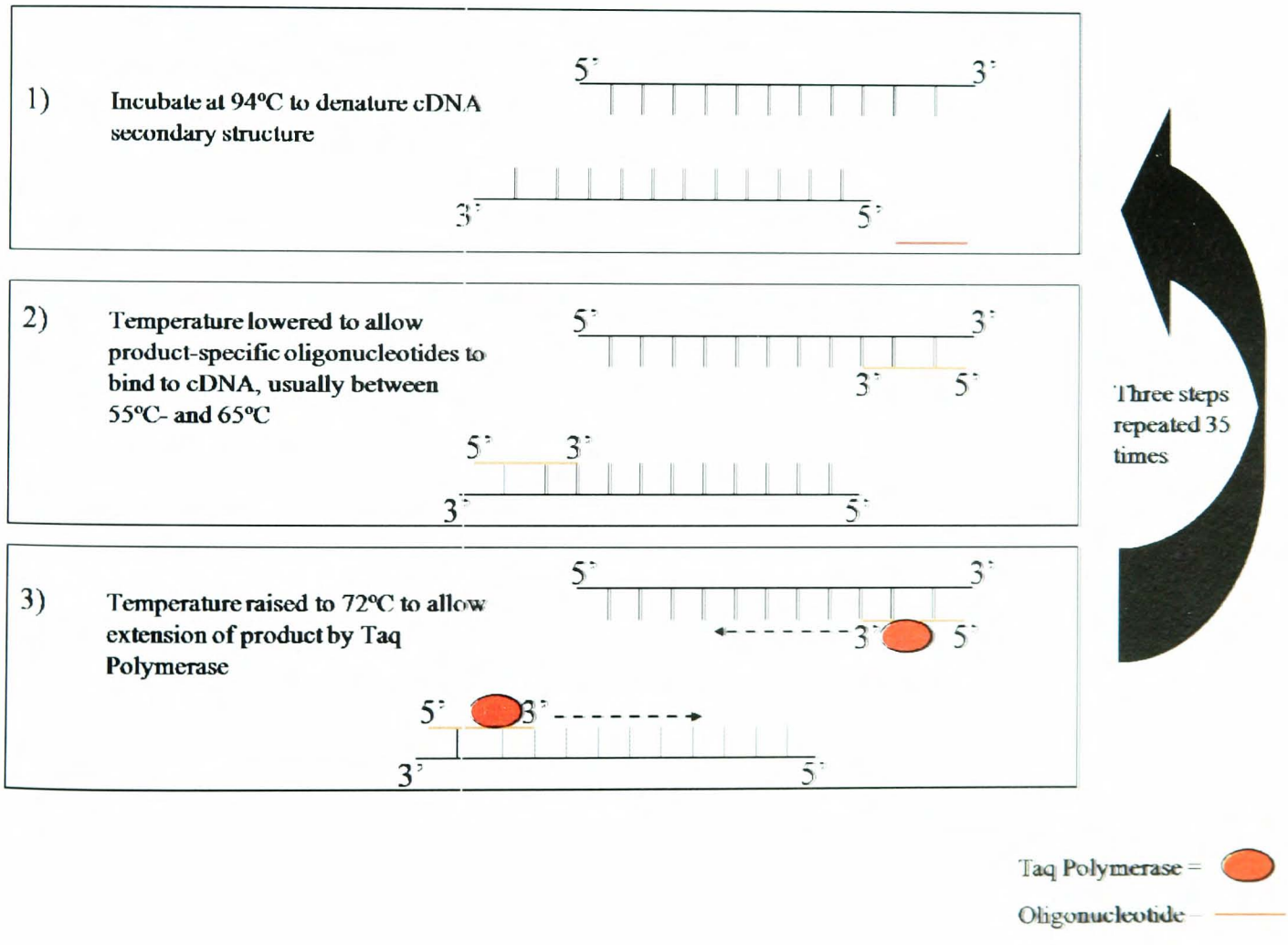


Figure 2.9-1 The three repeated cycles of the Polymerase Chain Reaction (PCR).

2.9.2 Electrophoresis of PCR products

To validate the primers and check the size of the nucleotide sequence amplified, the semi-quantitative PCR products were separated by horizontal electrophoresis in agarose gels and visualised using eithidium bromide staining. 10µl PCR product was diluted with 2µl loading dye and electrophoresed in 1.5% w/v agarose gel made in 1 X TAE buffer at 100V for 1 hour. The separated products in the gel were then stained for 20 minutes in 0.5 µg/ml ethidium bromide (in 1 X TAE buffer) and imaged under UV light using the Gel Doc (Gel Doc 2000) and Multi-Analyst software (BioRad Laboratories; CA, USA).

2.9.3 Quantitative PCR

Quantitative PCR uses similar thermal cycling conditions as used in the general PCR method, except after every cycle of cDNA synthesis; the quantity of cDNA is measured. To measure the quantity of cDNA an additional oligonucleotide is included in the reaction mixture, referred to as a dual-labelled fluorescent probe due to it being modified with two additional fluorescent dyes conjugated at either end of the specific sequence, at the 5' end is the reporter dye and the quencher is situated at the 3' end. These two dyes have differential energy properties, the reporter having high energy and the quencher having low energy, and so, when the two dyes are in close proximity (i.e. both bound at either end of the probe) the quencher absorbs the energy from the reporter and thus there is no release of fluorescent energy from the probe. This phenomenon is referred to as Flourescence Energy Resonance Transfer (FRET) and is the basis for cDNA quantification during quantitative real-time PCR using dual-labelled fluorescent probe technology.

The probe has a higher annealing temperature than the primers and thus binds before the primers to a specific sequence in the middle region of the amplicon. The DNA polymerase enzyme used in the PCR reaction has 5'nuclease activity which allows it to digest any obstructions in its path, and thus will degrade the probe as it copies the template cDNA between the two primers. During the quantitative PCR reaction the DNA polymerase copies the template cDNA strand and degrades the probe as it comes to find it in its path and due to the orientation of the two dyes, the quencher is released first. The removal of the quencher from the 3' end of the probe releases the inhibition on the reporter dye's fluorescence and the fluorescence can be detected. This reaction is summarised in a schematic diagram in figure 2.9-2. The level of fluorescence after each cycle is proportional to the amount of cDNA template being copied and thus the quantity of amplicon copied after every amplification cycle can be related to the level of fluorescence released.

The theory behind quantifying cDNA using the Polymerase Chain Reaction is that the amount of cDNA in the sample should double with each cycle of the reaction, giving an optimum efficiency of 2. The reaction causes an exponential rise in the quantity of the amplified product (amplicon) and the quantitative PCR method measures the rise in cDNA after every cycle. After every cycle the polymerase enzyme releases a fluorescent signal from the labelled probe, which is directly proportional to the quantity of template present, and it is this signal which was measured by the light-cycler equipment. The machine records the number of cycles taken for each sample to reach threshold fluorescence (Ct) and this is proportional to the amount of cDNA template at the start of the reaction. To quantify the relative concentration of unknown samples a pool of cDNA was diluted into a standard curve to be used as a reference for relative quantification, the dilution method is outlined in table 2.9-1.

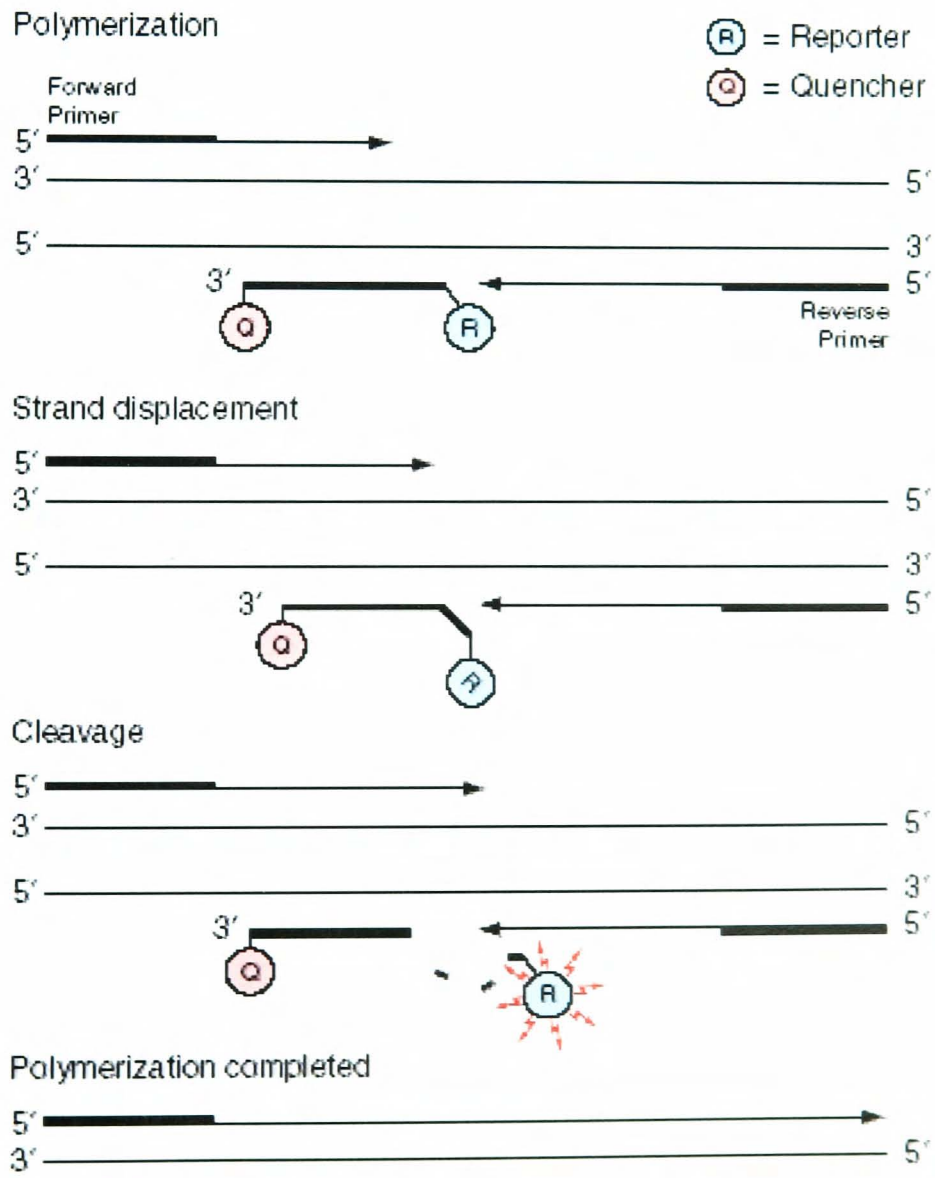


Figure 2.9-2 Schematic diagram of the release of the fluorescence signal during quantitative PCR. Image from TaqMan® PCR Reagent Kit With AmpliTaq Gold® DNA Polymerase Protocol manual Applied Biosystems (2002).

Table 2.9-1: Diluting cDNA to create a standard curve for relative quantification of unknown samples

Standard Curve	Relative Quantity			
	1	0.75	0.5	0.25
Volume Stock cDNA (µl)	100	75	50	25
Volume Water (µl)	0	25	50	75
Dilute these 1:10 (10µl Standard Curve + 90µl water)				
1:10	0.1	0.075	0.05	0.025
1:100	0.01	0.0075	0.005	0.0025
1:1000	0.001	0.00075	0.0005	0.00025

The q-PCR machine's software (Sequence Detection Systems V 2.2.2.; 2004 Applied Biosystems, Foster City, CA, USA) then calculates the efficiency of the reaction by plotting the number of cycles to reach threshold against the relative quantity of cDNA template in the standard curve. Figure 2.9-3 demonstrates the typical relationship between the fluorescence value generated at each cycle number, generated by the amplification of a cDNA standard curve, the threshold value of fluorescence is shown as a red line (measured using 7900HT Fast Real-Time PCR System, Applied Biosystems) and the corresponding cycle number is the Ct value. The software then calculates the equation of the standard curve by plotting the number of cycles required to reach threshold (Ct) plotted against the Log_{10} of the relative quantity of cDNA in the standard curve, shown in figure 2.9-4. Assuming that the quantity of DNA doubles after each cycle, the theoretical number of cycles required for the amount of cDNA to increase by a factor of 10 is Log_2 10, and therefore when the standard curve is plotted displaying the relationship between Ct value and log_{10} value of the template quantity (total RNA equivalents), theoretically the gradient is approximately 3.322 (3 dp) when the reaction is near 100% efficiency (DNA quantity doubling per cycle). Most reactions do not reach maximum efficiency as a result of a variety of factors, for example different binding capacities of the oligos to the cDNA strand. As a result of these factors each primer and probe set was evaluated for its efficiency across the standard curve and the unknown sample templates were diluted to lie where the reaction had a linear response, an example of unknown samples amplifying in the linear phase of the reaction is shown in figure 2.9-4.

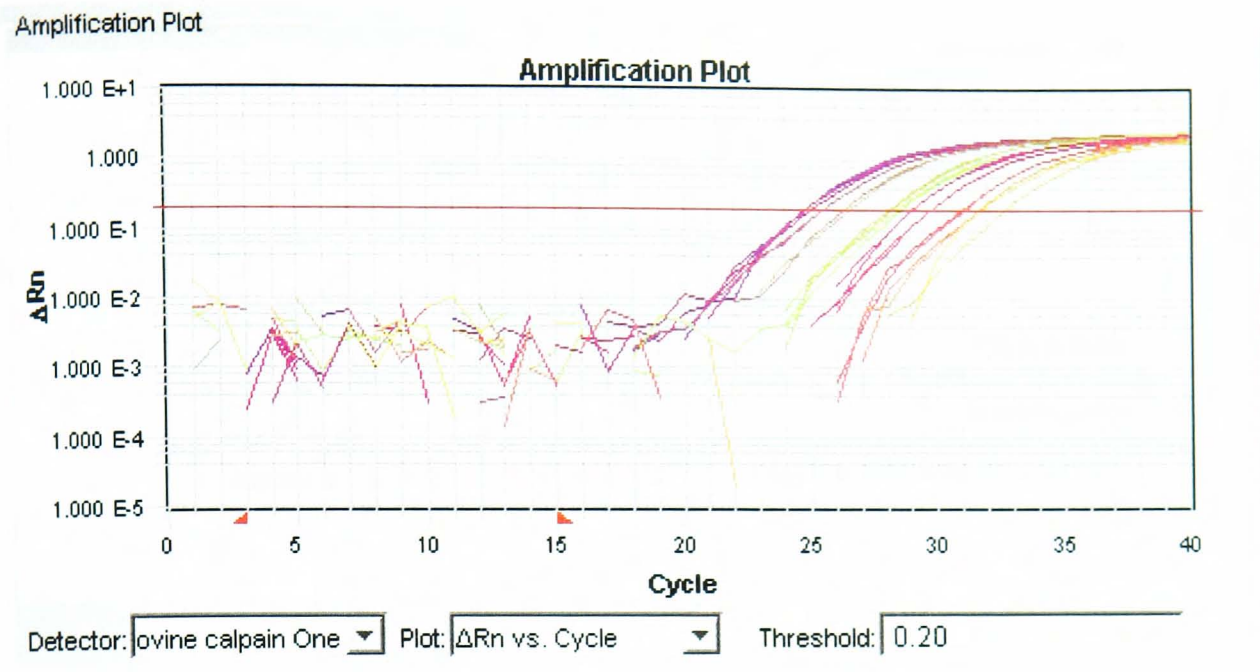


Figure 2.9-3 Amplification plot of standards used to measure Ovine Calpain I expression in LD. As concentration of cDNA in the starting template decreases, the time taken to reach threshold detection is longer; shown as a red line.

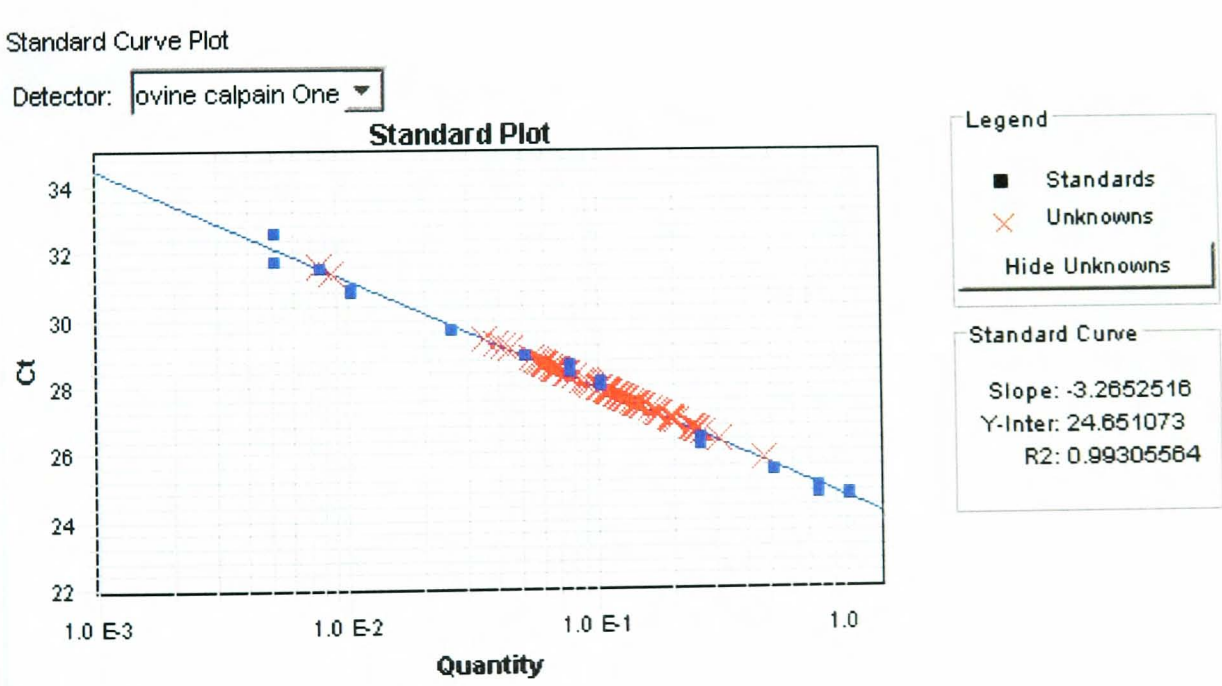


Figure 2.9-4 The number of cycles required to reach threshold (Ct) plotted against the log of the relative quantity of cDNA in the standard curve. The equation of the standard curve is calculated and the gradient of the line describes the efficiency of the reaction, 3.3 being optimum. The samples, denoted in red crosses, were diluted 1:10 and are shown lying across the linear phase of the reaction.

2.9.3.1 q-PCR in Sheep Trials

Expression of calpain system and calbindin –D28K mRNA in sheep trials was quantified using real-time quantitative PCR using the Fast-Taq system and analysed using Sequence Detection System software (SDS) (Applied Biosystems, Foster City, CA, USA). Primers for the ovine calpains, calpastatin and calbindin were designed as described in section 2.8 and are summarised in appendix B. The myosin heavy chain (MHC) genes were analysed using the LC480 system (Roche) using primers and probes designed by Miss Krystal Hemmings (University of Nottingham; Appendix B). A standard curve of either LD or kidney was generated from pooled cDNA taken from each trial sample and serially diluted to generate a range of dilutions against which the trial samples could be quantified. All trial samples of cDNA were diluted 1:10 before measurement to ensure samples were quantified against the linear phase of the standard curve. To correct for variation in sample preparation an internal control gene expected to be unchanged by vitamin D treatment was also quantified under the assumption that expression of this gene was not altered by treatment and that total RNA content of samples was constant. For this purpose β-Actin was measured and utilised as an internal standard for all genes. All sample values were calculated relative to the standard curve in arbitrary units and then divided through by the corresponding value for β-Actin; giving results expressed as a ratio to β-Actin.

For each reaction, cDNA diluted 1:10 was added into a well of the PCR thermal cycling plate and added to it were PCR Mastermix, dual labelled probe and forward and reverse primers before the final volume was adjusted with RNAse/DNAse free water, table 2.9-2 summarises the volumes and final concentrations of the components of each reaction system and the thermal cycling conditions used. For the calpain system analysis, the Applied Biosystems 7900 HT Fast Real-Time PCR system was used and the master mix was Taqman Fast Universal PCR 2X mastermix (Applied Biosystems, Warrington, UK) and each sample was measured in duplicate reactions in a 96-well reaction plate; raw data was analysed using Sequence Detection System (SDS, V2.2.2) software. For the analysis of the MHC genes the Roche Light Cycler 480 system was used, the master mix was LightCycler ®480 probes Master 2 X Mastermix (Roche Diagnostics GmbH, Germany) and each sample was measured in triplicate reactions in a 384-well plate; raw data produced was analysed using LC480 Software (LightCycler® 480 SW V 1.5, Roche Diagnostics, Germany).

Immediate analysis of the raw data was performed by each system's specific software, whereby the relative concentration (an average of replicates) was calculated against the standard curve, followed by the export of data to Microsoft Excel (version 2007) for further analysis investigating effects between treatment groups prior to statistical analysis performed in Genstat software (V11).

Table 2.9-2. Reaction volumes and thermal cycling conditions of two Q-PCR systems used in mRNA expression analysis in sheep trials.

	Q-PCR 7900HT Fast Real-Time PCR System		Q-PCR LightCycler ® 480 System	
Reagents	**Volume (µl)**	**Final Concentration (µM)**	**Volume (µl)**	**Final Concentration (µM)**
cDNA Template	8.3		2.5	
Equivalent RNA (µg)	0.0415		0.025	
Mastermix	12.5		7.5	
Dual-labelled probe	0.5	0.2	0.3	0.2
Forward primer	0.75	0.3	0.45	0.3
Reverse Primer	0.75	0.3	0.45	0.3
Water	2.2		3.8	
Total Volume	25		15	

Cycling Conditions	Temperature °C	Duration (seconds)	Temperature °C	Duration (seconds)
Denaturation	95	20	95	600
	95	1	95	10
Amplification	60	20 → Acquisition	60	50
			72 → Acquisition	1
		→ repeated for 40 cycles		→ repeated for 45 cycles
Cooling	None		40	10

2.9.4 Normalisation of data to β-Actin

Total RNA samples were normalised for RNA concentration prior to the RT step to ensure each cDNA sample was created from similar amounts of total RNA prior to PCR, however, to further correct for variation in the sample preparation a house keeping gene was used, which was β-Actin in case of the ovine experiments. Expression of β-Actin at mRNA level was measured using primers and probes designed for sheep by Dr. Zoë Daniel (University of Nottingham), for normalisation of the calpain genes the data from Fast Taq ABI system was used and for the Myosin Heavy Chain genes the data from the Roche LC480 was used. The level of expression of this gene was compared between treatment groups using a paired T-test in Genstat software and the data is shown in table 2.9-3. The results in table 2.9-3 show that the two real time PCR machines produced similar results for the expression of β-Actin in LD and kidney in both trials and that there were no effects of dietary treatment on β-Actin expression in LD and kidney of both trials ($P>0.05$ in all cases).

Table 2.9-3 Effect of Vitamin D_3 supplementation on mRNA expression of β-Actin, as measured by the Fast Taq ABI system, and again in the LD as measured by the Roche LC480 system. cDNA created from samples with constant RNA concentration from either LD or Kidney tissues from animals fed either a control or vitamin D supplemented diet.

Trial 1: 4 Days Vitamin D						
Housekeeping Gene Beta Actin		**Control**	**Vit D**	d.f.	S.E.D.	**P**
Fast Taq (ABI) (µg RNA equivalent)	**LD**	**1.631**	**1.608**	16	0.0855	**0.766**
	Kidney	**1.544**	**1.530**	17	0.0389	**0.68**
LC 480 (Roche) (relative expression)	**LD**	**0.1197**	**0.1066**	17	0.0177	**0.460**
Trial 2: 7 Days Vitamin D + Ca						
Housekeeping Gene Beta Actin		**Control**	**Vit D**	d.f.	S.E.D.	**P**
Fast Taq (ABI) (µg RNA equivalent)	**LD**	**1.358**	**1.427**	17	0.0676	**0.234**
	Kidney	**1.676**	**1.728**	17	0.0716	**0.389**
LC 480 (Roche) (relative expression)	**LD**	**0.0774**	**0.0785**	17	0.0133	**0.897**

2.10 Measurement of Proteins by Western Blotting technique

Analysis of specific protein levels in samples was performed by denaturing electrophoresis of extracted proteins followed by transfer to a nitrocellulose membrane followed by immunoprobing with antibodies specific to the protein targets. This method allows relative comparison of protein levels between control and treatment groups.

2.10.1 Extraction of proteins from animal tissues

Snap tissue samples taken during carcass sampling and stored at -70°C until extraction when samples were crushed in liquid nitrogen in a crucible before weighing out 1g (±0.05g) into a 20ml sterilin tube. 5ml of extraction buffer with protease inhibitors (containing 100mM Tris-HCl pH 5.5, 10mM enthylenediamine tetra-acetic acid (EDTA), 0.5mM 2-(4-aminoethyl)- benzenesulphonyl fluoride (AEBSF), 0.01mM leupeptin and 0.01mM pepstatin) was then added to the crushed sample. The extraction buffer contains three protease inhibitors to prevent proteolysis. The mixture was then homogenised (Polytron, The Nertherland) using 2 x 30 second bursts, allowing 30 seconds between to allow time for the blade to cool. Sterilin tubes were kept in an ice bath to prevent heating of the samples during homogenisation.

Following homogenisation 100µl of the mixture was removed into a 1.5µl tube containing 900µl 0.1M NaOH for protein concentration analysis (Lowry assay, see section 2.10.2). Another 250µl was removed and added to an equal volume of SDS-mix DTT (20% v/v glycerol, 12.5% v/v 1M Tris/HCl pHed to 6.8, 4% w/v SDS (Sodium Doecyl Sulphate), 15.4% w/v Dithiothreitol (DTT), and a few grains

bromophenol blue) for use in SDS-PAGE analysis. This solution contains strong reducing agents which denature the tertiary structure of proteins which allows optimum separation of proteins via electrophoresis through acrylamide gels. A further two 1.5ml tubes were filled with the homogenate and centrifuged at 15,000 rpm (Microfuge 22R Centrifuge, Beckman Coulter) for 15 min in a pre-cooled centrifuge (4°C). Following centrifugation, 100µl of the supernatant was added to 900µl 0.1M NaOH, and another 250µl supernatant added to 250µl SDS-mix DTT. These two aliquots of the supernatant protein fraction were stored at -20°C for protein quantification and electrophoresis. A further aliquot of supernatant was boiled on a 1.5µl tube heater at 100°C for 5 minutes before a final centrifugation (15,000 rpm for 15 min at 4°C). The resulting boiled supernatant, containing the endogenous calpain inhibitor calpastatin, was aliquoted into 0.1M NaOH and SDS-mix DTT as described for the supernatant above. All prepared extracts were then stored at -20°C until required, approximately 6 months later.

2.10.2 Determination of Protein concentration by Lowry Assay

The Lowry protein assay (Lowry *et al.*, 1951) was performed on extracts from sheep samples which had been stored in 0.1M NaOH at 1:10 dilution. The assay uses two solutions made up fresh for each assay; solution 1 (5ml 2% w/v Na_2CO_3 in 0.1M NaOH, 0.5ml 1% w/v $CuSO_4$, 0.5ml 2% w/v KNa Tartrate) followed by equal volume of solution 2 (5ml 0.1M NaOH and 0.5ml Folin's Ciocalteau's Reagent (FSA Lab Supplies, England).

In alkaline solution the copper III ions will complex with the proteins, this then reacts with the Folin-phenol reagent, reducing the acids to tungsten and molybdenum which produce blue solutions. The more protein present, the more acids are reduced thus producing a darker blue solution. The intensity of colour is detected colorimetrically by absorption at 620 nm. Protein standards were made up using Bovine Serum Albumin (BSA); six known concentration solutions were made up, containing 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mg/ml, stored at -20°C between assays. In a 96 well micro titre plate 50µl of each standard solution was added in duplicate. 0.1M NaOH solution was used as the 0 µl solution for background comparison. Again, in duplicate, 50µl of each sample was pipetted onto the plate, before all wells were made up to 200µl with 0.1M NaOH. 50µl of solution 1 (described above) was added to all wells and left for 5 minutes at room temperature. 50µl of solution 2 was then added and the plate incubated at room temperature for 20 minutes before the absorption at 620nm was read using the micro titre plate reader Dynatech MR5000 (Dynatech Laboratories Ltd, Guernsey, Channel Islands, UK). The absorption output was used to equate protein concentration by Biolinx 1.1a software, (Dynatech UK) comparing the unknown samples to the known concentrations of the BSA standards. Following determination of protein content within each

sample, those samples stored in SDS-mix DTT were diluted to constant protein concentrations. A standard mix was prepared by taking 20µl of each diluted sample and mixing in a 1.5ml tube. This standard was then loaded three to five times across gels to measure variation between and within gels.

2.10.3 Protein separation by SDS-PAGE (Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis)

Prepared denatured protein samples in SDS-mix, as described in section 2.10.1, were separated by vertical electrophoresis in Sodium Dodecyl Sulphate Polyacrylamide gels (SDS-PAGE). Polyacrylamide gels were prepared using variable acrylamide content keeping the ratio of acrylamide to bis-acrylamide constant (37.5:1 acrylamide:bis-acrylamide), quantities described in Appendix C. Appropriate concentrations of acrylamide in the gel were used according to molecular weight (MW) of protein to be investigated, varying from 8% w/v for proteins of size 250kDa to 75 kDa, to 12% w/v for smaller, 100kDa to 20kDa. Prior to loading, all samples were boiled on a heat block for approx 1 minute at 100°C to ensure denaturation of proteins and spun in a centrifuge (3 minutes at 15000 rpm at room temperature) to ensure a homogenous mix. 10µl pre-stained molecular weight marker (Biorad, USA) was loaded to demonstrate the distance moved by known molecular weight protein standards, ranging in size from 10 to 250kDa. Gels were run at 200V constant until the blue dye of the SDS-mix DTT began to run off the bottom of the main gel, usually around 45 minutes for a 10cm long gel.

2.10.4 Western Blot Protein Transfer

After running, the gels were laid in western blot buffer (5% (w/v) isopropanol, 0.4M Glycine and 2.5mM Tris Buffer, all Fischer Scientific Ltd, UK) for 5 minutes. A piece of 0.45 µm nitrocellulose membrane (GE Healthcare, UK) was cut to size just larger than the gels, then soaked in distilled water for 10 minutes to hydrate before immersion in western blot buffer ready preparation of the western blot stack. The stack was prepared on a sponge soaked in western blot buffer, followed with a layer of two sheets of Whatman 3MM paper (Fisher scientific, UK) also soaked in western blot buffer. The gel was laid on the 3MM paper and western buffer poured over to keep the gel moist before the nitrocellulose membrane was laid covering the gel. The layers were rolled with a glass rod to ensure no air bubbles were trapped between the gel and membrane. Two more layers of 3MM paper were added and a final sponge on top with additional buffer poured over the stack after every layer to ensure all components remained wet and rolled with the rod to remove any air bubbles. This ensures all proteins migrate evenly across the whole membrane, any air bubble between the gel and the membrane prevents protein migration to the membrane. The stack was compressed in a plastic

cassette then immersed in an electro-blotting Trans-blot tank (BioRad, Hemel Hempstead, UK) filled with western blot buffer. The gel was placed toward the negative electrode as proteins are negatively charged and move toward the membrane in the direction of the positive electrode. A constant current of 350mA was applied across the stack for 2 hours for the large gels used to run all experimental trial samples, and 200mA for 90 minutes for small gels, where fewer samples were used for antibody optimisation.

2.10.5 Ponceau Stain of Membrane

Following the blotting process the membrane was stained using Ponceau stain (0.5% w/v Ponceau S stain, 5% (w/v) trichloroacetic acid) to ensure protein transfer. The membranes were submerged in Ponceau stain for approximately 1 minute and washed in distilled water to reveal protein bands. The stain was removed using TBS-T (20mM Tris HCl pH 7.5, 150mM NaCl & 0.1% (w/v) Tween 20) once transfer of protein was confirmed. At this stage position of the molecular weight markers was denoted with a permanent marker.

2.10.6 Immunodetection of proteins

Once transferred to the nitrocellulose membrane, specific proteins were visualised using immunoprobing technique. The first step of this method removes all possible non-specifc binding sites still available on the nitrocellulose membrane. This was done by blocking the membrane in a solution of 5% (w/v) non-fat dried milk (Marvel®) in TBS-T for at least 30 minutes on a platform shaker at room temperature. Following the initial blocking, the membrane was probed by the protein-specific primary antibody overnight at 4°C. After the first antibody incubation, the membrane was washed for 30 minutes with 1% (w/v) non-fat dried milk in TBS-T with 6 x 5 minute washes using fresh wash solution each time. The membrane was then probed for 1 hour with a secondary antibody, usually anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase (HRP) or alkaline phosphatase enzyme, depending on the source of the primary antibody in use. Full details of antibodies and conditions used for detection are summarised in Appendix D. Following incubation with secondary antibody the membrane was washed again with 1% (w/v) non-fat dried milk in TBS-T for a further 30 minutes, again with 6 x 5 minute washes. A final 5 minute wash in TBS-T was the last step before the chemiluminescence reaction.

The calpain enzymes were visualized at the protein level using antibodies developed to porcine calpains (produced by Tim Parr, University of Nottingham) which were verified to cross react with sheep (Sazili, 2003; pp32). All samples were diluted to constant protein concentration of 5µg/µl before loading 20µl volume sample to give 100µg protein/lane. For the calpain enzymes the

proteins in the supernatant fraction were loaded and Calpain II was detected first before the membrane was stripped (as described in section 2.10.8) and re-probed with Calpain I antibody. For calpastatin detection the whole homogenate fraction was used and again samples were loaded to the same constant protein concentration of 5µg/µl so that a constant volume of 20µl could be loaded, giving a 100µg protein per lane. The calpastatin protein was detected using a monoclonal antibody from Sigma (details in Appendix D) following which the membrane was stripped using stripping buffer (Pierce, described in section 2.10.8) and was re-probed with an anti-Desmin antibody.

2.10.7 Chemiluminescence detection of proteins

If a secondary antibody conjugated with horse radish peroxidase was used, the luminescence solution required was EZ-ECL (Biological Industries, Israel), whereas for secondary antibodies conjugated with alkaline phosphatase enzyme the developing solution required was CDP star (GE Healthcare, UK). For EZ-ECL detection equal volumes of solutions A and B were added together depending on the surface area of the membrane (a total volume of 8mls was used for each large membrane containing around 25 samples), poured over the protein bound surface of the membrane and incubated at room temperature for 1 minute. For CDP-Star 6mls reagent was added per large 10cm gel, left to incubate for 5 minutes. The detection solutions were removed briefly using 3MM Whatman blot paper before the membrane was wrapped in cling film before securing into an autoradiograph cassette. Application of the detection reagents initiates a light reaction between the conjugated enzyme of the secondary antibody and the development solutions, the light emitted from this reaction was detected using autoradiography film (hyperfilm, GE Healthcare, UK). Exposure of the film to the membrane was dependant on strength of luminescence and usually varied between 15 seconds to 5 minutes. The film was then developed in a dark room by immersion in Kodak X-ray Developer solution for 2 minutes, a brief rinse in water then fixed by immersion in Ilford Hypam rapid fixing solution (both solutions were diluted 1 in 5 from stock solution with water, supplied by Calumet Photographic Co. (Nottingham, UK) for 2 minutes followed by final wash in water.

2.10.8 Stripping and re-probing with a second protein antibody

Following immunoprobing with the initial protein specific antibody, membranes were stripped of the bound primary and secondary antibodies to allow immunoprobing for a different protein. This allowed the same blot to be probed for a number of test proteins without repeating the initial electrophoresis and transfer. For all protein work performed using sheep tissue, the membrane was

washed in a pre-prepared stripping buffer (Pierce, UK) for 10 minutes followed by a brief wash in TBS-T. The blot was then blocked with 5% (w/v) marvel TBS-T and probed with another antibody.

For membranes used for protein detection in cell culture work where antibodies to both total protein and phosphorylated proteins were used to detect bands of the same size, the first antibody was removed from the membrane using 100mls of a stronger stripping buffer (0.09M SDS, 0.6M Tris and β-Mercaptoethanol added immediately prior to use to give 0.01M) added for each large membrane and heated in a 50°C water bath for 30 minutes. The membrane was then washed once in distilled water at room temperature on a bench top shaker for 5 minutes and twice in TBS-T solution for 5 minutes before the membrane was treated with detection reagents and developed to ensure there were no traces of the first antibody remaining. Following the second incubation with detection reagents, the membrane was washed again in TBS-T at room temperature to remove any remaining developing solutions, before the membrane was ready to be blocked with 5% marvel-TBS-T solution and re-probed with another primary antibody.

2.10.9 Quantification of protein blots

The protein bands on the developed blot were quantified using Bio Rad software (Gel Doc 2000, USA) and optical density of bands was measured using Multi Analyst image analysis software (BioRad, USA). Variation in signal detection (which included gel and blotting variation) across gels was corrected for using the protein standard replicate loaded on gels. Data was expressed as optical density of the band per weight of protein loaded per sample.

2.11 Cell culture methods

The final experiments of this study were performed in rodent skeletal muscle cell models in culture which were treated directly with active forms of vitamin D at differing time points. From these cell models total RNA and protein samples were prepared for analysis of vitamin D's direct signalling mechanisms within muscle cells. The scheme of this work followed three phases, a microarray analysis to identify novel targets of 1α,25 dihydroxyvitamin D_3 using primary skeletal muscle cells, selection of available muscle cell types on the basis of their myosin heavy chain gene expression and a final vitamin D_3 experiment on the chosen cell line to verify targets of the microarray and to evaluate the active form of vitamin D's possible role in fibre type maintenance. Primary skeletal muscle cell cultures were prepared from neonatal male rat pups as described below. These cells were removed directly from newborn rat muscle tissue and were allowed to proliferate in culture plates before addition of differentiation media which initiates formation of myotubes. These primary cell lines are most closely related to cells *in vivo* and provide a large number of identical samples

which can be treated directly with active vitamin D_3. High quality mRNA was extracted from these cells following treatment with vitamin D_3 and was analysed by microarray for novel targets in skeletal muscle tissue.

Although the microarray experiment was performed in the primary skeletal muscle cells, it was necessary to identify the myosin heavy chain genes which were expressed by this model as the purpose of the final experiment was to investigate vitamin D's effects in both developing and mature adult muscle. For the final experiment the muscle cells needed to express both the fast and slow myosin heavy chain genes so that any fibre type switching could be observed. Two other skeletal muscle cell models were assessed for their range of MHC expression, these were immortalised cell lines derived from rat, the L6 Aston, and from mouse, the C2C12 cell line. After identifying the cells which expressed the most appropriate range of MHC genes (see section 3.5 of this thesis), the final experiment of treating muscle cells directly with a vitamin D_3 analogue was performed at a range of doses and time points.

2.11.1 Preparation of primary skeletal muscle myoblasts from rat pups

This method of cell preparation generates proliferative myoblasts cultured from muscle tissue taken from neonatal male rat pups which can be differentiated into myotubes and treated with agents. This element of work was performed within Pfizer Ltd (Sandwich, UK) with the help of Mr Adrian Thompson and Dr. Christine Berger. Three day old male Wistar rat pups were killed by cervical dislocation and were placed in industrial methylated spirit (IMS) for approximately 1 minute to sterilise animal surfaces. Hind legs were then removed from the pups and the muscle tissue dissected and placed in falcon tube containing Dulbecco's modified eagle's medium (DMEM) until all tissue was harvested. All of the following preparation steps were performed in a sterile laminar flow hood using autoclaved equipment and sterile consumables. DMEM was poured off the tissue, and then the remaining liquid removed with Pasteur pipette. The tissue was then washed with 40mls tissue wash solution (4mls of Antibiotic-Antimycotic solution (Invitrogen Ltd.) added to 36mls of sterile phosphate buffered saline (PBS) with 40µl 1% w/v DNAse solution (deoxyribonuclease I type IV (Sigma, Ayrshire, UK) diluted with sterile deionised water to give a 1% w/v solution; stored in 100µl aliquots at -80°C to be thawed just before each preparation) by inversion three times. The tissue wash solution was decanted as before and the tissue washed again in 40mls sterile PBS. Finally the PBS was removed and the muscle tissue was poured onto the inverted petri dish lid. Muscle tissue was chopped using sterile scalpel blades on the petri dish lid for at least five minutes to break up the myofibrils and release single cells. The chopped tissue was then poured into the base of the petri dish and 10mls of tissue digestion solution was added (1.5mls Trypsin enzyme (concentration

of 25g/L in Hank's balanced salt solution (HBSS) Sigma, UK) was added to 13.5ml sterile PBS with 15µl 1% w/v DNAse solution). The lid was replaced and the dish placed into 37°C incubator for 30 minutes to allow the enzyme to digest proteins between the cells and release myoblasts further. Following the 30 minute incubation period with the tissue digestion solution, the cells were further disrupted by passing the solution through a 16G needle into a sterile syringe and dispelling again. This action was repeated 7 times each time returning the solution to the petri dish before drawing back into the syringe.

The dish was then returned to the incubator for another 10 minutes before the cells were passed through the syringe needle a further 7 times. The cells were then once more incubated for 10 minutes at 37°C and the syringe step repeated a final time. After the final disruption step the cells were retained in the barrel of the syringe, while 10mls of cell growth medium (125mls foetal calf serum (FCS) added to 500mls DMEM (20% FCS DMEM) with 7mls Antibiotic-antimycotic solution and warmed in a 37°C water bath prior to use) was used to wash the base of the petri dish and also drawn into the syringe barrel. This suspended cell solution was then filtered through a Swinnex-47 filter unit with a fitted wire mesh. The cell mixture was pushed through the filter and collected in a 50ml falcon tube. Following the cell solution a further 10mls cell growth media was passed through the filter unit and again collected in the 50ml falcon tube. This filter step was then repeated using another filter fitted with lens tissue, first passing the cell suspension through followed by another wash with 10mls cell growth solution, again collected into the final mixture. This solution was centrifuged at 1500rpm (Centaur 2, MSE) at 4°C for 5 minutes, before the majority of the supernatant was discarded leaving the cells in approximately 5mls media collected in base of the tube. A further 5mls of cell growth media was added to the cells and the pellet re-suspended by drawing into a pipette and releasing again.

2.11.1.1 Counting, Diluting and Plating Cells

1ml of the prepared cell solution was counted using a cell counter (Vi-Cell XR 2.03, Beckman Coulter). According to the number of viable cells, the solution was diluted with cell growth media to give a concentration of 5×10^4 cells per ml. Cells were then seeded at this density. In a 6-well plate format, 2.5mls of the solution was added to each well, giving a total of 12.5×10^4 cells per well, and a total of 7.5×10^5 cells per plate. Prepared primary myoblasts were plated into gelatine-coated 6-well plates (Biocoat, Becton Dickinson Labware, USA) at the above density and the solution swirled around in a figure of 8 to ensure even distribution of myoblasts, before incubation at 37°C with 5% CO_2 until confluent.

2.11.1.2 Fusing Cells

Once cells had proliferated to almost 90% confluence the cell growth media was substituted for cell fusion media (13mls donor horse serum (DHS) was added to 500mls DMEM (2.5% v/v DHS DMEM) with 6mls Antibiotic-Antimycotic solution (Sigma, UK) and warmed in a 37°C water bath prior to use). Cell growth media was removed with a disposable pipette and the cells washed gently with warmed sterile PBS, using 2mls PBS per well. PBS was then removed and the fusion media added, allowing 2.5mls per well. Cells were then returned to the incubator until required.

2.11.2 Culture of skeletal muscle cell lines

Two cell lines were used during this study, the rat-derived L6 Aston (provided by John Brameld, University of Nottingham) and the mouse derived C2C12 cell line (provided kindly by Philip Rhodes, University of Nottingham). All media, PBS and trypsin enzyme were warmed in a 37°C waterbath before use, unless stated otherwise. All work for cell line culture was performed in a sterile laminar flow hood and reagents were sterile and equipment autoclaved.

2.11.2.1 Thawing cells

Cells were frozen in 1ml freezing media (1ml dimethyl sulfoxide (DMSO) in 9mls FCS (10% DMSO FCS) and were defrosted quickly in a 37°C water bath. Upon thawing the cell solution was added to 10mls cell-line growth media (50ml fetal calf serum (FCS) into 500ml DMEM (10% FCS DMEM), supplemented with 10mls 200mM L-Glutamine and 10ml 10mg/ml Antibiotic/Antimycotic solution, Sigma, UK), pipetted up and down to ensure cells were dispersed before leaving to adhere in a 75ml flask. Media was changed for fresh cell-line growth media every 48 hours until cells were almost 90% confluent at which point cells were ready to be harvested.

2.11.2.2 Splitting and re-seeding cells

Cells were allowed to grow to 90% confluence in a 75ml flask before harvesting and re-seeding into 6-well plates for experiments. To harvest the adhered cells media was removed and cells washed by running PBS over the base of the flask. This PBS was removed and 2mls trypsin added to the flask before returning to the incubator for approximately 5 minutes. This allowed trypsin to digest the proteins which adheres the cells to the surface of the flask so that cells were free to be manipulated in suspension. After 5 minutes the flask was removed from the incubator and knocked slightly to aid release of cells, examination of the cells under a microscope confirmed cells were dislodged before continuation to next step.

When almost all cells had been released, 8mls cell-line growth media was added to the flask to neutralise the trypsin enzyme and prevent further damage to cells. This cell suspension as then moved to a sterile 50ml flacon tube and centrifuged at 700rpm (Centaur 2, MSE) at 4°C for 4 minutes. The supernatant from this step was decanted and cells re-suspended in 2mls fresh cell-line growth media. 20µl of this cell suspension was counted in a haemocytometer and the number of cells harvested was calculated. Cells were diluted in cell-line culture media and re-seeded at a density of 3×10^4 cells per well, requiring 1.8×10^5 cells per 6-well plate. Following passaging, cells were left to proliferate, renewing growth media every 48 hours until 90% confluent, at which point media was substituted for cell-line fusion media (10ml donor horse serum (DHS) into 500ml DMEM (2% v/v DHS DMEM), supplemented with 10mls 200mM L-Glutamine and 10ml 10mg/ml Antibiotic/Antimycotic solution) to promote myoblast differentiation. Cells were incubated in 37°C incubator with 5% CO_2 until required.

2.12 Cell Culture Experimental Procedures

To investigate the effect of 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and 1α(OH)-D_3 on cultured muscle cells, myoblasts of either cell lines or primary cells were required to have been grown in growth media until almost 90% confluent. The day of addition of cell fusion media was denoted as day 0 of differentiation and all time points post differentiation were counted from this day.

2.12.1 Diluting Vitamin D_3

Two vitamin D analogues were used in this study; for the microarray primary rat muscle cells were treated with the active 1α,25-dihydroxyvitamin D_3 and in the mouse C2C12 skeletal muscle cell line experiment, the analogue 1α hydroxyvitamin D_3 was used. Both forms of vitamin D_3 are biologically active and were purchased from Sigma (Germany), their structures are shown in figures 2.12-1 and 2.12-2.

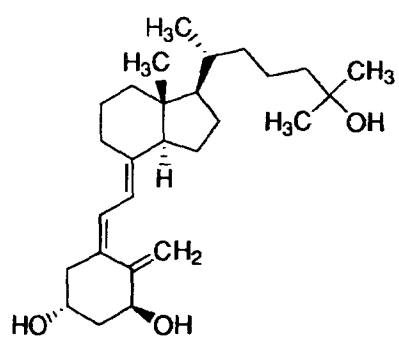


Figure 2.12-1 Structure of vitamin D_3 analogue used in rat primary microarray experiment; 1α,25-$(OH)_2$ vitamin D_3 also known as calcitriol.

Vitamin D_3 used in the rat primary muscle cells to produce mRNA for the microarray experiments was the activated form, 1α,25-dihydroxycholecalciferol , structure shown in figure 2.12-1, purchased from Sigma (Germany) and was first suspended in DMSO (1mg supplied, 1α,25-OH_2D_3 suspended in 249.5µl DMSO giving a 0.01M (10^{-2}M) vitamin D_3 solution),this solution did not require storage as all cells were treated on the same day for a 24 hour period.

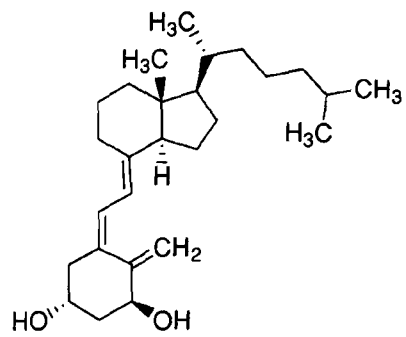


Figure 2.12-2 Structure of vitamin D_3 analogue used in muscle cell line experiment; 1,α-hydroxyvitamin D_3 also known as alfacalcidiol.

Vitamin D_3 used in the skeletal muscle cell lines to investigate vitamin D_3 direct signalling effects and to verify targets from the microarray was 1α-hydroxyvitamin D_3, structure shown in figure 2.12-2; a synthetic active analogue of vitamin D_3. This solution was stored at -20°C in the dark between treatment days, and this same stock was stored for up to 7 days over the course of the myoblast development experiment.

To treat cells using decreasing concentrations of 1,25$(OH)_2D_3$ or 1α(OH)-D_3, a series of dilutions was made, the first at 10^{-4}M was made taking 20µl of the 10^{-2}M vitamin D_3 analogue/DMSO solution and diluting 10 fold into 2mls cell fusion media (DMEM with 2% v/v DHS DMEM for cell lines, 2.5% v/v DHS DMEM for primary cells) or in cell growth media (DMEM with 10% v/v FCS) for treating myoblasts. Further dilution steps were made taking 20µl of the vitamin D_3/DMEM solutions and diluting 10 fold into another 2mls DMEM. These steps resulted in four dilutions of vitamin D_3 in DMEM; 10^{-4}M, 10^{-6}M, 10^{-8}M and 10^{-10}M. In addition; a 6-well plate of cells was treated with 0.1% v/v DMSO/DMEM solution (20µl DMSO added to 20mls DMEM, then 2.5mls added to cells per well) to be used as control samples.

2.12.2 Treatment of primary muscle cells with Vitamin D for microarray analysis

Three treatments were included in the microarray; DMSO/vehicle control, 1α,25 dihydroxyvitamin D_3 at 10^{-5} M, and 1α,25 dihydroxyvitamin D_3 10^{-9} M. Primary myoblasts were prepared as described in section 2.11.1 and were plated into gelatin coated 6-well plates at density of 2.5 X 10^4 cells/well and cultured for 4 days until 90% confluent. Cell fusion media (2.5% v/v DHS DMEM) was prepared with either 10^{-5}M, 10^{-9} M of 1α,25 dihydroxyvitamin D_3 (Sigma, Germany) or DMSO (0.1%

w/v)(preparation of vitamin D dilutions is described in section 2.12.1) and the original growth media on three 6-well plates was substituted for each treatment. Cells were then incubated in the treatment media for 24 hours. The following day cells were harvested in either protein extraction buffer (section 2.13.5; three replicates per treatment group) and frozen at -20°C or RLT extraction buffer (cell lysis buffer containing guanidinium thiocyanate, supplied with RNeasy midi kit described in section 2.16.1; three replicates for each treatment) and frozen at -80°C. Total RNA was prepared for the microarray using an RNEasy mRNA midi kit (Qiagen) and quantified on a nanodrop (ND-1000 Nanodrop Products; Thermo Fischer Scientific, Wilmington, USA) before checking the integrity using a Bioanalyser (Agilent). The microarray was performed by Mr. Thomas Wilson of Pfizer (Kalamazoo, MI, USA), using an Affymettix Genechip Microarray for Rat V.2.0. For analysis, relative expressions of genes in each $1,25(OH)_2D_3$ treatment group were subtracted from the relative expression of genes in the DMSO control group. The genes that were then changed with each vitamin D_3 treatment could be expressed as a fold change relative to DMSO controls.

2.12.3 Characterisation of Myosin Heavy Chain Gene expression in skeletal muscle cells.

To evaluate the cell lines for their use as a model to investigate vitamin D's possible effects in fibre type switching/maintenance, the expression profile of the MHC genes in each cell line as they differentiated was assessed. All cells were grown to 90% confluence in their respective cell growth media before differentiation was induced by substituting cell growth media for cell fusion media (2.5% v/v DHS DMEM for rat primary cells, 2% v/v DHS DMEM for cell lines). Cells were grown in 6-well plates and at each time point three wells were used to produce replicates of total RNA and three wells were used for protein samples. All RNA samples from all cell types were prepared for analysis using TRIzol reagent (section 2.13.4) and protein samples were prepared as described in section 2.13.5.

The day of addition of cell fusion media was denoted as day 0 and at this point the majority of cells were still myoblasts undergoing proliferation. Total RNA and protein was extracted from cells at this point before differentiation media was added and were denoted as Day 0 samples. Following addition of differentiation media, cells were allowed to differentiate into myotubes for up to 7 days and samples were extracted at days 2, 4 and 7 producing four time points at which the MHC expression could be validated.

Extracted total mRNA, prepared as described in 2.13.4 and reverse transcribed using MMLV RT enzyme (section 2.7.5) and using previously published MHC primers (see Appendices E and F for rat and mouse primers respectively), the expression of each MHC gene was validated. All cDNA was amplified for each MHC gene using the semi-quantitative PCR method (section 2.8.1) and

electrophoresed in agarose gel before imaging, described in section 2.8.2. As a positive control, total RNA prepared from adult mouse or rat LD was used to demonstrate positive bands in corresponding cell lines. To verify the expression of the MHC genes at mRNA level, the expression at protein level was detected using the protein samples and the western blotting technique (section 2.10) identifying MHC fast and slow proteins using monoclonal antibodies developed to each isoform (Nova Castra) summarised in Appendix D.

2.12.4 Treatment of Skeletal muscle cells with 1αOHD$_3$ for gene and protein expression analysis

Once the skeletal muscle cell line was chosen on the basis of MHC gene expression, a final experiment using 1αOHD$_3$ was performed. The cells were treated directly with 1αOHD$_3$ at a range of concentrations made up as described before in section 2.12.1. Skeletal muscle cells were treated at four developmental stages, the first stage was during myoblast proliferation where cells were treated with 1αOHD$_3$ solutions made in cell growth media. Three stages during differentiation were then used as time points for the differentiating cells, day 0 when myoblasts were the majority of cells present, at day 3 when myotubes were beginning to form and finally at day 7 when differentiated myotubes were present (see figure 3.6-1). At all time points, 1αOHD$_3$ treatment was added within the corresponding media and incubated for 48 hours before protein and total RNA extraction. Prior to harvest, cells were imaged using Leica Application Suite Software (version 2.5.0, Leica Microsystems, Switzerland) through a Leica DMIL microscrope (Leica Microsystems, Switzerland).

2.12.4.1 Treatment of cells at proliferating myoblast stage

Undifferentiated mouse C2C12 myoblasts grown to 75% confluence were treated with either 0.1% v/v DMSO or 1αOHD$_3$ at decreasing concentrations in cell growth media (10% v/v FCS DMEM) for 48hours. Cells were not induced to differentiate by addition of cell fusion media. 1αOHD$_3$ solutions were made up as described in section 2.12.1 and cells were cultured in treated growth media for 48 hours before total RNA and protein extraction. Each 1αOHD$_3$ dose required a full 6-well plate of cells, giving three wells to be extracted for proteins (three biological replicates) and three for total RNA extraction. To treat cells with 1αOHD$_3$, any media the cells had previously been incubated in was removed and 2.25mls fresh cell growth media was added. 225µl of the diluted 1αOHD$_3$ was then added to the fresh media in each well giving a final 10 fold dilution and the subsequent concentration of 1αOHD$_3$ on the cells were 10^{-5}M, 10^{-7}M, 10^{-9}M and 10^{-11}M. Myoblasts were

incubated for 48hours in the treatment media before extraction of protein and total RNA as described in section 2.13.

2.12.4.2 Treatment of cells at different stages of differentiation

Skeletal muscle cells were grown in cell growth media until 90% confluent at which point cell fusion media was added, denoted as Day 0. For each time point 5 x 6-well plates were required for each $1\alpha OHD_3$ dose and a DMSO control. For treatment of cells with $1\alpha OHD_3$ at day 0, growth media was removed from the cells and 2.25mls cell fusion media (2% v/v DHS DMEM) added, before 250µl treatment solutions (DMSO or $1\alpha OHD_3$ in cell fusion media) were added as described in section 2.12.1. Cells were incubated in treatment media for 48hours before media was removed and total RNA and protein samples extracted as described in section 2.13. For cells to be treated at later stages of differentiation, cell fusion media was added as normal without $1\alpha OHD_3$ treatment, when cells reached required age at either day 3 or day 7 post differentiation, media was removed and cell fusion media containing $1\alpha OHD_3$or DMSO was added and the cells incubated for 48 hours prior to protein and total RNA harvest.

2.13 Extraction of RNA and protein from cultured cells

To determine the expression of genes and proteins in cultured cells the total RNA and protein was extracted. For both extractions media was removed and extraction solutions were added quickly to minimise cell damage.

2.13.1 Harvest of mRNA from Primary cells for Microarray analysis

Total RNA was extracted from primary cell cultures using RNeasy mini kit (Qiagen) and supplied buffers and consumables were used as guided by the manufacturer's instructions. Media was removed immediately prior to extraction and 350µl of cell lysis solution, Buffer RLT, was added to aid cell dislodging and lysis. Removal of cells from the growth surface was further encouraged by repeat pipetting of Buffer RLT across the surface in addition to scratching the surface with the pipette tip. Samples were then frozen in Buffer RLT at -80°C until further extraction could be carried out, where the protocol according to manufacturer's instructions was followed. Extracted total RNA was dissolved in 40µl RNAse/DNAse free water, subjected to DNAse digest (DNAse I enzyme supplied with RNeasy kit, Qiagen) and quantity and quality of extracted RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies).

2.13.2 RT step with SuperScript III RT enzyme– expression of MHC using PCR

The Superscript III First-strand Synthesis System (SS-III) for RT-PCR (Invitrogen, UK) was used to generate cDNA for analysis of gene expression in rat primary muscle cells. All reagents required for cDNA synthesis were supplied in the SS-III kit and were used at the manufacturer's recommended proportions. All RNA was diluted to 0.1µg/µl according to total RNA concentration.

2.13.2.1 Denaturation of RNA and annealing of Primers

1µl random hexamers (at concentration of 0.5µg/µl) with 1µl 10mM dNTP mix was added to 5µl total RNA in a thin-walled microcentrifuge tube and the mixture made up to 10µl with water, before incubating at 65°C for 5 minutes. This mixture was then immediately moved to ice for quick cooling, this promoted annealing of primers to denatured RNA, and the samples were left on ice for 1 minute and briefly centrifuged to collect at the base of the tube before addition of the RT enzyme.

2.13.2.2 cDNA synthesis

The following reagents were added to each sample tube; 2µl 10 x RT buffer, 4µl 25mM $MgCl_2$, 2µl 0.1M DTT, 1 µl RNAse Inhibitor RNAse OUT (40 units, supplied with SS-III kit) and 1 µl SS-III reverse transcriptase enzyme (100 units), and the final volume of the reaction mix made up to 20 µl with water. The contents of the tube were then vortex mixed for approximately 15 seconds and briefly centrifuged to collect at the base of the tube, before incubation at room temperature for 10 minutes to allow the reverse transcriptase to begin extension from the random primers. The samples were then incubated at 50°C for one hour to complete cDNA synthesis. The reaction was inactivated by heating to 85°C for 5mins before collection by centrifugation and removal to ice. The reaction mixtures were diluted 1:5 with water to create a 100 µl stock solution of cDNA for each sample which was stored at -20°C.

2.13.3 Microarray

Total RNA prepared for the microarray was shipped to Pifzer Ltd in Kalamazoo in USA to be analysed by microarray, performed by Mr. Thomas Wilson using the Affymetrix Rat 230_2.0 array.

Briefly there are five steps to the reaction,

1. Target preparation: double stranded cDNA is created from the mRNA. An *in vitro* transcription (IVT) reaction is then performed to create biotin-labelled cRNA from the cDNA, this cRNA is fragmented before the hybridization step.

2. Target Hybridization: A cocktail of reagents for hybridization including the fragmented target and the probe array controls was prepared before incubation for 16 hours to hybridize the reagents to the probe array.

3. Fluidics station set up.

4. Probe array washing and staining: the probe array was washed and stained immediately after hybridization.

5. Probe array scan: the probe array must be scanned

6. Data Analysis

2.13.4 Microarray Analysis

The microarray used an affymetrix Rat 2.0 chip and measured the relative expression of mRNA of 31,099 genes. The data was analysed by Dr Neil Graham of the Arabidopsis Stock Centre, University of Nottingham, using Genespring software (Strand Life Sciences R&D Division, Bangalore, India). The relative expression of genes in each vitamin D_3 treatment group were subtracted from the relative expression of genes in the DMSO control group, in a T-test performed in Genespring. This analysis gave a list of genes which had changed more than 1.5 fold, either up or down, with a significant P value of $P<0.05$). The list of changed genes was then uploaded into Ingenuity Pathway Analysis (IPA Version 7.1; Ingenuity Systems Inc, 2000-2008) software to determine the functions and related pathways of these genes to further understand the mechanism of vitamin D_3 signalling in muscle cells.

2.13.5 Harvest of total RNA and protein from skeletal muscle cell lines

2.13.5.1 Harvest of total RNA

TRIzol® Reagent (Invitrogen, UK) was used to harvest the mRNA from cultured cell lines. Media was removed and 1ml TRIzol® reagent added to each well; usually three wells per plate were used as biological replicates. The reagent was left for approximately 30 seconds and the plate rocked slightly to ensure coverage of all cells. To encourage removal of all cells, the surfaces of the wells were scraped with the pipette tip and the reagent repeat pipetted over the surface of the well. Once all the contents of the well were dispersed in the TRIzol® the solution was moved to a sterile 1.5ml tube and stored on ice until all wells were harvested, at which point samples were frozen at -80°C until further steps of the extraction could be carried out from the phase separation stage, as described in section 2.7.

2.13.5.2 Harvest of proteins

1xSDS-DTT mix (20% v/v glycerol, 12.5% v/v 1M Tris/HCl pHed to 6.8, 4% w/v SDS (Sodium Doecyl Sulphate), 15.4% w/v Dithiothreitol (DTT), without addition of bromophenol blue) was used to extract the proteins and store them until further analysis. 1ml 1xSDS mix without bromophenol blue was added to each well and left for approximately a minute on the cells, rocking the plate slightly to ensure even coverage. The plate was then scraped using a pipette tip and the mixture repeat pipetted, passing over whole surface area of the well to aid cell dislodging and lysis. Once all contents of the well were suspended the solution was transferred into a sterile 1.5ml tube and kept on ice until all samples were harvested. Protein samples were then stored at -20°C until further analysis. Proteins were quantified using the Plus-One 2-D quant kit (Amersham, Buckinghamshire, UK) and diluted to constant protein concentration.

2.13.6 Determination of protein concentration by 2-D Quant Kit

Samples taken from cell cultures were extracted directly into denaturing SDS protein running buffer and needed to be quantified using a technique that would tolerate a high concentration of SDS and DTT in the sample. The 2-D quant kit (Amersham Biosciences, UK Ltd., Buckinghamshire, UK) overcomes the interference from interfering substances as it precipitates the protein out of the solution containing possible interfering compounds and re-dissolves it into an alkaline solution containing copper ions for analysis. The copper ions bind to the protein in the solution and the strength of blue colour of the solution is relative to the quantity of unbound copper, measured using a spectrophotometer. The kit supplies all solutions required for the assay, these are BSA stock protein solution, precipitant, co-precipitant, copper solution and colour reagent. Colour reagent solution must be prepared before the assay is carried out by addition colour reagent B to colour reagent B at a ratio of 100:1, allowing 1ml per sample. A set of Bovine Serum Albumin (BSA) protein standards were created by taking a set volume of a stock BSA at 2mg/ml (provided with kit)into an eppendorf, to give a known quantity of protein over a range from 10µg to 50µg (table 2.13-1).

Table 2.13-1 Creation of protein standards for 2-D Quant Assay

Standard Curve						
Volume of 2mg/ml Stock BSA Solution (µl)	0	5	10	15	20	25
Equivalent protein quantity, µg	0	10	20	30	40	50

2.13.6.1 Precipitation of the protein

500µl Precipitant solution (provided in kit) was added to 10µl of the samples in SDS-Mix DTT in an eppendorf (each sample in duplicate). The tubes were then vortex mixed and the left to incubate at room temperature for 3 minutes. 500µl of the co-precipitant solution was then added and the contents mixed by inversion before the protein was pelleted by centrifugation at 13, 000 rpm at 4°C for 5 minutes. The supernatant was removed without disturbing the protein pellet before addition of 100µl copper solution with 400µl distilled water. The contents of the tube were vortex mixed to encourage the precipitated protein to dissolve. Once the protein was dissolved in the copper solution, 1ml of colour reagent was added to each sample and the samples mixed by vortex immediately to allow the solutions to mix rapidly. The solutions were then incubated at room temperature for 20 minutes before the absorbance was read at 480nm. A standard curve was plotted in Microsoft Excel (V. 2007) and the equation of the line was used to calculate the relative protein concentration of the unknown samples.

2.13.7 Western Blotting of C2C12 cell samples

Two proteins were measured in the C2C12 cells using SDS-PAGE and western blotting analysis, described in sections 2.10.3 to 2.10.9; these were 4EBP1 and MAPK ERK1/2. Product information, SDS-PAGE conditions and development solutions for these antibodies are shown in Appendix D. Cell protein samples were diluted to a constant concentration of 0.25 µg/µl so that a volume of 20µl loaded resulted in a load of 5µg protein for SDS-PAGE. Both protein targets were detected using the total antibody before stripping and re-probing with the antibody to phosphorylated protein using the ECL advance detection kit. To ensure complete removal of the initial total antibody the stripped membranes were treated with detection reagent and hyperfilm detected as for usual luminescence detection, this ensured any remaining luminescence would be detected and expended. In both proteins the removal of the total antibody by the stripping technique described in section 2.10.8 was successful and so membranes could be re-probed with the phosphorylated antibody with assurance there would be no interference from the previous antibody. The western blots produced were analysed as described before (section 1.10.9) and a ratio for the level of phosphorylation was calculated, dividing the absorbance for phosphorylated protein by the absorbance for the total protein.

2.13.8 Real Time PCR using LC480 system

Primers and probes for MHC analysis were published by da Costa *et al.* (2007; oligonucleotides purchased from Sigma), mRNA sequences and oligonucleotide information are summarized in

appendix F. Oligonucleotides purchased from Sigma were first checked using semi-quantitative PCR and validated by electrophoresis using agarose gel electrophoresis described in section 2.8.1 and 2.8.2. Further targets were investigated using assay on demand kits purchased from Applied Biosystems (in Appendix G). A primer and probe set for Desmin was created using Primer Express Version 1.5, shown in Appendix F.

In total three housekeeping genes, 7 targets of vitamin D_3 signalling and 6 isoforms of MHC were measured by real time PCR, summarized in Table 2.13-2 (expression of neonatal mRNA was too low to quantify satisfactorily and was removed from analysis).

Table 2.13-2 Summary of gene targets and their sources measured in C2C12 experiment and the standard curve and cDNA dilution used for analysis.

Gene	Primer/Probe Source	Standard Curve	Dilution
Beta Actin	da Costa *et al*., 2007	LD Muscle & Day 10 Cell	1:50
Cyclophilin A	ABI Assay on Demand	LD Muscle & Day 10 Cell	1:50
GAPDH	ABI Assay on Demand	LD Muscle & Day 10 Cell	1:50
Myf5	da Costa *et al*., 2007	Day 10 Cell	1:50
Myogenin	ABI Assay on Demand	LD Muscle	1:50
Desmin	Primer Express V5.0	LD Muscle	1:4
Metallothionein 2A	ABI Assay on Demand	Day 10 Cell	1:50
Parvalbumin	ABI Assay on Demand	LD Muscle	1:50
C/EBPβ	ABI Assay on Demand	Day 10 Cell	1:50
c-Myc	ABI Assay on Demand	Day 10 Cell	1:50
Embryonic MHC	da Costa *et al*., 2007	Day 10 Cell	1:50
MHC I/β	da Costa *et al*., 2007	LD Muscle	1:4
MHC IIA	da Costa *et al*., 2007	LD Muscle	1:4
MHC IIX	da Costa *et al*., 2007	LD Muscle	1:50
MHC IIB	da Costa *et al*., 2007	LD Muscle	1:50

2.13.8.1 Standard curves used in analysis of C2C12 mRNA transcripts

For each gene that was analyzed, two separate standard curves were run on the plate with the unknown samples allowing the possibility to quantify the results relative to either set of standards. A standard curve was made from pooled cDNA created from mRNA extracted from adult mouse LD tissue. A second standard curve was created from cDNA from C2C12 myotubes grown for 10 days after onset of differentiation, denoted the Day 10 standard curve. The use of two standard curves was necessary owing to the nature of the expression pattern of certain genes during the differentiation process, in particular the MHC genes. It was found that the developmental MHCs were not expressed highly in the LD and therefore needed a standard curve created from C2C12 cDNA, whereas in contrast the adult MHC genes appeared over the course of differentiation and the result was that unknown samples did not lie in the linear range of the C2C12 cDNA curve. The

differences between the positions of the unknown samples relative to either the LD or day 10 standard curve in the example of the MHC IIA gene is demonstrated in figure 2.13-1, showing that sample data would need to be extrapolated should the Day 10 standard curve be used.

Therefore, the C2C12 Day 10 standard curve allowed the measurement of genes which were highly expressed in differentiating muscle cells such as the myogenic regulatory factor Myf5, and the embryonic and neonatal isoforms of MHC; whereas the LD standard curve was required for the measurement of genes whose expression appeared over the course of differentiation where all samples would not lie in the linear range of the Day 10 standard curve, such as the adult MHC isoforms. The comparison of the mRNA expression changes induced by vitamin D treatment were made only within each individual gene and there were no comparisons made between different genes. All three housekeeping genes which were investigated lay on the linear range of both standard curves and hence all results produced could be normalized to a housekeeper, however there were significant effects of vitamin D_3 treatment on these internal standards and the results were not normalized to any housekeeping gene, taking the normalisation of total RNA prior to RT step into consideration instead. The preparation of the RNA was of a high quality shown in Appendix H and total RNA concentration had been adjusted to a constant concentration prior to the RT step.

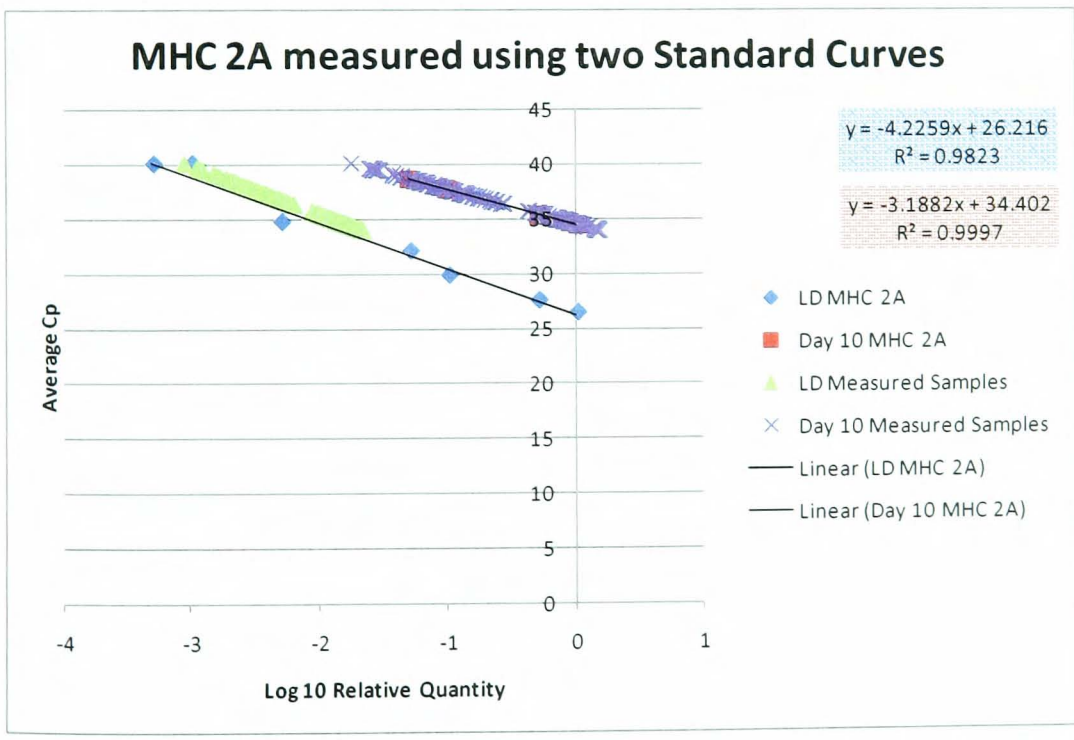


Figure 2.13-1. Position of unknown sample MHC IIA (2A) expression when measured relative to 2 cDNA standard curves, mouse LD and C2C12 cell aged 10 days.

2.14 Statistical analysis

All data produced in the two sheep trials were analysed by paired T-tests, except for the shear force data across the aging period, which was analysed by two-way ANOVA using Genstat V.11. In the event of missing samples both the lost animal and its opposite treatment pair were removed from the analysis. For the microarray data a T-test was performed using Genespring software. For the cell culture work, data was analysed by either ANOVA or two-way ANOVA using Genstat V.11. In the case of a significant P value result from ANOVA analysis ($P<0.05$ or below), a Dunnett's test (Dunnet C.W., 1964) was performed *post hoc* using least significant difference values (LSD) from the Genstat ANOVA output and the relevant T value to calculate the treatment values which were significantly changed in comparison to the DMSO control.

Results:Vitamin D & Meat Quality Trials

3.1 Trial 1: 4 Days Vitamin D

Previous published work in sheep has investigated the supplementation of vitamin D immediately prior to slaughter as a mechanism to increase calcium levels in the muscle to activate the calpain proteolytic system post mortem. Neither of the two published studies fully investigated the calpain system to verify the mechanism of the potential effects and were inconclusive in terms of evaluating vitamin D's capability in activating the calpain system via increased calcium status. The first trial performed by Wiegand *et al.* (2001) did provide data to suggest that a high dose of vitamin D at 2 x 10^6 IU Vitamin D_3/day was sufficient to increase calcium levels, but this study focused on reducing the effects for the callipyge genotype on shear force, a breed with characteristically high protein growth through decreased protein degradation resulting in tough meat (Koohmaraie *et al.*, 1995), and this study found there was no significant effect of dietary vitamin D on shear force in this genotype. This study did observe a reduced shear force when comparing between the vitamin D Vs control diet regimes in normal genotype animals, but these results were not significant. There was no additional work to investigate effects on the calpain system despite finding calcium levels were significantly higher in the serum and numerically higher in the muscle. Work in this laboratory has previously investigated the effect of a short supplementation period of 4 days of vitamin D_3 at 2 x 10^6 IU Vitamin D_3/day with the additional effect of a calcium bolus on the final day. This trial did not observe any improvement of LD chops, nor effect on the calpastatin levels at 0 and 24 hours post mortem. This previous study found a significant increase in serum calcium levels by nearly 7% ($P<0.001$) but did not investigate further effects on muscle calcium levels or the calpain enzyme.

The aim of this trial was to investigate the effects of supranutritional level of dietary vitamin D for a short duration immediately prior to slaughter. The hypothesis was that a short, high level of dietary vitamin D would cause an increase in biologically active form of vitamin D which would lead to a response in calcium homeostasis of the animals before, presumably, the active hormone was catabolised by the kidney. In addition the large dose of vitamin D would increase calcium uptake from the gut and this would increase calcium levels in the muscle and subsequently activate the calpain system post mortem. Therefore the objective of this study was to investigate any signalling effects that vitamin D_3, and/or the change in calcium status, could have on the calpain system at the mRNA and protein level, as well any effect on meat quality.

3.1.1 Trial Outline

Thirty six Mule X Charolais wether lambs at approximately 7 months of age were individually penned and allocated to two treatment groups consisting of control (n=18) and high vitamin D_3 (n=18). All animals were housed and fed the basal diet as described in section 2.2. The basal diet period lasted 3 weeks and allowed for acclimatisation to the housing and diet before subsequent treatment. The animals were weighed once a week to ensure a weekly weight gain, and feed intake was recorded daily. A week before the supplementation period the paired sheep were reallocated to pens according their size and age so that they could be slaughtered in weight order, beginning with the heaviest and finishing with the lightest, as described by the housing layout shown in diagram 2.2.2-A. A period of 4 days before slaughter date the VIT D_3 group (n=18) were fed the basal diet supplemented with vitamin D_3 (Rovimix D_3 500, DSM Ltd, Derbyshire UK), supplying 2 x 10^6 IU Vitamin D_3/day split between two feeds, whilst the control group (n=18) still received the same basal diet. The composition of the diets is shown in table 2.2.2.; there were no differences in the energy, nitrogen or calcium levels of the diets. Animals were allocated into three slaughter groups (n=12) according to age and size with vitamin D (n=6) and control (n=6) paired animals slaughtered alternately across each slaughter day (total of 12 animals/day). The start of vitamin D_3 supplementation for each group was staggered across three days, shown in the timeline of the trial in figure 3.1-1. Animals were slaughtered, carcass measurements taken and samples collected according to procedure previously described in chapter 2 of this thesis, section 2.2. LD chops were vacuum packed and left to age for 3, 7, 14 days at 4°C according to latin square arrangement presented in Appendix A, this allowed the length of the LD to be represented in each day of aging, this was important as tenderness across the LD can vary greatly (Kerth *et al.*, 2002). Due to varying sizes of the animals, some LD muscles did not yield 4 chops and so the first three chops of all animals were sampled in a latin square arrangement to assess tenderisation across the 14 day aging period. The LD of larger animals did yield four chops and so the extra chops, denoted chop #4, were sampled after 21 days of aging to assess ultimate shear force, denoted in the diagram in Appendix A.

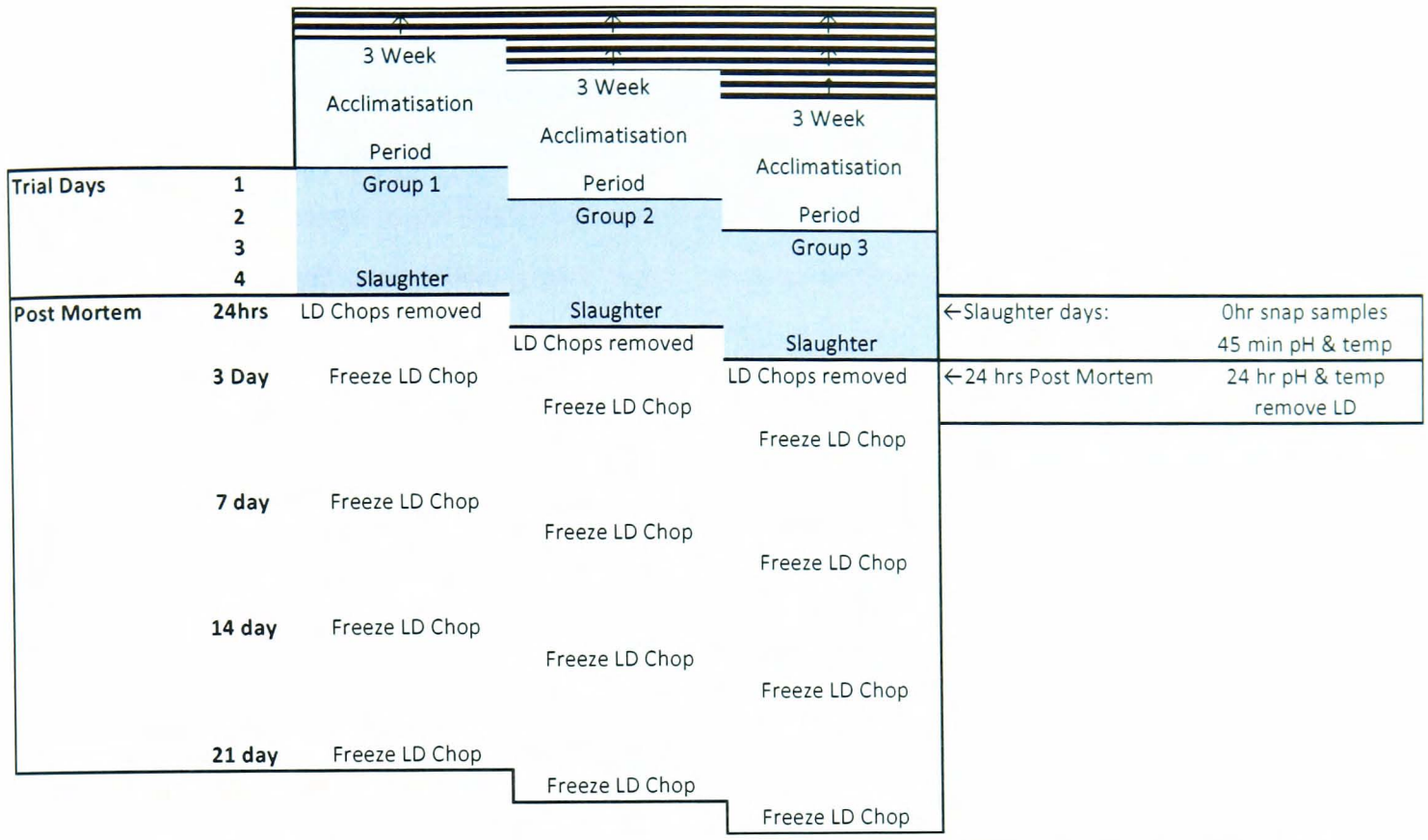


Figure 3.1-1 Timeline for Trial 1: 4 days vitamin D immediately prior to slaughter. Shown in darker blue box is the duration of vitamin D supplementation at 2 x 10^6 IU/day, duration of 4 days immediately prior to slaughter.

3.1.2 Meat quality results: Trial 1; 4 days vitamin D_3.

Animals were weighed weekly for the duration of the trial and the morning before slaughter. Feed intake was monitored by recording any refusals from the daily ration of 1.4Kg feed, and on the slaughter days three animals fed the vitamin D diet in each slaughter group refused less than 350g (25%) of their daily ration. Across the four day supplementation period, thirteen animals in total refused some feed, nine of these were vitamin D-supplemented animals and this amounted to between 2 and 17% of the total trial feed fed across the four days. Of the nine sheep which refused feed on the slaughter days, eight were vitamin D diet group and one was from the control group, although all animals ate at least 75% of their diet on this day. These refusals were not considered to be significant as they were not associated with effects on the liveweights, carcass weights or pH of the carcasses and the level of feed refusal did not associate with plasma vitamin D or serum calcium levels (data not shown) and all vitamin D fed animals had significantly higher plasma vitamin D than control animals. There were no significant differences between treatment groups in liveweight before and after, growth and feed conversion ratio (FCR), results shown in table 3.1-1. Carcasses were weighed immediately after slaughter and dressing percent calculated from final liveweight and hot carcass weight (taken immediately after dressing); there were no significant effects of diet on carcass weight or dressing percent. Any differences in dressing percent would be attributed to changes in gut fill and so any significant effects of the feed refusals during the trial week would

affect the dressing percent of the carcasses, however there were no differences between the animals and the refusals can be considered insignificant. pH of the carcass was measured in the LD at the region of the initial snap sample (demonstrated in Appendix A) 45 minutes after slaughter and 24 hours later, the change in pH (ΔpH) was calculated from the two values. As described in chapter 1, muscle pH decreases post mortem as a result of anaerobic glycolysis and the extent of this pH change is affected by glycogen levels pre-slaughter (Pösö & Puolanne; 2005). If the animals had been stressed prior to slaughter or had been fasted then it would be expected that the development of muscle pH would have been affected (Peterson *et al.*, 1997). However, there was no effect of vitamin D_3 supplementation for 4 days immediately prior to slaughter on carcass pH or subsequent pH changes (table 3.1-1); suggesting no effect of diet on pre-slaughter glycogen depletion which could have had detrimental effects on carcass and meat quality.

Table 3.1-1 Effect of 4 days vitamin D_3 supplementation on animal growth, feed intake and carcass characteristics, n=18.

		Control	Vitamin D	S.E.D.	P
Initial Lamb Weight (Kg)		**35.49**	**35.87**	0.801	**0.428**
Final Lamb Weight (Liveweight) (Kg)		**41.08**	**41.03**	0.855	**0.914**
Total Weight Gain (4 weeks) (Kg)		**5.594**	**5.161**	0.636	**0.516**
Average Daily Gain (ADG) (Kg/Day)		**0.1868**	**0.1725**	0.0212	**0.512**
Feed Conversion Ration (FCR)		**13.59**	**12.6**	1.54	**0.529**
Carcass Weight (Kg)		**19.14**	**19.13**	0.514	**0.966**
Dressing Percent (%)		**46.59**	**46.61**	0.774	**0.978**
LD pH	**0 Hr**	**6.289**	**6.198**	0.0604	**0.194**
	24 Hr	**5.839**	**5.828**	0.048	**0.452**
LD ΔpH		**0.450**	**0.371**	0.0667	**0.249**

Plasma samples were analysed for 25 hydroxy-vitamin-D_3 (25(OH)-D_3) and -D_2 levels using tandem mass spectrometry by Professor Bill Fraser (University of Liverpool). 25(OH)-vitamin D is routinely used to assess overall vitamin D status of animals as circulating $1,25(OH)_2D_2$ levels are tightly regulated (Li *et al.*, 2009). 25(OH)-Vitamin D_3 levels were significantly raised ($P<0.001$) by the 4 days vitamin D_3 supplementation, and as expected 25(OH)-Vitamin D_2 levels were unaffected (table 3.1-2). The form of vitamin D supplemented in the diet was vitamin D_3, and these results demonstrate the changes in vitamin D status were implicated by the diet. This is evidence to show that four days of vitamin D_3 supplementation at 2×10^6 IU Vitamin D_3/day was effective in altering 25(OH)-vitamin D_3 status of the animals; increasing the treated animals circulating D_3 levels by a factor greater than

10. Serum and muscle calcium were analysed using Atomic Absorption Spectroscopy by Dave Bozon (University of Nottingham) as described in section 2.5. There was no effect of 4 days vitamin D_3 supplementation on calcium levels in the serum despite the significant increase in plasma vitamin D_3 levels (table 3.1-2) and subsequently there was no effect on muscle calcium levels, results expressed as mg/g wet tissue.

Table 3.1-2 Effect of 4 Days Vitamin D_3 Supplementation on plasma Vitamin D, serum and LD calcium levels.

	Control	Vitamin D	S.E.D.	P
D_3 ng/ml	**27.8**	**319.6**	18.85	**< 0.001**
D_2 ng/ml	**10.1**	**8.2**	1.62	0.309
Serum Calcium (mg/L)	**119.4**	**121.7**	4.479	0.633
LD Calcium (mg/g wet tissue)	**58.34**	**52.91**	5.472	0.342

Vitamin D_3 and D_2, n=17; serum and muscle calcium, n=18.

LD chops were aged for 3, 7, 14 and 21 days at 4°C before shear force was determined by Warner-Bratzler method (Wheeler *et al.*, 1997). Chops aged between 3 and 14 days were sampled by latin square arrangement as described previously (section 3.1, trial outline) and were analysed by two-way ANOVA; chops aged for ultimate shear force after 21 days were sampled separately due to smaller n of samples and were analysed by t-test. Shear force of LD chops was significantly changed by time during the aging process as was expected (P<0.05, table 3.1-3), however, there was no difference between the treatment groups on shear force of LD chops. Shear force following 21 days of aging was not affected by diet (P=0.603).

Table 3.1-3 Effect of 4 days vitamin D_3 on shear force (Kg) of LD, n=18 for aging period analysed by two-way ANOVA, n=12 for 21 day LD chops analysed by t-test.

	Time (days)					
Diet / Age	**3**	**7**	**14**		**S.E.D.**	**P**
Control	4.87	4.50	4.30	**Diet**	0.201	0.351
Vitamin D	5.17	4.77	4.30	**Age**	0.247	**0.017**
				Diet*Age	0.349	0.808
	Control	**Vitamin D**				
21 day shear force LD	4.32	4.50			0.381	**0.603**

Fat content of the LD was determined for a subset of 6 samples, taking two pairs from each slaughter group. Muscle fat was extracted by the Soxtherm Method and calculated as a percentage from the initial weight of muscle from which the fat was extracted as described in section 2.4. There was no significant effect of 4 days vitamin D_3 supplementation on fat content of the LD (table 3.1-4).

Table 3.1-4 Effect of 4 Days Vitamin D_3 Supplementation on LD Fat Content, n=6.

	Control	Vitamin D	S.E.D.	P
LD Fat Content (%)	**9.02**	**8.39**	1.614	**0.568**

3.1.3 Vitamin D effects on gene expression: Trial 1; 4 days vitamin D_3.

Despite no effect of the vitamin D_3 supplementation on the immediate vitamin D responses observed at the calcium homeostasis level, there were significant changes in the circulating 25(OH)-vitamin D_3 levels which demonstrates an increase in vitamin D status and so there could be potential direct signalling effects of vitamin D on gene expression in target tissues. To determine whether the circulating vitamin D_3 had been able to mediate effects via gene expression, specific targets were measured at both the mRNA and protein level using reverse-transcriptase real-time PCR and western blotting respectively. Expression of mRNA of calbindin D-28K was measured in the kidney, a gene known to be up-regulated by vitamin D in its role in mineral homeostasis (Minghetti & Norman, 1988), and the levels of mRNA of the calpain system were measured in LD to investigate whether the dietary vitamin D_3 could modulate shear force via this mechanism. Expression of β-actin at mRNA level was measured and was found to be unchanged by diet treatment in both LD and kidney tissue (data shown in table 2.9-3 in chapter 2), therefore all data was normalised to β-actin mRNA and is expressed as a ratio of transcript mRNA: β-actin mRNA. Expression of calpastatin at the mRNA level was not affected by 4 days vitamin D_3 supplementation, however, expression of the calpain enzymes was significantly altered. There was a trend for expression of calpain I (μ-calpain) to be increased by 3.7% ($P<0.1$) whereas calpain II (m-calpain) expression was significantly increased by 10% ($P<0.05$) following 4 days vitamin D_3 supplementation (table 3.1-5). The increase in both these enzymes at the mRNA level presents the possibility of an effect at the protein level, however, data shown in table 3.1-5 indicated this was not translated to the protein level. It is possible that the short term vitamin D_3 supplementation was not sufficient to cause overall changes in meat quality but was beginning to exert effects on the enzymes responsible for the tenderisation process by increasing their expression at the gene level. However, protein products from changes in gene expression take time to be translated and it is likely that the short period of four days prior to slaughter is not long enough to translate to changes at the protein level; an experiment by Antrobus *et al.* (1995) reported responses in protein levels of kidney cells to 1,25 $(OH)_2D_3$ treatment took between 1 to 3 days to be slightly increased but after 7 days their expression was much stronger.

In order to further investigate the possible effects the short term supplementation of dietary vitamin D_3 may have on gene expression, it was decided to investigate responses in the known target tissue

of vitamin D, the kidney. In the kidney responses to calcium homeostasis are mediated by the vitamin D receptor (VDR) and the calcium binding protein, calbindin D-28k and it is known that vitamin D_3 upregulates calbindin D-28K and its receptor, the VDR (Minghetti & Norman, 1988). Calbindin D-28k expression at the mRNA level was measured using real-time RT-PCR and expression was normalised to β-actin mRNA, results expressed as a ratio of calbindin: β-actin in table 3.1-6. In addition the levels of calbindin D-28k and VDR were measured at the protein level using western blotting, data shown in table 3.1-6 and representative blots are shown in figure 3.1-2. There was no effect of vitamin D supplementation on the levels of calbindin D-28k at both mRNA and protein level, although the protein levels of VDR were approaching a trend (P=0.103) which could indicate a potential down-regulation of the VDR. This data suggests that a short supplementation period of vitamin D of four days is not sufficient to affect significant changes at either the gene or protein level of known vitamin D response genes.

Table 3.1-5 Effect of 4 Days Vitamin D_3 supplementation on mRNA and protein expression of calpain system in LD.

LD mRNA	Calpain Genes: Beta Actin Ratio			
	Control	Vitamin D	S.E.D.	P
Calpain I	1.020	1.058	0.0260	0.099
Calpain II	0.967	1.064	0.0376	0.014
Calpastatin	0.956	0.987	0.0357	0.382
	Absorbance/ 100 µg protein			
LD protein	Control	Vitamin D	S.E.D.	P
Calpain I	8.668	7.687	1.083	0.279
Calpain II	7.784	8.121	0.550	0.785
Calpastatin	15.79	17.99	2.553	0.339
Desmin	5.744	6.027	0.630	0.468

Calpain system mRNA n=17, calpain I protein n=15, calpain II, calpastatin and desmin protein n=18.

Table 3.1-6 Effect of 4 Days Vitamin D_3 supplementation on mRNA and protein expression of vitamin D targets in kidney.

Kidney mRNA	Control	Vitamin D	S.E.D.	P
Calbindin : Beta Actin ratio	0.995	1.060	0.0689	0.260
	Absorbance/ 100 µg protein			
Kidney protein	Control	Vitamin D	S.E.D.	P
Calbindin	9.468	8.889	0.822	0.504
VDR	10.221	6.846	1.934	0.103

Calbindin mRNA n=18, calbindin protein n=17, VDR protein n=9.

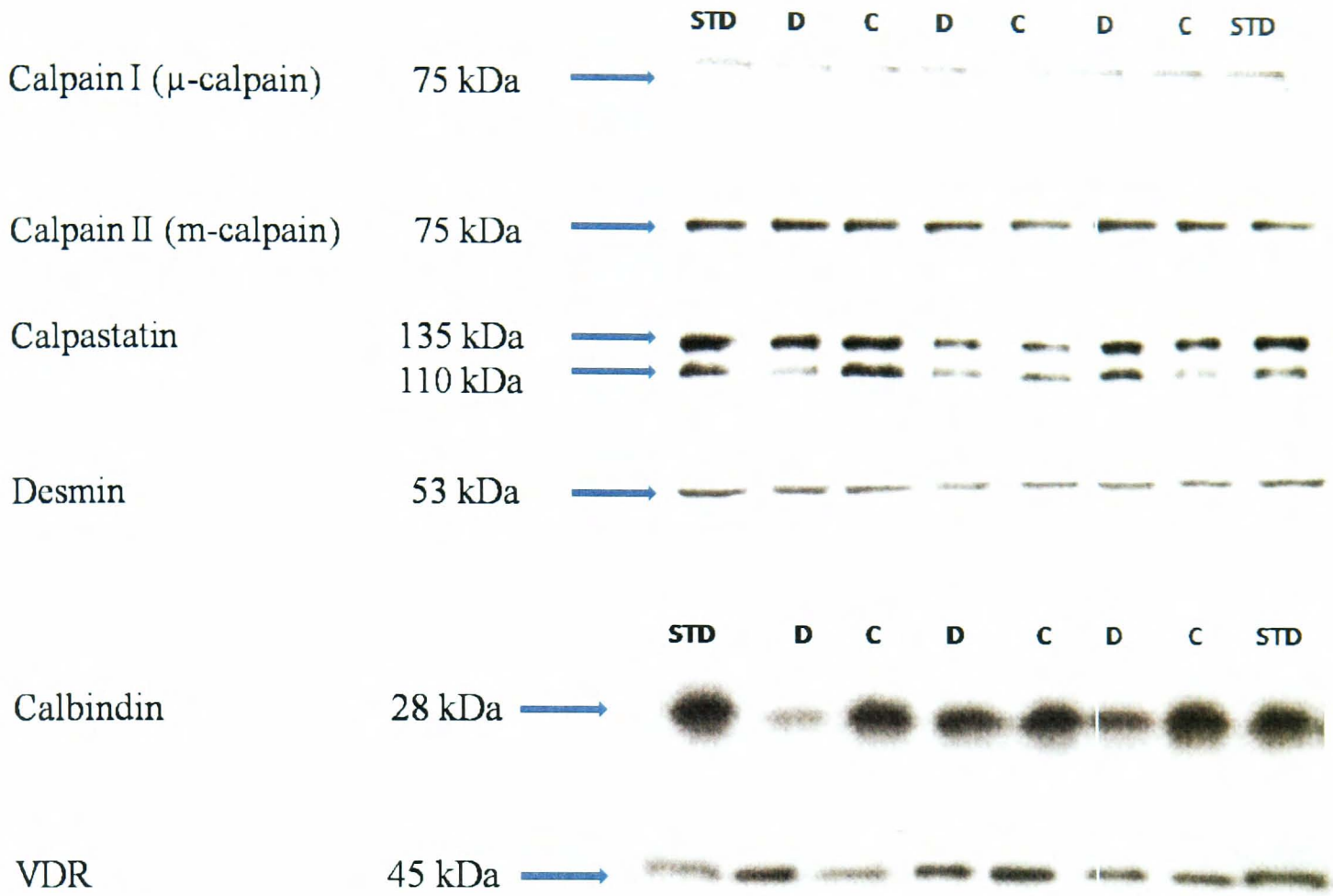


Figure 3.1-2 Representative blots of the calpain system in LD and vitamin D responsive targets in kidney protein extracts from sheep in trial 1: 4 Days vitamin D_3. All protein samples were loaded with constant weight of 100μg protein/lane, treated (D) and control (C) samples were loaded alternately between a repeated standard sample (STD). Calpain I and II: 100μg LD supernatant fraction separated by SDS-PAGE using 10% acrylamide gel, Calpastatin and desmin: 100μg LD whole homogenate fraction separated by SDS-PAGE using 8% acrylamide gel. Calbindin and VDR: 100μg whole homogenate kidney protein extract separated by SDS-PAGE using 12% acrylamide gel. Further antibody information presented in Appendix D, calpain I n=15, calpain II, calpastatin and desmin n=18, calbindin n=17 and VDR n=9.

3.1.4 Trial Summary

The aim of this trial was manipulate meat quality using a short supplementation period of 4 days vitamin D_3 immediately prior to slaughter in order to maximise the effect of the supplementation before the vitamin D homeostasis system would be expected to begin down regulating circulating levels. While this supplementation regime was successful in increasing the circulating vitamin D_3 levels of the treated animals, it did not translate into a change in calcium concentration of the serum or muscle. In turn there was no effect on the shear force of chops from the LD and so it can be concluded that a 4 day vitamin D_3 supplementation period immediately prior to slaughter does not affect meat quality in sheep. A simple hypothesis associated with the effects of supplementing feed with vitamin D is that high circulating levels of Vitamin D will increase body Ca^{2+} concentrations which would in turn lead to an increased activation potential for the calpain system post mortem; as the activity of the calpain enzymes and their affinity to bind to their inhibitor, calpastatin, is regulated by calcium concentration of the muscle. However in the current study there was no change in the calcium status of the treated sheep so, based on this hypothesis, it is not surprising that there was no effect on the tenderisation process in this study.

However in addition to the Ca^{2+} homeostatic effects Vitamin D is known to have effects on gene expression on proteins that are not associated with Ca^{2+} homeostasis through the nuclear receptor VDR. The elevated vitamin D_3 levels did have a significant effect on the expression of the calpain II enzyme at the mRNA level, and indicated a trend to increase calpain I, which is evidence to suggest that vitamin D_3 may directly moderate the expression of these enzymes which are responsible for the majority of post mortem proteolysis. This is in agreement with a study performed in cattle, whereby Cho *et al.* (2006) fed cows a high vitamin D (125mg/day) diet for 6 days prior to slaughter and reported significant increases in the mRNA of both calpain I and calpain II with significant decrease in calpastatin mRNA in the LD. However, effects in gene expression need to be translated into changes at the protein level and it is likely that 4 days was not enough time to observe responses at the protein level. In light of the results in this study, a second trial was conducted using a longer supplementation period with an additional calcium bolus. The purpose of this regime was to attempt to increase the calcium status of the sheep and to allow time for the vitamin D_3 to mediate potential gene responses.

3.2 Trial 2: 7 Days Vitamin D & Calcium Bolus

The hypothesis of this trial was that a longer supplementation period of vitamin D_3 would produce a response in calcium uptake via the gut which could be further optimised as a result extra calcium available in the diet. The expectation was that the increase in the calcium status of the animals would then lead to an increase in calpain activities, causing increased proteolysis post mortem and subsequent improvements in the quality of the meat in terms of tenderness. Previous trials performed in this laboratory have used a short 4 day dose period with calcium bolus and a longer 7 day dose period without calcium; both trials saw significant changes in calcium status but no significant effects on meat quality. The objective of this trial was to try to combine these treatments and investigate whether a longer supplementation period of dietary vitamin D_3, with a calcium bolus, could produce significant effects in calcium status and improve meat quality via increased activation of the calpain system.

3.2.1 Trial Outline

Thirty six Mule X Charolais wether lambs at approximately 7 months of age were individually penned and allocated to two treatment groups consisting of control (n=18) and high vitamin D_3 (n=18). All animals were allocated to pens and paired into treatment-control pairs according to age and weight, as shown in figure 2.2-2. All animals were initially fed 700g of a basal diet with no supplemented vitamins or minerals twice a day (half daily allowance of 1.4 kg/animal/day), as described in section 2.2. The basal diet period lasted around 5 weeks to allow for acclimatisation. A period of 7 days before slaughter date the VIT D_3 group (n=18) were fed the basal diet supplemented with 2×10^6 IU Vitamin D/day (Rovimix D_3 500, DSM Ltd, Derbyshire UK) whilst the control group (n=18) still received the same basal diet without vitamin D_3. Due to the animals being allocated to one of three groups (each containing VIT D_3 n=6 and control n=6) according to age and size, commencement of vitamin D_3 supplementation was staggered slaughter days. The day before slaughter the high vitamin D_3 animals were fed a diet with the same high vitamin D_3 content plus an extra calcium bolus mixed into the feed to give double the calcium content compared to the control diet; as analysed by Frank Wright Ltd (calcium propionate suppliers), composition of the diet is shown in table 2.2.3. The overall timings and subsequent sample points are summarised in figure 3.2-1.

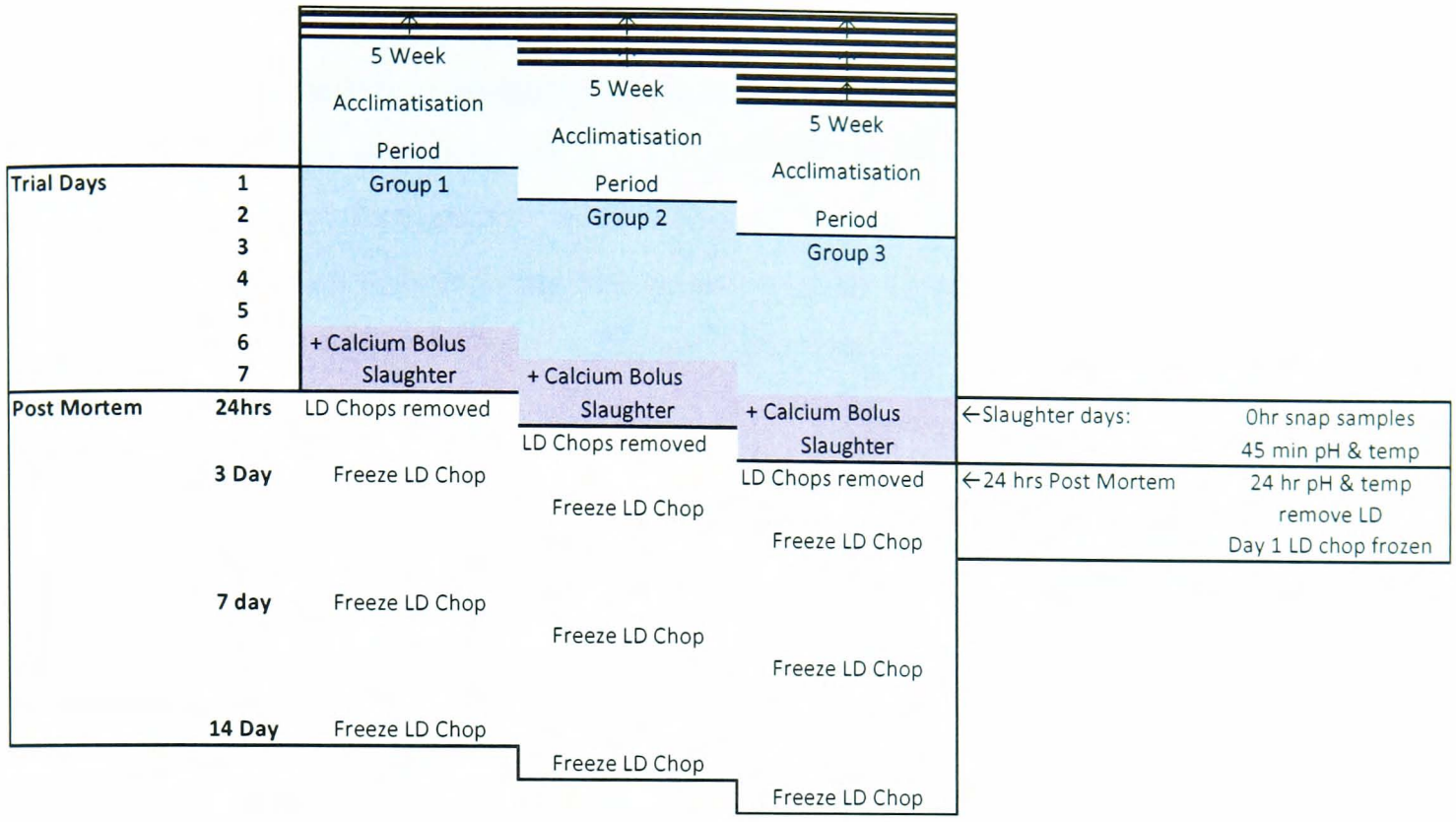


Figure 3.2-1 Timeline for Trial 2: 7 days vitamin D + calcium bolus immediately prior to slaughter. Shown in dark blue is the period of vitamin D only supplementation for 5 days before diets are supplemented with vitamin D and an additional calcium bolus for the final 2 days immediately prior to slaughter, shown in purple box.

A group of four lambs not included in the trial, all healthy and eating 1.4kg feed/day of the basal diet were used to assess the palatability of the high vitamin D plus calcium propionate diet. These animals were fed the trial diet for two days to assess palatability of the high vitamin D/Calcium content, specifically as animals had eaten the vitamin D only diet during trial 1, it was decided to check the calcium propionate affected palatability. There were no refusals from this group over the two days and there were no ill effects seen afterwards as all animals continued to eat the basal diet and based on these observations the study was continued using the diet. Animals were slaughtered, carcass measurements taken and samples collected according to procedure previously mentioned in chapter 2, section 2.2. LD chops were vacuum packed and left to age for 1, 3, 7 and 14 days according to latin square arrangement in Appendix A. Aging of samples was reduced to 14 days aging period as the recommended aging period for lamb is between 7 and 14 days (Warris, 2000) and work by Dransfield (1994) states that 80% of tenderization of lamb takes place over 7.7 days. The chops set aside for the final aging time point of 14 days were taken posterior to the first sample point along the LD (according to sample collection outlined in Appendix A).

All animals were weighed twice a week to ensure weekly weight gain and their feed intakes monitored. Despite the preliminary trial, where four animals had eaten the trial diet without any ill effects, during the trial week there were varying amounts of feed refused by the treatment group.

Ten animals out of a total of eighteen left from 100g up to 3kg across the course of the week (out of a total of 9.1kg presented to them during the week), data shown in Appendix K. This is a significant amount of feed refusal when in comparison only 2 control lambs left feed (0.9kg each across the week). The amount of feed refused by the treatment group on calcium days was even more pronounced, where two animals which had always consumed 1.4kg/day left up to 1.2kg of feed. There were 8 animals in the treatment group who ate all food presented, compared to 16 control animals who did the same.

Weights were not significantly different at the beginning of the trial, whereas on slaughter date the treated animals had significantly lower liveweights than controls ($P<0.01$), shown in table 3.2-1. These reduced weights are likely to be caused by the reduced feed intakes during the trial week as this was the only period when animals saw weight loss between their weekly weight checks and is likely to be due to reduced gut fill. Overall there was no reduction in ADG and FCR over the course of the trial, shown in table 3.2-1, and the difference in pre-slaughter liveweight between treatment groups is presumed to be a result of the Vitamin D_3 plus calcium diet being possibly less palatable, as the majority of refusals were on days when calcium was given. This is further supported by the carcass weight and dressing percent data, table 3.2-1, as there are no differences between the overall carcass weights after dressing. The dressing percent data is not significantly different but numerically the dressing percent is higher in the vitamin D group, due to their lower liveweights prior to slaughter. There was a significant difference between carcass pH at 45 minutes post mortem and in ΔpH ($P<0.05$), shown in table 3.2-1. However there was no difference in final pH as measured at 24 hours post mortem.

To further evaluate the effect of the feed refusal on the carcass traits and the subsequent plasma vitamin D_3 and serum calcium levels, the data for the animals of the vitamin D treatment group was analysed for differences between those that ate all feed and those that refused. It was found that there was no effect on feed refusal on plasma D_3 levels nor serum calcium, nor on the pH at 45 minutes post mortem. There was a significant effect on the pH at 24 hours post mortem ($P=0.01$) and on the liveweight of the animals immediately prior to slaughter ($P=0.038$) however there was no effect on the weights of the dressed carcasses, the data is shown in appendix I. Overall this data is evidence to show that the differences observed in liveweight is a factor of gut fill and the actual carcasses were no different in size. The difference in ultimate pH 24 hours post mortem is not indicative of dark, firm, dry (DFD) meat as a result of reduced glycogen stores pre-slaughter as the values for the animals which refused feed were significantly lower than those which ate all feed. Overall there was no difference in the plasma levels of vitamin D_3 and serum levels of calcium which

were the hypothesized mediators of effects in calcium and calpain activity; therefore it was decided to continue to include all 18 vitamin D treated animals in the analysis of the subsequent data presented in this section.

Table 3.2-1 Effect of 7 days vitamin D_3 + Calcium supplementation on animal growth, feed intake and carcass traits, n=18.

		Control	Vitamin D	S.E.D.	P
Initial Lamb Weight (Kg)		**42.99**	**42.55**	0.801	0.255
Final Lamb Weight (Liveweight) (Kg)		**49.68**	**48.23**	0.855	**0.002**
Total Weight Gain (6 weeks) (Kg)		**6.683**	**5.678**	0.636	0.120
Average Daily Gain (ADG) (Kg/Day)		**0.163**	**0.1386**	0.021	0.120
Feed Conversion Ration (FCR)		**9.135**	**10.863**	1.540	0.146
Carcass Weight (Kg)		**25.46**	**25.25**	0.514	0.479
Dressing Percent (%)		**51.28**	**52.4**	0.774	0.108
LD pH	**0 Hr**	**6.60**	**6.46**	0.060	**0.050**
	24 Hr	**5.50**	**5.51**	0.048	0.664
LD ΔpH		**1.10**	**0.95**	0.067	**0.044**

As there was no effect on the carcass weight and pHu was not affected by the reduced feed intake in the final days of the trial it was decided that all animals should be included in the analysis. The purpose of this study was to ascertain whether a vitamin D_3 and calcium supplementation regime immediately prior to slaughter was able to raise calcium status of the animals in both the serum and muscle which would serve to activate the calpain activity post mortem and subsequently improve the tenderisation process. Plasma samples were analysed using HPLC by Stuart Jones at Nottingham City Hospital and results are shown in table 3.2-2. 25(OH)-vitamin D_3 levels were significantly raised by the treatment diet ($P<0.001$), and this equates to almost a 25 fold increase (2364%) compared to controls. 25(OH)-vitamin D_2 levels were also changed between groups ($P<0.001$) as measured by this assay, with Vitamin D_3 plus calcium treated animals having a 10 fold increase in their circulating levels of vitamin D_2. Serum calcium was significantly increased by the dietary supplementation regime, with the treated group ($P<0.001$; table 3.2-2), with vitamin D_3 plus calcium bolus giving a 10% increase in circulating levels of calcium in the serum of sheep. In addition to significant changes in the serum calcium there was a significant increase in the muscle calcium levels in the treated group ($P=0.024$; table 3.2-2), with the calcium levels in the muscles of treated animals increased by 10%.

Table 3.2-2 Effect of 7 Days Vitamin D_3 + calcium bolus on plasma 25(OH)-vitamin D, serum & LD calcium levels.

	Control	Vitamin D	S.E.D.	P
D_3 ng/ml	49.9	1230	55.82	< 0.001
D_2 ng/ml	24.8	250.8	18.35	< 0.001
Serum Calcium (mg/L)	129.1	142.7	3.367	<0.001
LD Calcium (mg/g wet tissue)	44.73	49.45	1.770	0.024

Plasma vitamin D_3 and D_2 and serum calcium n=18, LD muscle calcium n=16.

Together, the data summarised in table 3.2-2 indicates that despite the variable feed refusal observed in the animals fed the treatment diet, there were still large and highly significant changes in the circulating levels of vitamin D_3 and calcium and this was translated into changes in the calcium levels in the LD. This is further justification to analyse further measurements using all animals in the trial as the changes in vitamin D_3 and calcium status were so large that any subsequent effects on the calpains and meat quality would be related to these parameters.

LD chops were aged for 1, 3, 7 and 14 days at 4°C before tenderness was determined by Warner-Bratzler method. Samples were aged at 4°C for 3, 7 and 14 days and then selected for freezing according to a latin- square arrangement (see appendix A), data was analysed using ANOVA. Chops aged for 14 days were sampled separately to provide data for shear force after 2 weeks of aging, shown in table 3.2-3. Shear force of all chops decreased with time (P<0.01) and there was no interaction between diet and aging period (P=0.808), however, shear force of chops taken from treated animals were significantly higher than those from controls (P<0.01). This data suggests that a supplemental regime of 7 days Vitamin D_3 plus calcium bolus treatment has a detrimental effect on shear force of LD chops.

Table 3.2-3 Effect of 7 days vitamin D_3 + calcium bolus on shear force of LD, n=18.

	Time (days)					
Diet / Age	1	3	7		S.E.D.	P
Control	4.083	3.66	3.322	Diet	0.1436	0.002
Vitamin D	4.338	4.09	3.82	Age	0.1759	0.007
				Diet * Age	0.2488	0.777
	Control	Vitamin D				
14 day shear force LD	3.685	3.944			0.191	0.133

To ascertain whether fat content of the LD was contributing to the change in shear force, the Soxhlet fat content was determined for a subset of 6 samples, taking two pairs from each slaughter group. Muscle fat was extracted by the Soxtherm method and calculated as a percentage of muscle weight. There was no significant effect of 7 days vitamin D_3 supplementation plus calcium bolus on the fat

content of LD (table 3.2-4) although numerically the vitamin D_3 group had lower fat content than that of the control group and the P value is approaching a significant trend of P=0.121.

Table 3.2-4 Effect of 7 Days Vitamin D_3 Supplementation plus calcium bolus on LD Fat Content, n=6.

	Control	Vitamin D	S.E.D.	P
Extracted LD Fat Content (% per g freeze dried tissue)	**12.03**	**9.58**	1.495	0.121

3.2.2 Vitamin D signalling results: Trial 1; 7 days vitamin D_3 + calcium bolus.

The longer supplementation of vitamin D_3 for 7 days in tandem with an extra calcium bolus was able to produce significant changes in the vitamin D_3 and calcium status of the treated sheep and it was hypothesised that these changes would cause changes in the calpain system and vitamin D signalling responses. There was a detrimental effect of this supplementation regime on the shear force of the LD chops and so the expression of the calpain system was measured to ascertain whether the increased toughness was related to their activities.

The expression of the calpain system at the mRNA level was measured using real-time RT-PCR, as described in chapter 2 of this thesis (section 2.9-3). There was no effect of diet on the expression of β-actin and so all data was normalised to this gene (table 2.9-3, chapter 2). Expression of calpain I or μ-calpain, in the LD was significantly decreased (P=0.04, table 3.2-5) in the treatment group compared to expression in the control animals although this difference was not apparent at the protein level (P=0.691, representative blots shown in figure 3.2-2). Expression of calpain II (m-calpain), and calpastatin at the mRNA level was not affected by diet treatment; however at the protein level there was a significant increase in the expression of calpain II (m-calpain) by 16%. There was also a trend for an increase in calpastatin at the protein level (P=0.087, table 3.2-5) by 17%, although these changes were not apparent at the mRNA level. Given that calpastatin is the calpain-specific endogenous inhibitor, this increase in its protein level could be responsible for the raised shear force observed in the LD chops from treated animals in this trial.

As mentioned in section 3.1.3, calbindin D-28K is a gene known to be up-regulated by vitamin D and its mRNA and protein levels were measured to qualify the effects of dietary vitamin D_3 supplement. There was a significant effect of 7 days dietary vitamin D_3 plus calcium bolus on the mRNA expression of calbindin D-28k in the kidney, where the calbindin D-28K expression was increased by 8.8% ($P< 0.05$, table 3.2-6). This change at the mRNA level was not translated into a change at the protein level as calbindin D-28k protein levels were unchanged (P=0.147) despite a higher numerical value in the vitamin D_3 treated group. However, there was a significant change in the VDR protein

levels in the kidney whereby VDR levels were significantly increased 2 fold (100%) by 7 days vitamin D_3 + calcium treatment ($P<0.001$, table 3.2-6). Taken together these data show that the supplementation of vitamin D_3 for 7 days + calcium did cause significant changes at the gene and protein levels and it is likely the changes in calbindin D-28k mRNA were the result of a direct signalling effect of vitamin D_3 at a gene transcription level in the nucleus.

Table 3.2-5 Effect of 7 Days Vitamin D_3 + calcium on mRNA and protein expression of Calpain System in LD muscle.

LD mRNA	Calpain Genes : Beta Actin Ratio			
	Control	Vitamin D	S.E.D.	P
Calpain I	1.004	0.951	0.0264	**0.04**
Calpain II	1.005	0.994	0.0292	**0.594**
Calpastatin	1.053	1.025	0.0465	0.513
	Absorbance/ 100 µg protein			
LD protein	Control	Vitamin D	S.E.D.	P
Calpain I	8.182	7.923	0.748	0.691
Calpain II	4.853	5.636	0.437	**0.050**
Calpastatin	11.690	13.720	2.201	**0.087**

Calpain system mRNA n=18, calpain I protein n=15, calpain II protein n=16 and calpastatin protein n=17.

Table 3.2-6 Effect of 7 Days Vitamin D_3 + calcium on mRNA and protein expression of vitamin D targets in kidney.

Kidney mRNA	Control	Vitamin D	S.E.D.	P
Calbindin : Beta Actin ratio	1.000	1.088	0.0716	**0.043**
	Absorbance/ 100 µg protein			
Kidney protein	Control	Vitamin D	S.E.D.	P
Calbindin	6.341	10.318	2.282	0.147
VDR	3.172	6.510	0.715	**<0.001**

Calbindin mRNA n=18, calbindin protein n=14, VDR protein n=17.

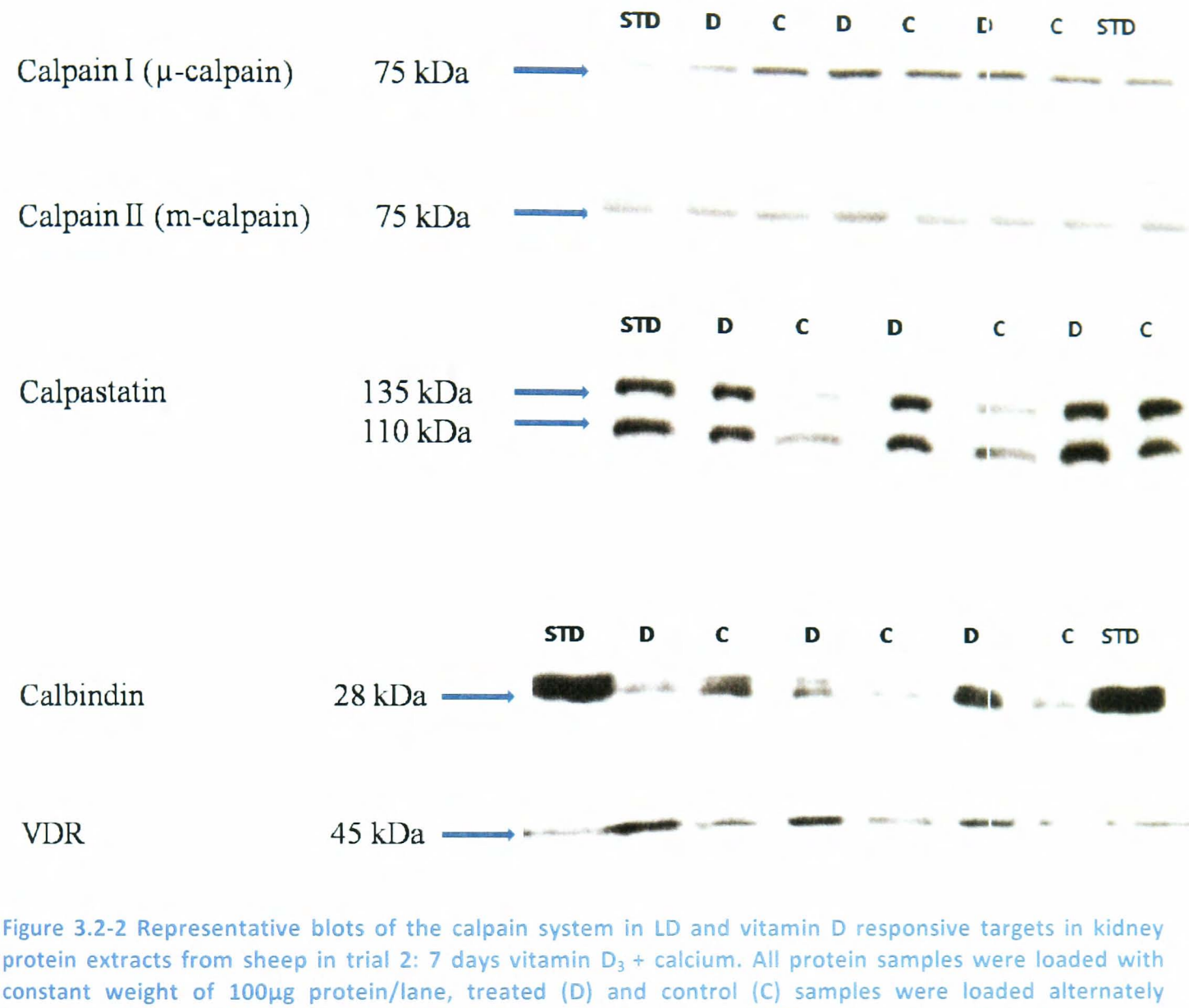


Figure 3.2-2 Representative blots of the calpain system in LD and vitamin D responsive targets in kidney protein extracts from sheep in trial 2: 7 days vitamin D_3 + calcium. All protein samples were loaded with constant weight of 100μg protein/lane, treated (D) and control (C) samples were loaded alternately between a repeated standard sample (STD). Note: there is no second STD on the calpastatin protein image. Calpain I and II: 100μg LD supernatant fraction separated by SDS-PAGE using 10% acrylamide gel, Calpastatin: 100μg LD whole homogenate fraction separated by SDS-PAGE using 8% acrylamide gel. Calbindin and VDR: 100μg whole homogenate kidney separated by SDS-PAGE using 12% acrylamide gel. Further antibody information presented in Appendix D, calpain I n=15, calpain II n=16, calpastatin n=17, calbindin n=14 and VDR n=17.

3.2.3 Trial Summary

The aim of this trial was to manipulate meat quality using a longer supplementation period of 7 days vitamin D_3 with an additional calcium bolus immediately prior to slaughter in order to maximise the changes in calcium status which would have subsequent effects on meat quality. This supplementation regime did confer significant changes in circulating vitamin D and calcium levels and subsequently calcium levels in the LD were significantly raised. Despite the changes in calcium status there was no improvement in the shear force of LD chops post mortem, which was instead increased by the dietary regime. The increase in shear force could be attributed to a number of parameters such as the slight reduction in fat within the LD indicated in table 3.2-4; however it is more likely that this increase in shear force is related to increase in the protein levels of calpastatin which would inhibit calpain activity.

However, the second aim of this trial was to allow a longer supplementation of vitamin D_3 with extra calcium which would translate into changes in gene expression as a result of direct signalling at gene transcription level, as it was thought 4 days of dietary vitamin D_3 in the previous trial was not long enough to observe gene expression changes. This longer supplementation of vitamin D_3 did cause an increase in mRNA of calbindin D-28k, a known target of vitamin D_3 in the kidney, a target organ of the vitamin D-calcium homeostasis mechanism. This increase in calbindin D-28k mRNA was not translated into changes at the protein level, although the values were numerically higher with vitamin D_3 group ($P=0.147$). In addition to changes in calbindin D-28k, expression of VDR at the protein level was significantly higher with vitamin D_3 treatment ($P<0.001$), which demonstrated that the circulating vitamin D_3 was having a direct signalling effect in its target organs and provides evidence to conclude 7 days vitamin D_3 supplementation is sufficient to confer gene expression changes.

3.3 Summary of Sheep Trials

The aim of the two trials performed in sheep in this study was to evaluate the efficacy of a pre-slaughter vitamin D supplementation regime on its ability to improve meat quality. The proposed mechanism by which the vitamin D supplementation could improve the development of tenderness post mortem was by increasing circulating calcium levels which would lead to raised calcium levels in the muscles. The purpose of raising the calcium levels in the muscle would be to increase the activity of the calpain enzymes, in particular calpain I (μ-calpain), which was shown to be activated when carcasses were infused with calcium chloride post mortem (Koohmaraie *et* al., 1989; Polidori *et al.*, 2000). If this could be achieved by vitamin D supplementation prior to slaughter it was hypothesised that this regime would provide an acceptable dietary manipulation technique to promote a tender meat product without the need to process carcasses in the abattoir. In light of the evidence produced from these two meat trials and from trials performed previously (Sazili 2003, Wiegand *et* al. 2001; Boleman *et al.*, 2004) it can be concluded that despite some regimes being competent in increasing calcium status of sheep, this does not lead to an improvement in shear force of the muscle. In the case of trial 2 of this thesis where animals were fed vitamin D with additional calcium bolus in the week prior to slaughter, the increase in calcium status of was attributed to tougher LD chops.

Interestingly there were changes in the expression of components of the calpain system as a result of 4 days vitamin D_3 supplementation alone, which caused an increase in both calpains I and II at the mRNA level but was not translated to changes at the protein level. However, this effect was not observed again where 7 days vitamin D_3 + calcium caused a decrease in the expression of calpain I at mRNA level although the protein levels of calpain II were increased. More importantly the detrimental effect of the 7 days vitamin D_3 + calcium on shear force of LD chops was supported by an increase in calpastatin at the protein level. A model to explain this increase in shear force could possibly be that the increase in calpastatin directly inhibited the activities of the calpain enzymes and therefore inhibited the tenderisation process.

Results: Vitamin D in Muscle cells

Introduction to muscle cell experiments

Endo *et al.* (2003) reported that knockout VDR -/- mice had abnormally raised levels of myogenic differentiation factors Myf5, myogenin, E2A, and the embryonic and neonatal MHC genes. In addition to observations in VDR -/- mice they reported that treatment of C2C12 cells with 1, 25 $(OH)_2$ vitamin D for 48 to 96 hours caused a decrease in Myf5, myogenin and neonatal MHC expression. As described in section 1.2 of this thesis, Myf5 is an early myogenic factor required to determine cells into the myogenic cell line whereas myogenin is expressed later in the process of muscle cell formation and is required for fusion and differentiation of myoblasts into myotubes. In light of the observations reported by Endo *et al.* (2003) it might be concluded that vitamin D, acting via its receptor VDR, is required for myogenesis and regulates the process by regulating the proliferation of early myoblasts via affects on Myf5 expression and on the process of myotube formation via myogenin expression. In addition to the effects on myogenic factors Endo *et al.* reported extended raised expression of the neonatal MHC isoform in VDR -/- mice which was down regulated in C2C12 cells treated with 1,25 $(OH)_2$ vitamin D, these findings suggest that vitamin D is able to alter the expression of MHC isoforms. In agreement with this are the reports that supplementation of elderly patients with 1,25 $(OH)_2$ vitamin D resulted in an increase in the number of type II muscle fibres (Sato *et al.,* 2005) and that supplementation with the active analogue 1α-(OH) vitamin D increased the number of fast oxidative fibres and decreased fast glycolytic fibre number (Sorenson *et al.*, 1979). With a number of reports describing loss of type II muscle fibres associated with vitamin D deficiency (Bischoff *et al.*, 2003; Bischoff-Ferrari *et al.*, 2006; Snijder *et al.*, 2006) there is strong evidence for vitamin D's role in maintaining the expression profile of the MHC proteins, in particular the fast isoforms.

In order to investigate vitamin D's possible role in regulating gene expression in skeletal muscle tissue, cell cultures were used as a model to be treated with active vitamin D_3 directly. Two experiments were performed in this section of work; beginning with a microarray performed to identify novel targets of vitamin D in muscle tissue. The latter experiment was designed to test the hypothesis that direct treatment of vitamin D in muscle cells can perturb the growth and differentiation of muscle cells and alter the MHC gene expression.

3.4 Microarray to indentify novel targets of $1,25(OH)_2D_3$

3.4.1 Trial outline

Primary skeletal muscle cells prepared from hind limb muscles of neonatal male rat pups as described in section 2.11.1 and myoblasts were seeded at a density of 12.5 $x10^4$ cell per well and cultured until 90% confluence, at which point cell growth media was substituted for cell fusion media supplemented with one of three treatments; either 10^{-5}M or 10^{-9} M of 1α,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) or 0.1% DMSO control. Cells were treated with the active form of 1,25 dihydroxyvitamin D_3 for 24 hours. Following treatment, three wells from a 6-well cell culture plate providing three replicates of total RNA were extracted using the RNeasy mini kit (Quiagen), a kit designed to consistently produce very high quality mRNA to prevent false discoveries. The microarray was performed by Thomas Wilson of Pfizer (Kalamazoo, MI, USA) using was the rat V.2.0 microarray chip, produced by Affymettix. The analysis was performed in two stages; firstly the raw data was processed using Genespring software (with kind help of Neil Graham of the Arabidopsis Stock Centre, University of Nottingham) before the list of up and down regulated genes were analysed using Ingenuity Pathway Analysis (IPA V7.1, Ingenuity Systems).

3.4.2 Quality of RNA used in the microarray

It was of extreme importance that the RNA used in the microarray was intact and of good quality in order to present the most reliable data from the microarray. The total RNA from the rat primary muscle cells was extracted as described in section 2.13.1 and the quality of the RNA was analysed using an Agilent 2100 Bioanalyzer (Agilent technologies) which is developed to accurately analyse RNA using very small sample sizes and saves the need to perform electrophoresis using an agarose gel, thereby reducing the amount of sample needed to check RNA integrity before it is utilised in the microarray. The Bioanalyzer grades the RNA out of 10, producing a Relative Integrity Number value (RIN value),and transfers the interpreted integrity of the RNA into a hypothetical gel in order to demonstrate the intact bands of 28S, 18S and tRNA as would be seen using agarose gel electrophoresis. For use in a microarray RNA needs to be wholly intact and to produce a RIN value of at least 9 out of 10. Data for the RNA prepared in this study is shown in table 3.4-1, showing that all samples scored 10 out of 10 except DMSO replicate 2, which still had an acceptable score of 9.2 out of 10. The relative band of 28S should be around twice the intensity of the 18S band, hence the ratio of the two bands should be as close to 2.0 as possible in good quality RNA and again all RNAs had acceptable 28S/18S ratios The hypothetical gel showing all intact 28S and 18S ribosomal RNA and

tRNA bands is presented in figure 3.4-1. From this data it can be assumed that the RNA was of high quality and would provide a valid template for the microarray analysis.

Table 3.4-1 Concentration and integrity of RNA used in microarray.

Sample	RNA Conc ng/µl	Ratio rRNA 28s/18s	RNA Integrity No. RIN
DMSO 1	350	1.9	1.0
DMSO 2	396	1.8	9.2
DMSO 3	367	1.9	10.0
10^{-5}M 1	109	1.9	10.0
10^{-5}M 2	283	1.9	10.0
10^{-5}M 3	337	2.0	10.0
10^{-9}M 1	399	2.1	10.0
10^{-9}M 2	478	2.0	10.0
10^{-9}M 3	570	2.0	10.0

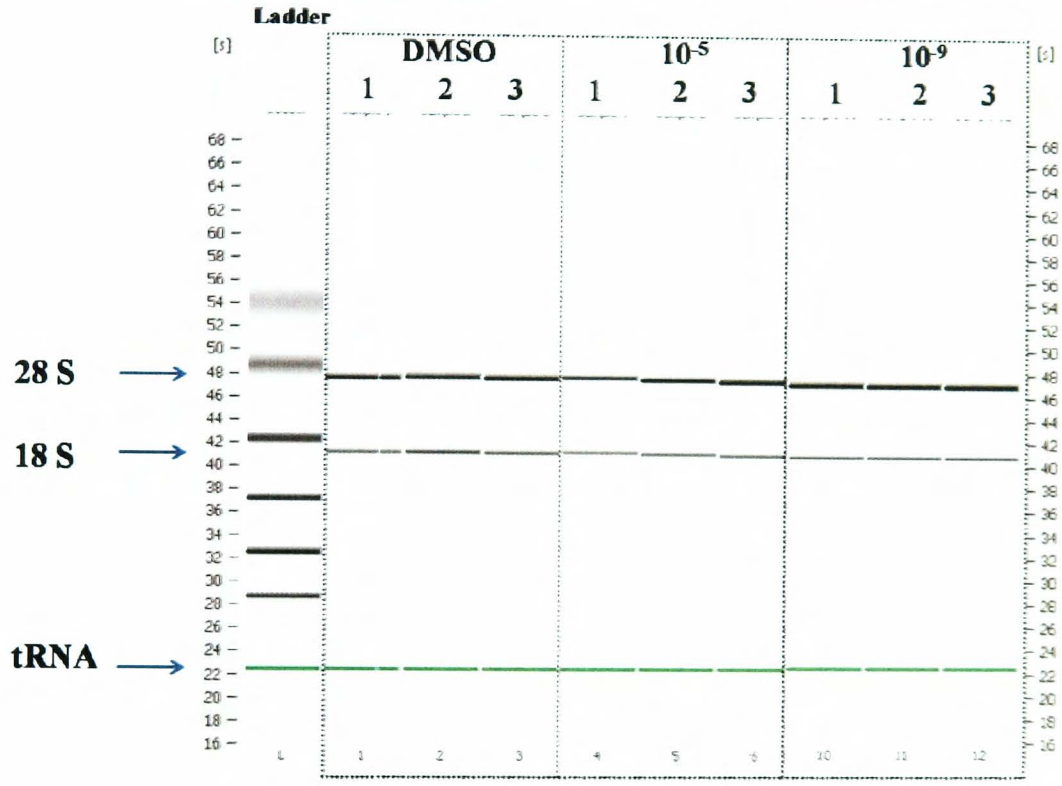


Figure 3.4-1 Effects of 1,25 Vitamin D_3 on quantity and quality of RNA extracted from rat primary muscle cells in culture. Hypothetical gel graph of band intensity of RNA produced by Agilent 2100 Bioanalyzer.

Table 3.4-2 Effect of $1,25(OH)_2D_3$ treatment on yield of RNA harvested for microarray analysis.

	DMSO	10^{-5}	10^{-9}	S.E.D.	P
RNA conc (ng/µl)	371^{a}	243^{ab}	482^{ac}	70	**0.039**

Values analysed by ANOVA, values with the same superscript are not significantly different, n=3.

There was a significant effect of treatment on RNA yield from rat primary myoblasts treated with $1,25(OH)_2D_3$, $P<0.039$ table 3.4-2. When the values were tested using least significant difference values from the ANOVA output, using a T value from the Dunnett's test, there was a significant difference between the concentrations of RNA harvested from cells treated with either dose of $1,25(OH)_2D_3$, but neither of these yields were significantly different to the yield of the DMSO controls. The effect was that RNA yields were significantly lower when cells were treated with $1,25(OH)_2D_3$ 10^{-5}M in comparison to the yield of RNA from cells treated with the dose of 10^{-9}M ($P<0.05$).

3.4.3 Analysis of microarray data

The changes affected by each dose of $1,25(OH)_2D_3$ were calculated by T-tests between each vitamin D dose and DMSO control, performed in Genespring software, identifying genes with expression changes over 1.5 fold or more in either up- or down-regulation. This produced a list of genes which had changed 1.5 fold or more in either direction with 95% significance. The individual gene targets included in the lists were analysed further using Ingenuity Pathway Analysis software (IPA, V.7.1; Ingenuity Systems) to identify their function and role in signalling pathways.

3.4.4 Genes changed by $1,25(OH)_2D_3$ 10^{-5} M treatment

Out of 31,099 targets measured by the microarray, the expression of 475 genes was significantly changed at least 1.5 fold ($P<0.05$). Of the total 475 genes changed 157 were up-regulated in comparison to 318 which were down-regulated. The role of these genes in cells was further analysed by the IPA software to identify the signalling changes that were being brought about by vitamin D at the highest dose of 10^{-5}M. The software identified signalling pathways in which the changed genes were present and produced colour coordinated diagrams (green is down-regulated, red is up-regulated, higher intensity of colour relates to higher fold change) of the network and the location of the changed molecules in those pathways. The list of the top 5 networks containing genes affected by $1,25(OH)_2D_3$ at the 10^{-5}M treatment is shown in table 3.4-3. The score related to each network is related to the number of changed genes in each network and a high score denotes a large number of changed genes located in the network.

Table 3.4-3 Top 5 signalling networks containing highest number of molecules changed by $1,25(OH)_2D_3$ 10^{-5}M treatment.

Networks	Associated Network Functions	Score
1	Genetic Disorder, Ophthalmic Disease, **Cellular Growth and Proliferation**	**48**
2	**Cellular Growth and Proliferation, Embryonic Development, Connective Tissue Development and Functio**	**38**
3	Cancer, **Cell Death**, Hematological Disease	**35**
4	Cardiovascular System Development and Function, Cardiovascular Disease, Cell Death	**31**
5	Cancer, Cellular Movement, Lipid Metabolism	**29**

Genes which were included in these pathways are summarised in tables 3.4-4 and 3.4-5 which are separated for those transcripts either up-regulated or down-regulated by $1,25(OH)_2D_3$ respectively. IPA produced diagrams for each of the top 5 networks, shown in figures 3.4-2 to 3.4-6, where known signalling pathways which contain or interact with those genes changed by treatment can be visualised. Figure 3.4.2 depicts network 1 (table 3.4-3), related to cellular growth and proliferation and the central signalling molecule of this pathway is the MAPK, ERK. The second network in table 3.4-3 is depicted in figure 3.4-3, presenting molecules involved in cell growth, proliferation and differentiation and is focussed around the Jun and RNA polymerase molecules which are related to gene transcription. The transcription factors C/EBP β and δ were up-regulated by 10^{-5}M $1,25(OH)_2D_3$ treatment and are members of this network. Network 3 from table 3.4-3 is depicted in figure 3.4-4, showing molecules related to cell death and cancer by IPA. Signalling pathways denoted in figure 3.4-4 are centred around the nuclear factors NFκB and NFAT which are regulators of gene transcription. The calcium-regulated calcineurin is part of this pathway and this presents a possible link between the action of $1,25(OH)_2D_3$ in skeletal muscle cells and their interaction with calcium signalling pathways. Other proteins of interest in this pathway are calpastatin (CAST) and creatine kinase which were both down regulated by $1,25(OH)_2D_3$ treatment at 10^{-5}M. The fourth network in table 3.4-3 is depicted in figure 3.4-5 produced by IPA and shows molecules related to cell death, centred around the MAPK, p38, and indicating a relationship with caspase-1. The relationship to caspase-1 is of interest in terms of early cell growth as the caspases are apoptotic enzymes. Genes changed by $1,25(OH)_2D_3$ at 10^{-5}M were two MAPK signalling proteins, MEK (ERK kinase) and MAPKK4, and MEF2C. Finally network 5 from table 3.4-3 is shown in figure 3.4-6 and is focussed around the transforming growth factor TGF β-1 and is related to cancer and cellular movement. The networks are useful to put the list of changed genes into related protein signalling pathways and it can be concluded that $1,25(OH)_2D_3$ signals were mediated by a number of mechanisms, of these the molecules of interest are the MAPKinases and transcription factors such as C/EBP β and δ.

Table 3.4-4 List of genes up regulated with $1,25(OH)_2D_3$ 10^{-5}M

Up Regulated		
Gene Name	**Protein Name**	**Fold Change**
Rbp4	retinol binding protein 4, plasma	20.91
Mt1a	metallothionein 1a	6.79
LOC682651 /// LOC689415	similar to Metallothionein-2 (MT-2) (Metallothionein-II) (MT-II)	4.30
Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	2.57
Gpr68_predicted	G protein-coupled receptor 68 (predicted)	2.39
Hsd11b1	hydroxysteroid 11-beta dehydrogenase 1	2.32
Ndrg2	N-myc downstream regulated gene 2	2.24
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	2.23
Fads1	fatty acid desaturase 1	2.21
Mme	membrane metallo endopeptidase	2.10
Agtr1a	angiotensin II receptor, type 1 (AT1A)	2.07
Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	1.95
Fads2	fatty acid desaturase 2	1.93
Notch2	Notch gene homolog 2 (Drosophila)	1.92
Rras2	related RAS viral (r-ras) oncogene homolog 2	1.86
Nqo1	NAD(P)H dehydrogenase, quinone 1	1.84
Fgfr1	Fibroblast growth factor receptor 1	1.84
Agtr1a	angiotensin II receptor, type 1 (AT1A)	1.83
Hsf1	heat shock transcription factor 1	1.80
Dapk1_predicted	death associated protein kinase 1 (predicted)	1.75
Gadd45a	growth arrest and DNA-damage-inducible 45 alpha	1.72
Junb	Jun-B oncogene	1.69
Srebf1	sterol regulatory element binding factor 1	1.60
Rab34	RAB34, member of RAS oncogene family	1.58

Table 3.4-5 List of genes down regulated by $1,25(OH)_2D_3$ 10^{-5}M

Down Regulated		
Gene Name	**Protein Name**	**Fold Change**
Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	-4.24
Igfbp3	insulin-like growth factor binding protein 3	-3.05
Cited2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	-2.68
Pawr	PRKC, apoptosis, WT1, regulator	-2.57
Rasl11b	RAS-like family 11 member B	-2.54
Tgfb3	transforming growth factor, beta 3	-2.34
Rgs2	regulator of G-protein signaling 2	-2.30
Rbp1	retinol binding protein 1, cellular	-2.25
Rgs2	regulator of G-protein signaling 2	-2.15
Cited2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	-2.15
Wisp1	WNT1 inducible signaling pathway protein 1	-2.13
Rxrg	retinoid X receptor gamma	-2.08
Atp1b1	ATPase, Na+/K+ transporting, beta 1 polypeptide	-2.01
Usp13_predicted	ubiquitin specific protease 13 (isopeptidase T-3) (predicted)	-1.96
Cacna1s	calcium channel, voltage-dependent, L type, alpha 1S subunit	-1.92
Tnni2	troponin I type 2 (skeletal, fast)	-1.90
LOC684533	similar to myosin light chain 1 slow a	-1.88
Tmod1	tropomodulin 1	-1.85
Igfbp6	insulin-like growth factor binding protein 6	-1.82
Smoc2_predicted	SPARC related modular calcium binding 2 (predicted)	-1.80
Cdh11	cadherin 11	-1.79
Ctsk	cathepsin K	-1.77
Myh8	myosin, heavy polypeptide 8, skeletal muscle, perinatal	-1.77
Myh1 /// Myh2	myosin, heavy polypeptide 1/// 2, skeletal muscle, adult	-1.75
Igfbp6	insulin-like growth factor binding protein 6	-1.71
Cast	calpastatin	-1.71
Capn6	calpain 6	-1.68
Casq2	calsequestrin 2	-1.66
Map2k4	mitogen activated protein kinase kinase 4	-1.63
Ndrg4	N-myc downstream regulated gene 4	-1.62
Casq2	calsequestrin 2	-1.61
Fzd2	frizzled homolog 2 (Drosophila)	-1.60
Atp1b1	ATPase, Na+/K+ transporting, beta 1 polypeptide	-1.55
Mybpc1	myosin binding protein C, slow type	-1.53
Rdh5_predicted	retinol dehydrogenase 5 (predicted)	-1.53
Plcb4	phospholipase C, beta 4	-1.52
Jun	Jun oncogene	-1.52
Myh10	myosin, heavy polypeptide 10, non-muscle	-1.51

Network 1 : Vit D -5 excel for IPA - 2009-05-06 10:21 AM : Vit D -5 excel for IPA.xls : Vit D -5 excel for IPA - 2009-05-06 10:21 AM

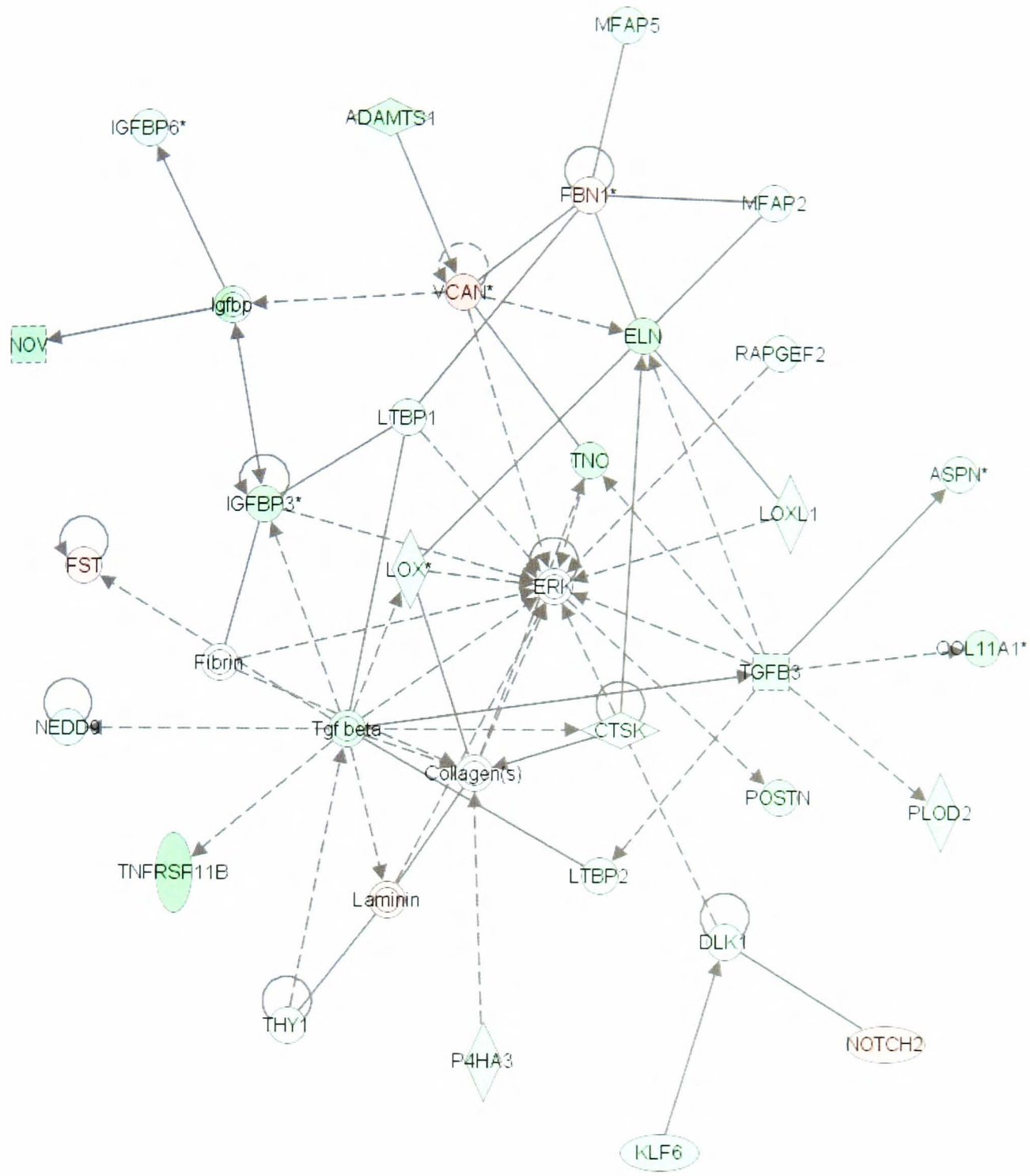


Figure 3.4-2 Diagram of molecules included in network 1: Associated with Genetic Disorder, Opthalmic Disease, & Cellular Growth and Proliferation; generated by IPA from analysis of $1,25(OH)_2D_3$ 10^{-5}M data. Green is down regulated, red is up-regulated, higher intensity of colour relates to higher fold change.

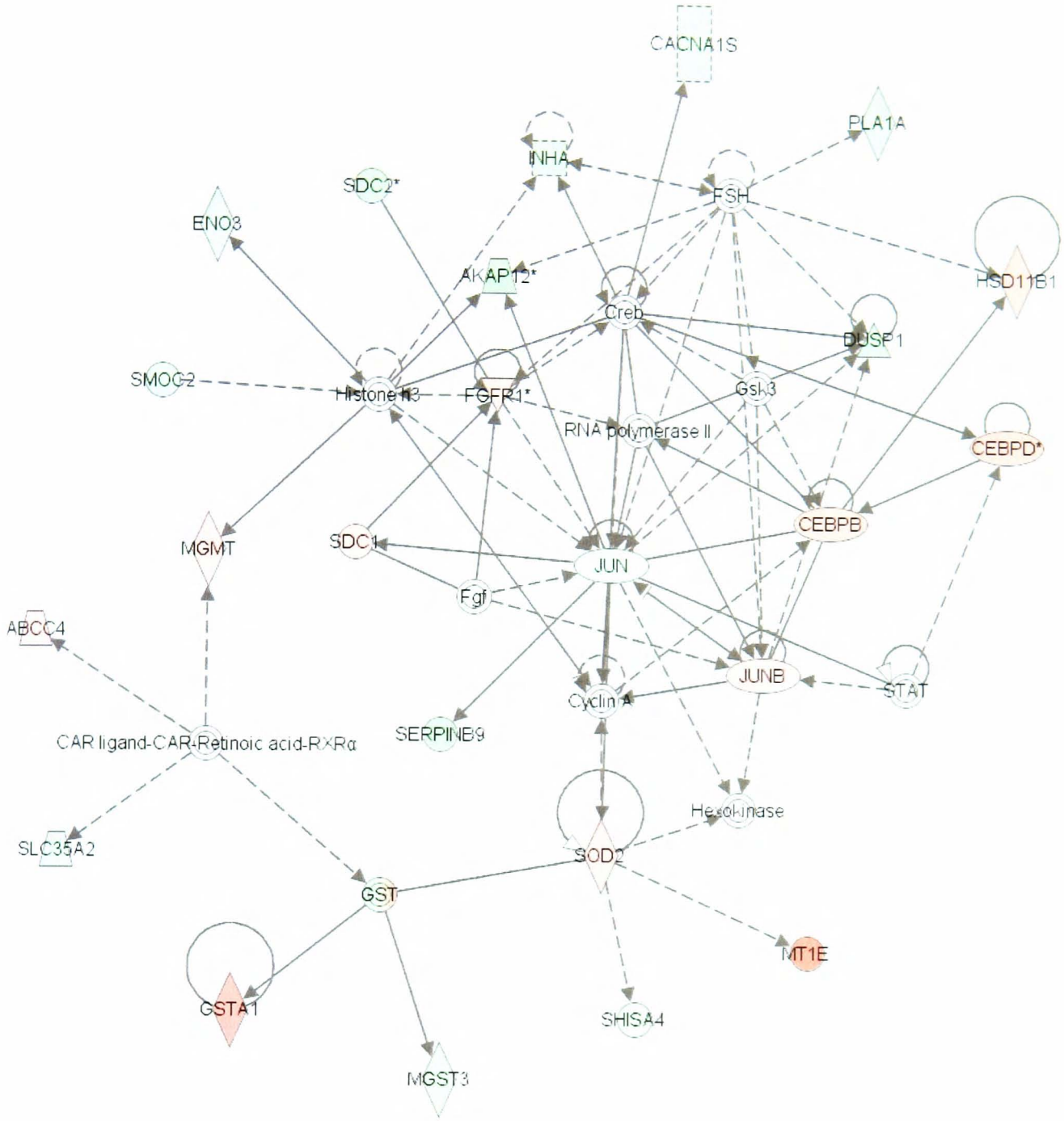


Figure 3.4-3 Diagram of molecules included in network 2: Associated with Cellular Growth and Proliferation, Embryonic Development & Connective Tissue Development and Function; generated by IPA produced from analysis of $1,25(OH)_2D_3$ 10^{-5}M data. Green is down regulated, red is up-regulated, higher intensity of colour relates to higher fold change.

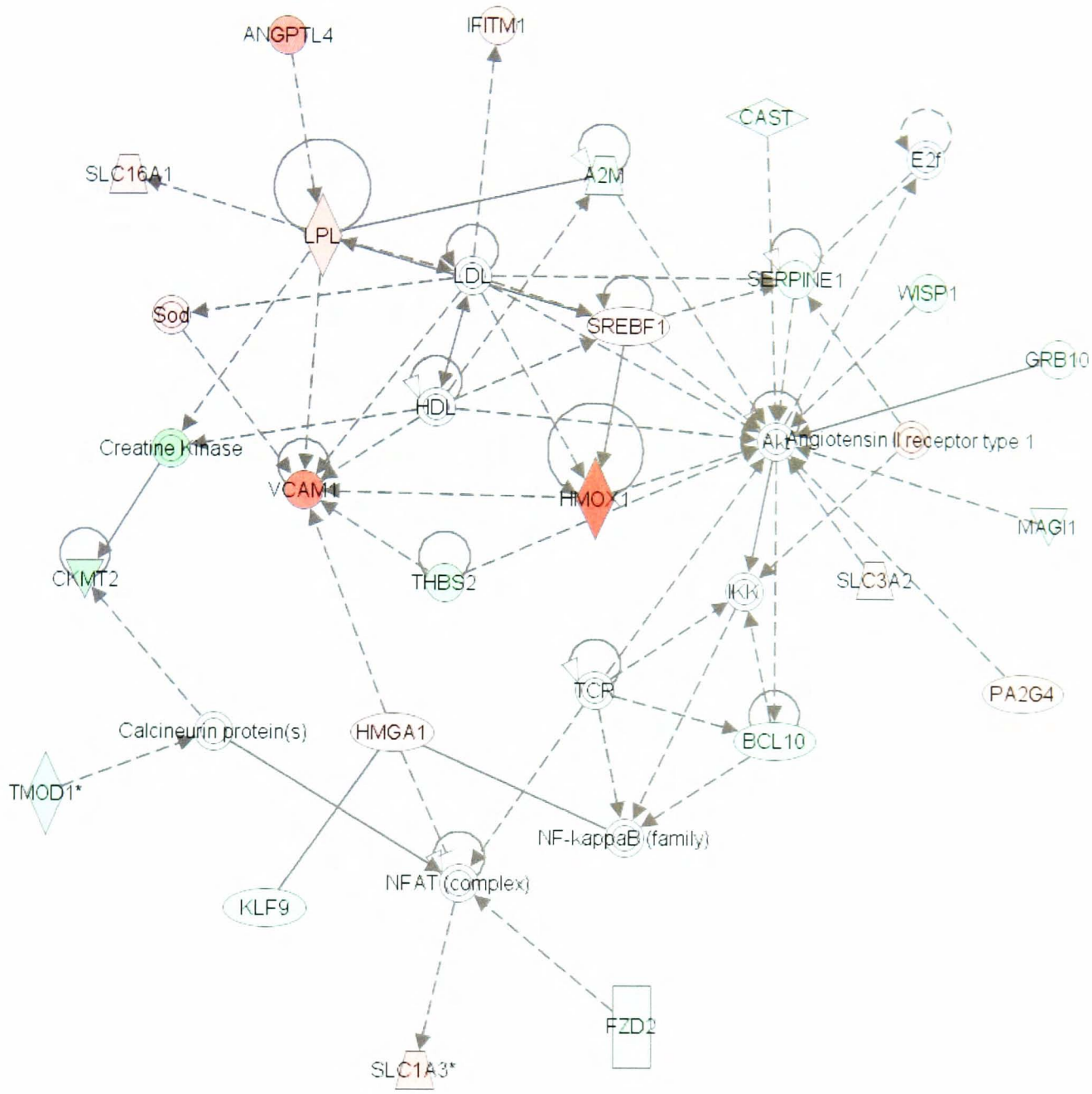


Figure 3.4-4 Diagram of molecules included in network 3: Associated with Cancer, Cell Death & Hematological Disease; generated by IPA produced from analysis of $1,25(OH)_2D_3$ 10^{-5}M data. Green is down regulated, red is up-regulated, higher intensity of colour relates to higher fold change.

Network 4 : Vit D -5 excel for IPA - 2009-05-06 10:21 AM : Vit D -5 excel for IPA xls : Vit D -5 excel for IPA - 2009-05-06 10:21 AM

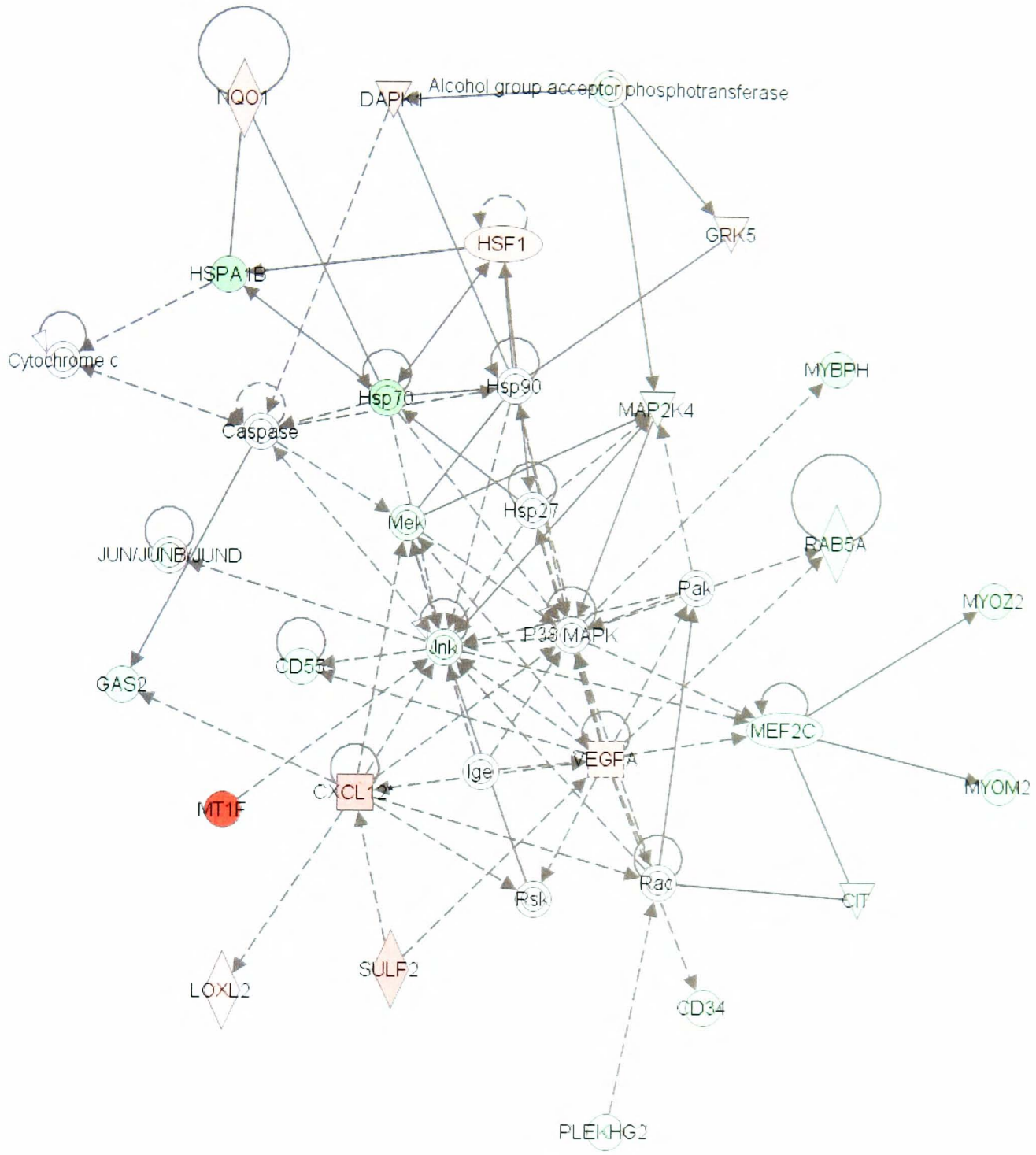


Figure 3.4-5 Diagram of molecules included in network 4: Associated with Cardiovascular System Development and Function, Cardiovascular Disease & Cell Death; generated by IPA produced from analysis of $1,25(OH)_2D_3$ 10^{-5}M data. Green is down regulated, red is up-regulated, higher intensity of colour relates to higher fold change.

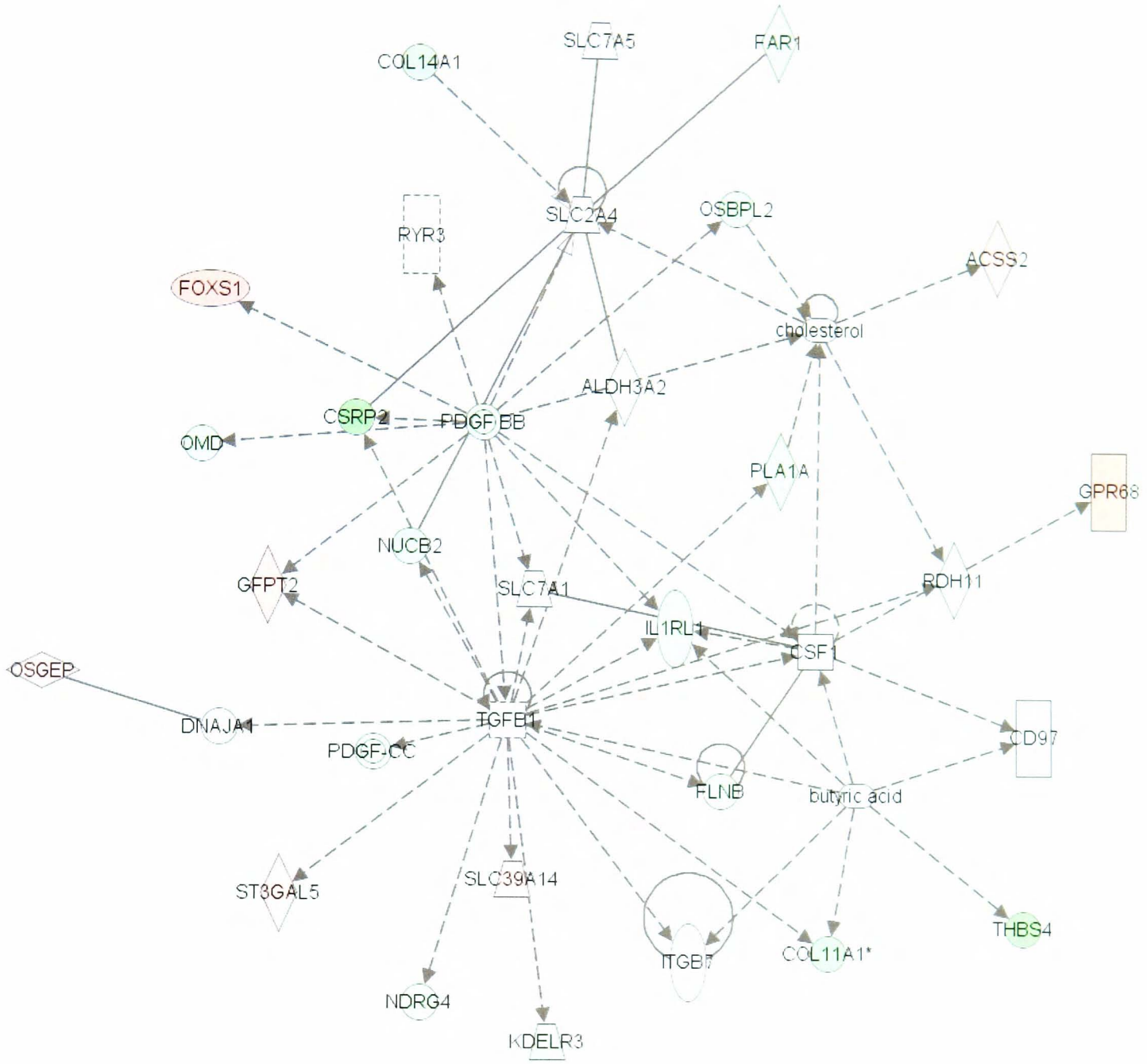


Figure 3.4-6 Diagram of molecules included in network 5: Associated with Cancer, Cellular Movement & Lipid Metabolism; generated by IPA produced from analysis of $1,25(OH)_2D_3$ 10^{-5}M data. Green is down regulated, red is up-regulated, higher intensity of colour relates to higher fold change.

3.4.5 Genes changed by $1,25(OH)_2D_3$ 10^{-9} M treatment

When rat primary skeletal muscle cells were treated with $1,25(OH)_2D_3$ at a lower dose of 10^{-9} M, 43 genes were changed at least 1.5 fold out of 31,099 targets measured by the microarray ($P<0.05$). Out of the total 43 changed genes, 27 genes were up-regulated by treatment in comparison to 16 which were down regulated. To further understand the mechanisms of $1,25(OH)_2D_3$'s action on skeletal muscles the changed proteins were analysed by IPA software which generated 3 networks associated with the changed molecules, summarised in table 3.4-6. Genes which were included in these pathways are summarised in tables 3.4-7 and 3.4-8 which are separated for those transcripts either up-regulated or down-regulated by $1,25(OH)_2D_3$ respectively. The networks generated by IPA listed in table 3.4-6, which contain the genes shown in tables 3.4-7 and 3.4-8, are shown in figures 3.4-7 to 3.4-9.

Table 3.4-6 Top 3 signalling networks containing highest number of molecules changed by $1,25(OH)_2D_3$ 10^{-9}M treatment.

Networks	Associated Network Functions	Score
1	Tissue Morphology, Connective Tissue Development and Function, Skeletal and Muscular System Developmer	33
2	**Cellular Growth and Proliferation, Cellular Development, Cancer**	26
3	Cell Death, DNA Replication, Recombination, and Repair, Cancer	16

Table 3.4-7 List of genes up regulated by $1,25(OH)_2D_3$ 10^{-9}M

Up Regulated		
Gene Name	**Protein Name**	**Fold Change**
Rbp4	retinol binding protein 4, plasma	12.07
Prom1	prominin 1	3.12
Hsd11b1	hydroxysteroid 11-beta dehydrogenase 1	2.82
Fst	follistatin	2.54
Cdc42ep2	CDC42 effector protein (Rho GTPase binding) 2	2.47
RGD1305645_predicted	similar to RIKEN cDNA 9230117N10	2.38
LOC682651 /// LOC689415	similar to Metallothionein-2 (MT-2) (Metallothionein-II) (MT-II)	2.15
Steap2_predicted	six transmembrane epithelial antigen of prostate 2 (predicted)	2.03
	Transcribed locus	2.03
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	2.02
Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	1.99
Sulf2	sulfatase 2	1.98
Crlf1_predicted	cytokine receptor-like factor 1 (predicted)	1.95
Spp1	secreted phosphoprotein 1	1.90
Sod2	superoxide dismutase 2, mitochondrial	1.89
Fkhl18	forkhead-like 18 (Drosophila)	1.85
	Transcribed locus	1.84
LOC679990 /// LOC687552	similar to phosphoglucomutase 5	1.81
Cct6a	Chaperonin subunit 6a (zeta)	1.79
Slc1a3	solute carrier family 1 (glial high affinity glutamate transporter), member 3	1.77
Crlf1_predicted	cytokine receptor-like factor 1 (predicted)	1.76
Gadd45a	growth arrest and DNA-damage-inducible 45 alpha	1.72
Pola2	polymerase (DNA directed), alpha 2	1.67
Loxl2_predicted	lysyl oxidase-like 2 (predicted)	1.65
Steap2_predicted	six transmembrane epithelial antigen of prostate 2 (predicted)	1.60
Syngr2	synaptogyrin 2	1.60
Notch2	Notch gene homolog 2 (Drosophila)	1.55

Table 3.4-8 List of genes down regulated by $1,25(OH)_2D_3$ 10^{-9}M

Down Regulated		
Gene Name	**Protein Name**	**Fold Change**
Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	-2.20
Cd276	CD276 antigen	-1.87
LOC498564	similar to integrin, beta-like 1	-1.84
	Transcribed locus	-1.80
Osbpl2	oxysterol binding protein-like 2	-1.79
	Transcribed locus	-1.77
Pcdh18_predicted	protocadherin 18 (predicted)	-1.70
RGD1305645_predicted	similar to RIKEN cDNA 1500015O10 (predicted)	-1.63
RGD1566401_predicted	similar to GTL2, imprinted maternally expressed untranslated (predicted)	-1.60
Gramd3	GRAM domain containing 3	-1.60
Angptl4	angiopoietin-like 4	-1.57
Pawr	PRKC, apoptosis, WT1, regulator	-1.57
LOC312678	similar to Retinoblastoma-binding protein 2 (RBBP-2)	-1.57
RGD1307397	similar to RIKEN cDNA 2810037C03	-1.53
Agpat5_predicted	1-acylglycerol-3-phosphate O-acyltransferase 5	-1.51

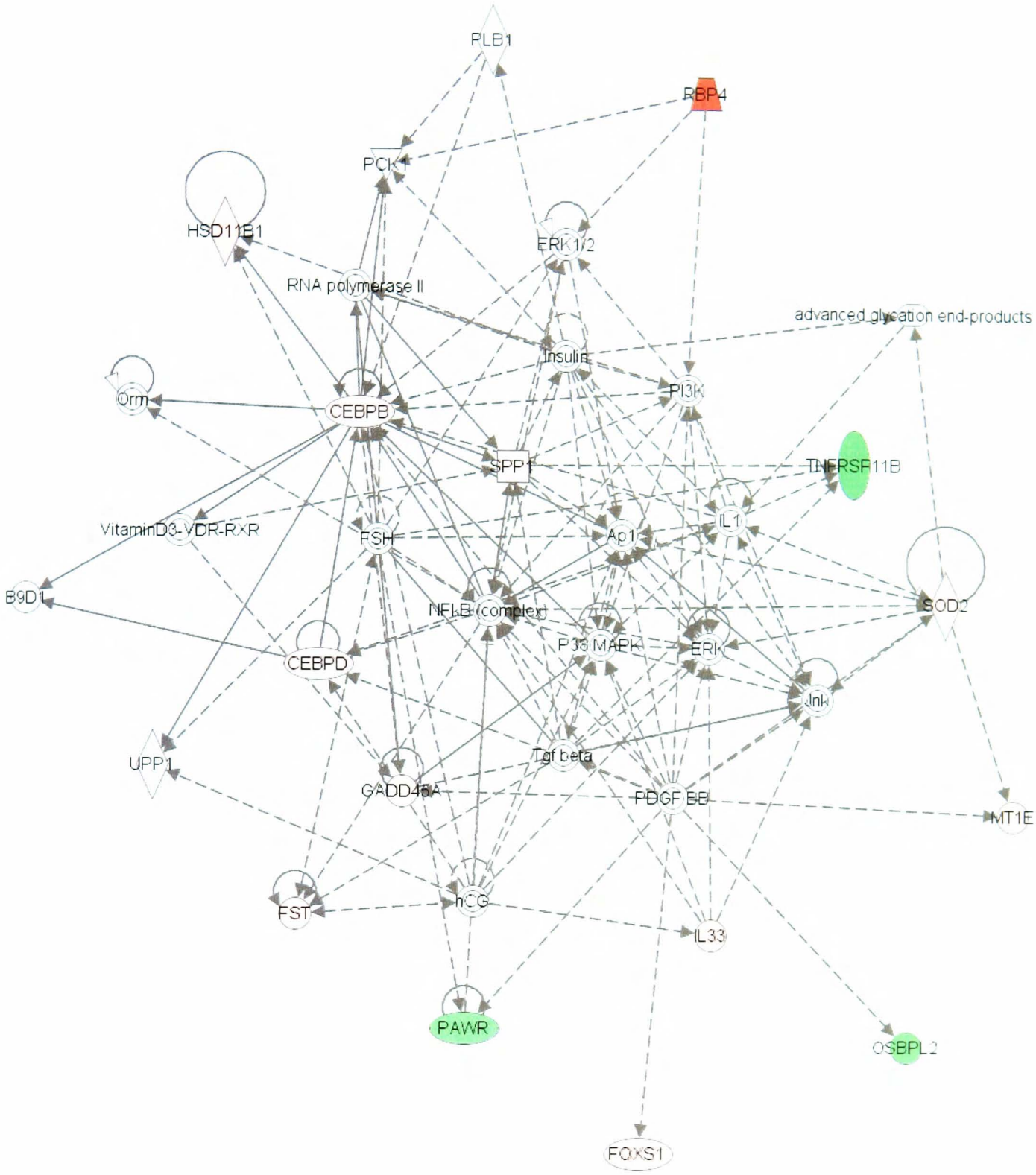


Figure 3.4-7 Molecules changed by $1,25(OH)_2D_3$ at 10^{-9}M concentration, network 1: Associated with Tissue Development and Function. Green is down regulated, red is up-regulated, higher intensity of colour relates to higher fold change.

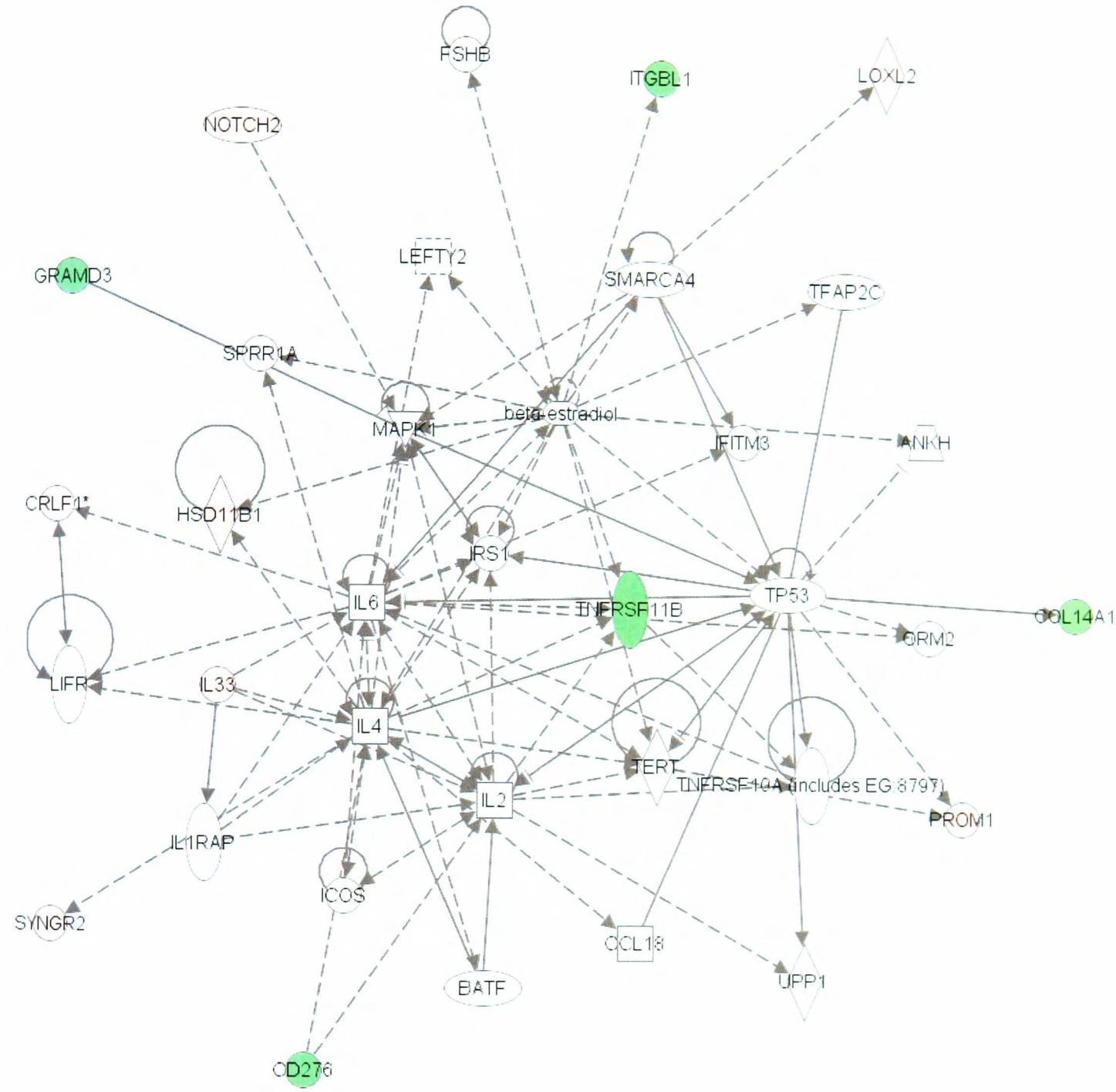


Figure 3.4-8 Molecules changed by $1,25(OH)_2D_3$ at 10^{-9}M concentration, network 2: Associated with Cellular growth and proliferation. Green is down regulated, red is up-regulated, higher intensity of colour relates to higher fold change.

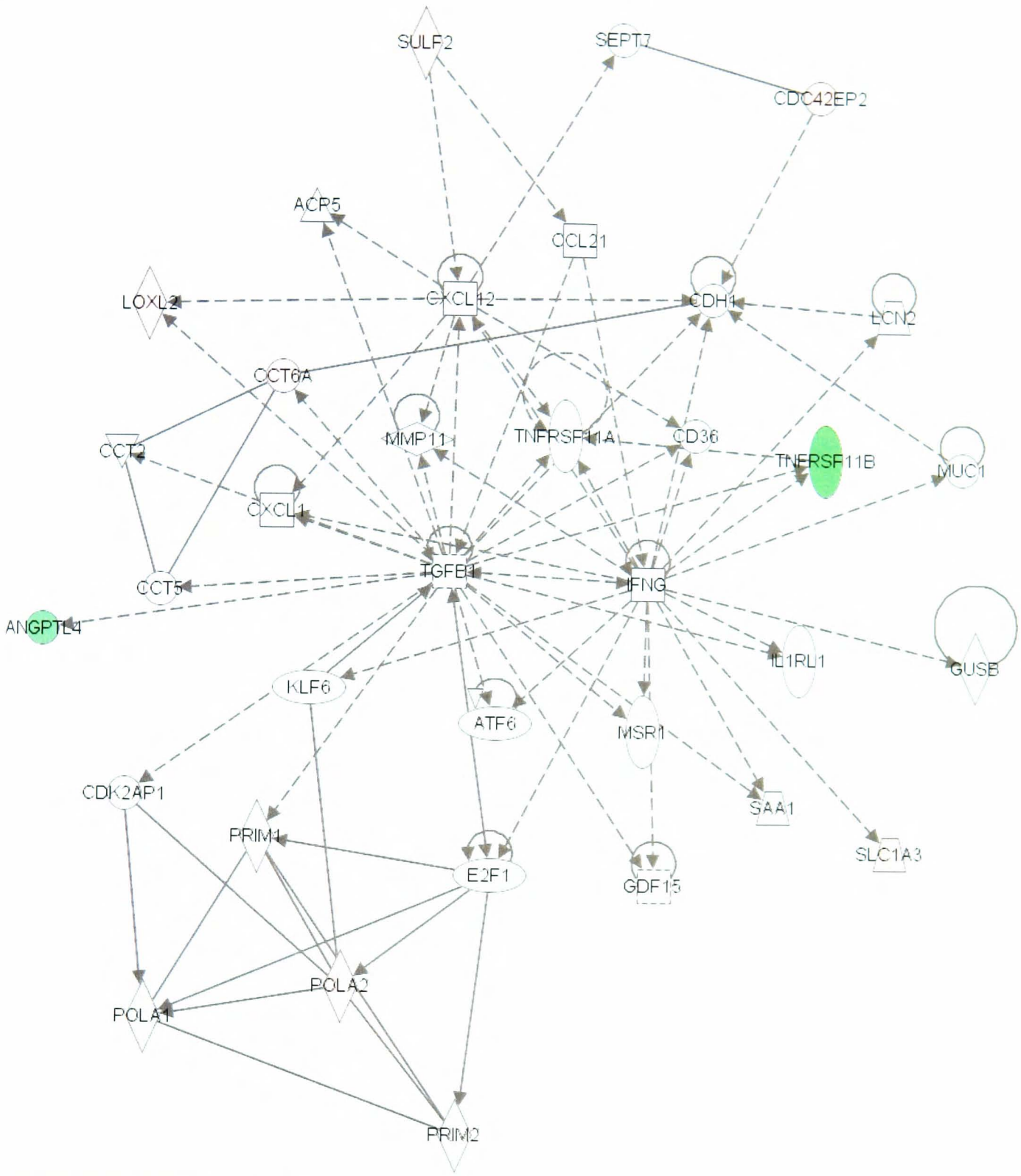


Figure 3.4-9 Molecules changed by $1,25(OH)_2D_3$ at 10^{-9}M concentration, network 3: Associated with Cell death & DNA replication, recombination and repair. Green is down regulated, red is up-regulated, higher intensity of colour relates to higher fold change.

3.4.6 Summary and discussion of microarray data

From 31,099 interrogated sequences, $1,25(OH)_2D_3$ treatment at 10^{-5}M caused significant changes in 475 genes and $1,25(OH)_2D_3$ at 10^{-9}M changed only 43 genes, meaning 1.53% and 0.14% of genes measured by the microarray were affected at each dose respectively. Overall there was an imbalance between the up-regulated and down-regulated genes changed by 10^{-5}M, where twice as many genes, 318, were down-regulated compared to only 157 which were up-regulated. This was the opposite to the gene expression changes observed with the lower dose of $1,25(OH)_2D_3$ where treatment at the 10^{-9}M concentration caused an increase in the expression of 27 genes and a decrease in 16. This did raise some concern that the higher dose of 10^{-5}M was reducing transcription to some extent, which could be related to reduced cell proliferation or apoptosis, and the large number of down-regulated genes could be a result of the cells beginning to cease normal transcriptional activity. This hypothesis was further supported by the list of networks related to those changed genes whereby IPA software identified 5 signalling networks, of which 3 were related to cell death, and also by the effect on RNA yield shown in table 3.4-2.

The primary aim of the microarray was to identify targets responsive to biologically active $1,25(OH)_2D_3$ in muscle cells to be validated in the final experiments, and these are identified in table 3.4-9. This is the first reported microarray performed to analyse $1,25(OH)_2D_3$'s direct effects on skeletal muscle, however Latres *et al.* (2005) performed an experiment in C2C12 cells using IGF-1 and dexamethasome (a glucocorticoid) to evaluate genes which were differentially regulated between the two treatments. Insulin-like growth factor-1 (IGF-1) is well known to promote muscle growth (Gerrard *et al.*, 1998), and on the contrary, glucocorticoids are known to promote muscle atrophy during sepsis and wasting and their effect in muscle is to promote muscle degradation via proteolysis by both the ubiquitin-proteasome pathway and the calpains (Yang *et al.*, 2005). There were some similarities between the results of the microarray performed in this thesis and the results reported by Latres *et al.* (2005) and this supports $1,25(OH)_2D_3$ acting to influence control of muscle growth. Genes which were significantly up-regulated by IGF-1 but down-regulated by a glucocorticoid included metallothionein 1 and 2, both of which were found to be up-regulated in myoblasts treated with $1,25(OH)_2D_3$ at the 10^{-5}M dose and metallothionein 2a was up-regulated by the lower dose of 10^{-9}M $1,25(OH)_2D_3$. A second study using microarray analysis to investigate gene expression changes during induced skeletal muscle atrophy in rats found metallothionein 1, 1B and 1L to be increased during atrophy (Lecker *et al.*, 2004) and does not agree with Latres *et al.*'s conclusions, but does highlight again the changes in metallothionein during changes in muscle growth, and it was decided to evaluate this protein as a target of vitamin D in skeletal muscle in the following final experiments of this thesis.

Table 3.4-9 Genes changed in both treatments with $1,25(OH)_2D_3$ exhibiting dose responses.

Up Regulated			
Gene Name	Protein Name	Fold Change	
		10^{-5}M	10^{-9}M
Rbp4	retinol binding protein 4, plasma	20.91	12.07
LOC682651 /// LOC689415	similar to Metallothionein-2 (MT-2) (Metallothionein-II) (MT-II)	4.3	2.15
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	2.23	2.02
Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	2.57	1.99
Notch2	Notch gene homolog 2 (Drosophila)	1.92	1.55

Retinol binding protein is involved in signalling via retinoic acid which mediates gene expression in a similar mechanism to $1,25(OH)_2D_3$, as the retinoic acid receptor (RAR) forms a heterodimer with RXR. The genes identified in table 3.4-9 had all been up-regulated by $1,25(OH)_2D_3$ in a dose response and were the chosen targets for real time PCR analysis in the last cell culture trial.

Further additional targets were chosen from their relevance in literature where parvalbumin was found to be down-regulated by IGF-1 and up-regulated by the glucocorticoid, therefore its expression showed association with muscle wasting (Latres *et al.*, (2005). Although in the $1,25(OH)_2D_3$ microarray of this thesis parvalbumin was not found to be affected, it is well known to be a gene responsive to $1,25(OH)_2D_3$ (De Viragh *et al.*, 1989). Additionally, the microarray results indicated that $1,25(OH)_2D_3$ was able to activate signalling pathways known to affect the maturation process of skeletal muscle cells. The MAPK pathways were affected by both doses of $1,25(OH)_2D_3$ shown by changes in genes associated with p38 MAPK and ERK 1/2. The MAPKinases are well documented to affect cell growth and differentiation and have been shown to be activated by $1,25(OH)_2D_3$ treatment in skeletal muscle cells (Lösel & Wehling, 2003, Buitrago *et al.*; 2001, 2006), described in section 1.1-4 of this thesis.

In light of this information, additional targets were chosen to be investigated in the last experiment of this thesis to assess vitamin D's effects on pathways known to affect muscle cell proliferation and growth; these were parvalbumin and c-Myc, measured at the transcript level, and the MAPKinase, ERK 1/2, and 4EBP1, assessed for their extent of phosphorylation at the protein level.

Trial 4: Vitamin D signalling in Skeletal Muscle cells in Culture

3.5 Characterisation of skeletal muscle cells for culture work

Vitamin D deficiency is related to loss of muscle strength and fewer type II fibres in aging muscle. In order to investigate vitamin D's role in regulating muscle function and maintaining fast fibre type it was decided to treat muscle cell cultures directly with a constitutively active analogue of vitamin D, 1α-hydroxyvitamin D (1α(OH)D, known as alfacalcidiol), a substance unlikely to be degraded over the treatment period of 48 hours. The hypothesis of the experiment was that treatment with 1α(OH)D would cause a shift towards a fast muscle fibre type in muscle cells. In order to observe fibre type switching between the slow and fast fibre types it was important that the cell model should express, if at all possible, the full range of mature myosin heavy chain genes including MHC I/β slow. In the event of fibre type switching, it was envisaged that the expression of MHC genes would undergo sequential changes from oxidative to glycolytic associated MHC gene expression in the sequence: MHC I/β → MHC IIA → MHC IIX → MHC IIB, or vice versa. Therefore it was necessary to evaluate the available cell lines for their MHC expression. Once characterised, the cell line which showed the largest range of MHC gene expression could then be used to monitor the changes in MHC expression, in particular it was important to find a cell line which expressed as large a range of MHC as possible from the slow MHC I/β isoform to the MHCIIB fast adult isoforms.

3.5.1 Outline of work

Three cell types were evaluated for MHC gene expression, to determine the maturity of the cell line for validity in comparisons to adult animal muscle tissue. Both the rat primary muscle cells and the L6 Aston cell line were rat derived cells and were evaluated using rat specific primers to the MHC genes developed by Sanchez *et al.* (2006) and Jaschinki *et al.* (1998), primers shown in Appendix E. The mouse derived C2C12 cells were evaluated using primers designed specifically to the mouse isoforms of the MHC genes by da Costa *et al.* (2002), shown in Appendix F. Semi-quantitative PCR reactions were performed using primers for each gene and products were electrophoresed in agarose gels before staining and imaging. Adult LD cDNA for each species was used as a positive control for MHC gene expression.

3.5.2 Expression of MHC in rat derived muscle cells (Rat Primary cells & L6 Aston cell line)

cDNA produced from mRNA extracted from rat primary cells at sequential stages of development was used to determine MHC gene expression. Rat primary muscle cells were prepared from neonatal Wistar male rat pups as described in section 2.11.1 and evaluated for their use in this experiment. Primary cells directly harvested from animal tissue where cells are not passaged between harvest and treatment are useful models for animal muscle tissue in vitro, with less risk of adaptation to in vitro environment as can happen over time with cell lines. Cell lines tend to retain most functions of their parent cells but can lose them as they undergo a number of passages (Harrison & Rae, 1997).

Cells were harvested at 4 time points from confluent myoblast phase (day 0) through to fully formed myotubes (day 7). The primers used to characterise the MHC gene expression are summarised in table 3.5-1 and the positive DNA bands produced by these primers in the cDNA produced from RNA extracted from 3-month old rat LD are shown in figure 3.5-1. The size of DNA products produced by these primers correspond to the predicted product size in table 3.5-1 and so these bands were used as positive results with which to compare the products produced by the cells.

Table 3.5-1 Basic PCR primers and predicted product sizes used to characterise rat-derived cells for MHC gene expression.

Rat MHC Pimers for Isotyping					
MHC	**Gene**	**Sequence**	**Region of sequence**	**Primers designed by**	**Product Length (bp)**
Embryonic	Myh 3	K02111	5' end	Sanchez *et al.* (2006)	181
Neonatal	Myh 8	NM_012604	3' end	Sanchez *et al.* (2006)	103
I / β	Myh 7	NM_017240	3' end	Jachinski *et al.* (1998)	288
IIa	Myh 2	L1360	3' end	Jachinski *et al.* (1998)	310
IIx	Myh 1	XM_213345	3' end	Jachinski *et al.* (1998)	120
IIb	Myh 4	BC113948	3' end	Jachinski *et al.* (1998)	197

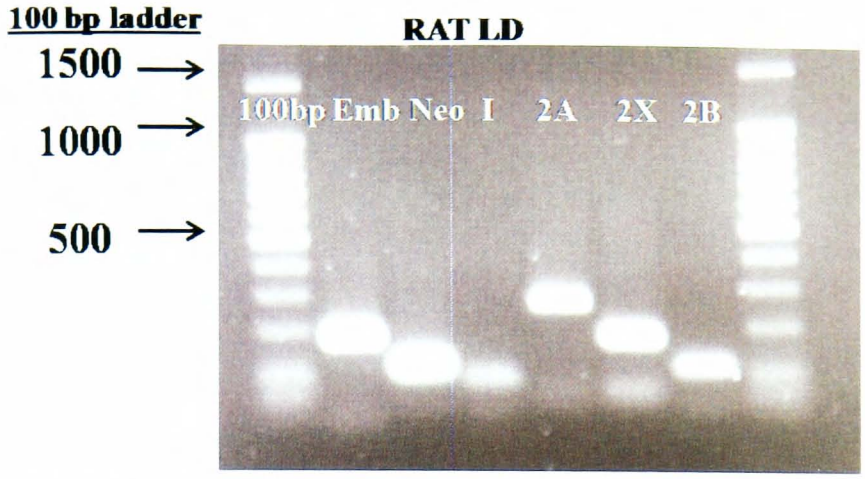


Figure 3.5-1 Expression of MHC in 3 month old rat LD verified using primers designed by Jaschinski *et al.* (1998) & Sanchez *et al.* (2006). PCR products electrophoresed in 1.5% agarose gel, 10µl of product synthesised from 5µl cDNA template (equivalent to 0.125µg RNA) loaded with 2µl dye, ladder is 100bp with major band sizes indicated.

In rat primary skeletal muscle cell cultures both embryonic and neonatal MHC genes were expressed throughout differentiation and were still expressed after 7 days of development (shown in figure 3.5 -2). Expression of MHC IIX (2X) is at a similar level to that of the neonatal and embryonic MHC genes and remains fairly constant throughout development, whereas expression of IIA (2A) appears to increase with age and IIB (2B) declines. There is no expression of MHC I in these cells, although there is a very light band of the correct product size beginning to appear at 7 days post induction of differentiation. The basic PCR products produced by cDNA samples from the rat derived L6 Aston muscle cell line are shown in figure 3.5-3; the L6 Aston cell line is derived from the standard rat L6 muscle cell line and the L6 Aston is a fusing derivative of this cell line. This image shows MHC expression is predominantly limited to the fast isoforms of MHC IIA (2A) and IIX (2X) at day 0 when fusion media was added to confluent myoblasts and these isoforms remain highly expressed throughout the course of differentiation. Around 2 days post differentiation, the cells begin to express embryonic and IIB (2B) MHC genes but there is no expression of neonatal or, crucially, MHC I/β.

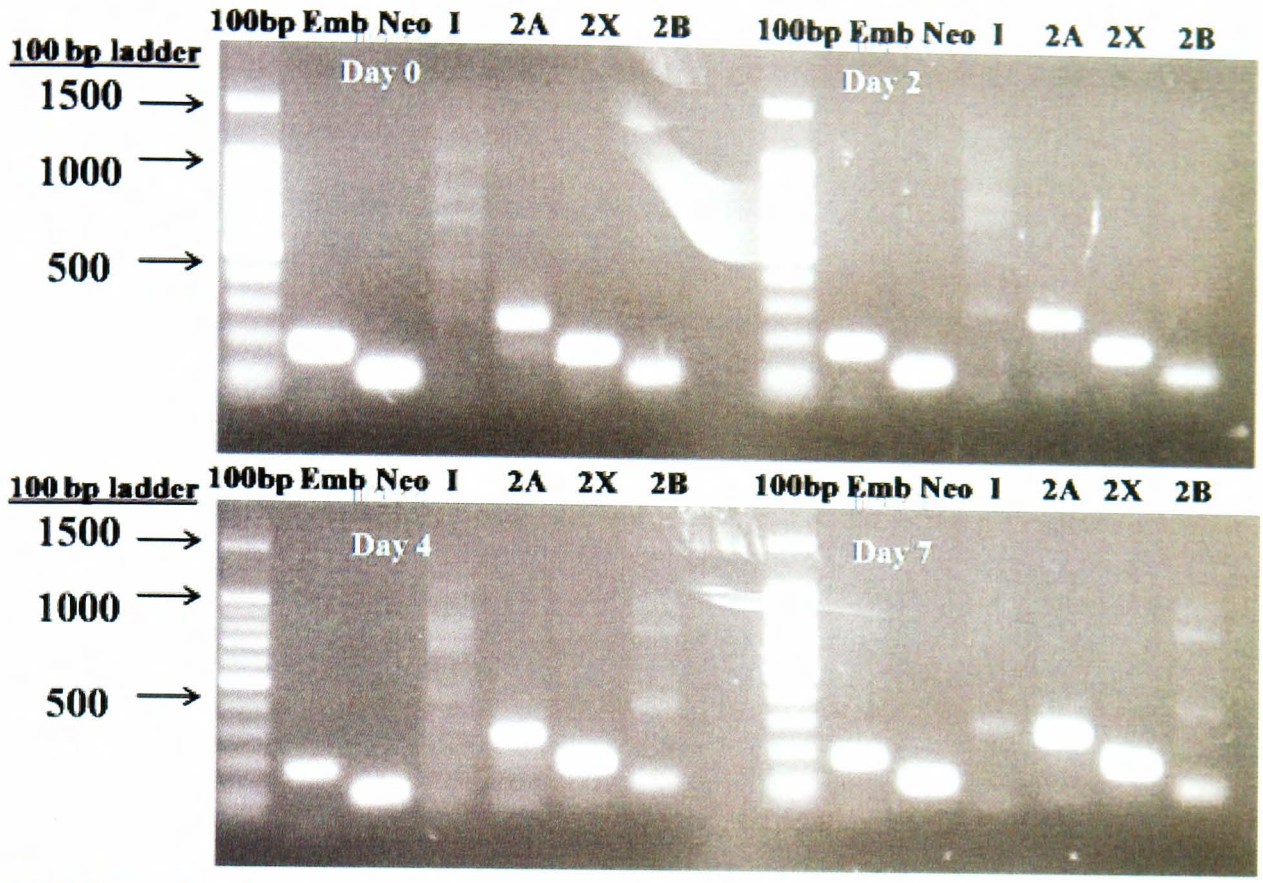


Figure 3.5-2 Expression of MHC in rat primary muscle cells at four different ages post differentiation (day 0 is day media is substituted to cell fusion media). PCR products electrophoresed in 1.5% w/v agarose gel, 10µl of product synthesised from 5µl cDNA template (equivalent to 0.125µg total RNA) loaded with 2µl dye, ladder is 100bp with major band sizes indicated.

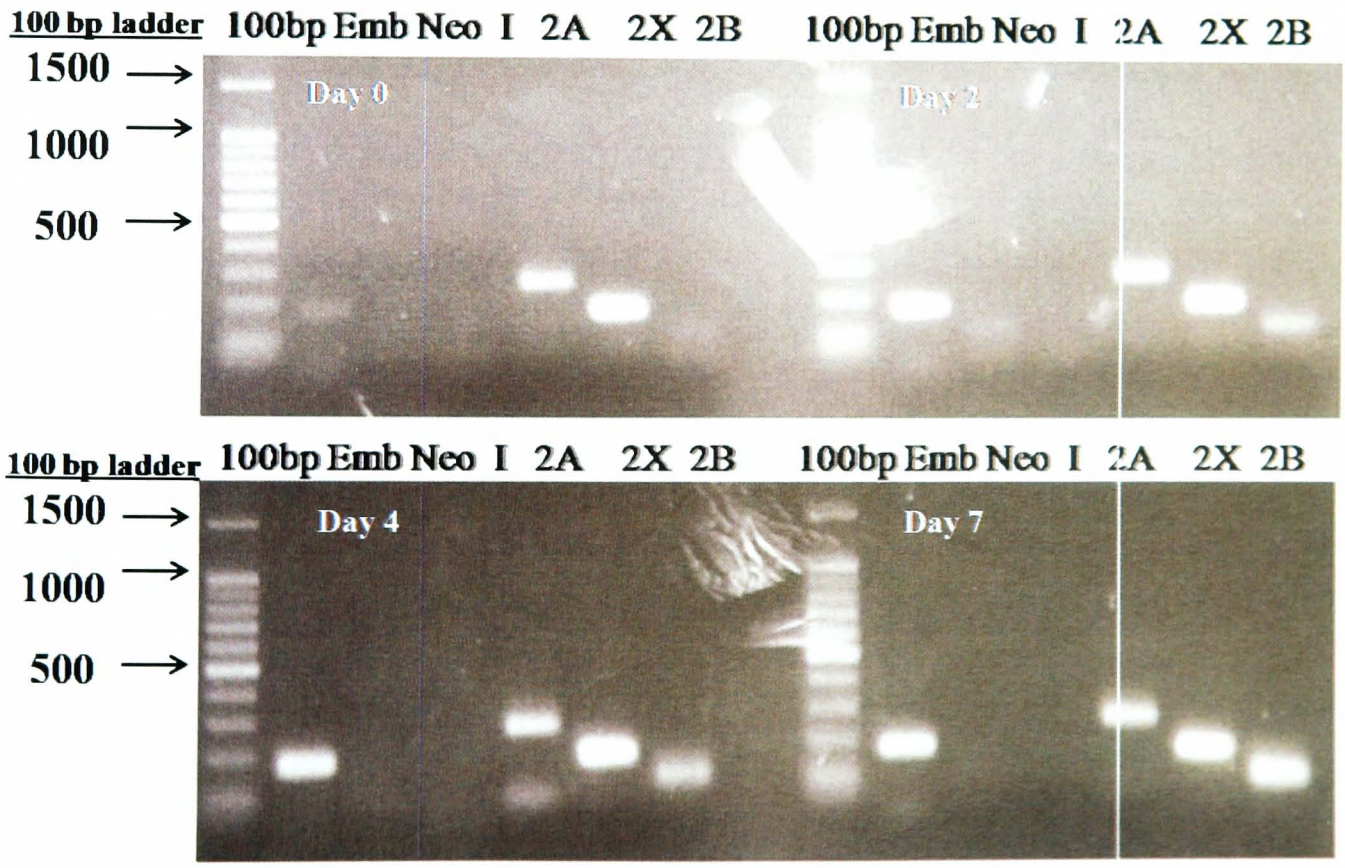


Figure 3.5-3 Expression of MHC in the L6 Aston muscle cell line at four different ages post differentiation (day 0 is day media is substituted to cell fusion media). PCR products electrophoresed in 1.5% w/v agarose gel, 10µl of product synthesised from 5µl cDNA template (equivalent to 0.125µg total RNA) loaded with 2µl dye, ladder is 100bp with major band sizes indicated.

3.5.3 Expression of MHC in Mouse Muscle cells

Mirroring the experiment performed in rat muscle cells, mRNA from C2C12 cells was harvested at the same stages of differentiation and were isoform-typed using primers designed specifically to mouse. Primers designed by da Costa *et* al. (2007) were used to evaluate the MHC expression in C2C12 cells, summarised in table 3.5-2 along with the predicted product sizes. Positive PCR products were generated using cDNA extracted from mouse LD and the resulting bands produced are shown in figure 3.5-4. Primers for β-actin and Myf5 were also produced by da Costa *et al.* and were tested in the same way to ensure they were expressed the cell line, positive products shown in figure 3.5-4. Information for all these primers is presented in Appendix F. These primers were designed for real-time PCR analysis for all of the MHC isoforms using a fluorescent probe for detection and so the relative intensity of the bands produced may not directly reflect the actual level of expression. These images can be assumed to be a guideline to represent the actual expression within the cell line.

Table 3.5-2 Information for primers used to characterise MHC gene expression of C2C12 cells.

Mouse MHC primers & probes for qPCR, designed by da Costa et al. *(2007)*				
MHC	**Gene**	**Sequence**	**Region of sequence**	**Product Length (bp)**
Embryonic	Myh 3	XM_908146	5' end	72
Neonatal	Myh 8	NM_177369	3' end	142
I / β	Myh 7	AY056464	3' end	116
IIa	Myh 2	NM_001039545	5' end	81
IIx	Myh 1	NM_030679	5' end	70
IIb	Myh 4	NM_010855	3' end	80

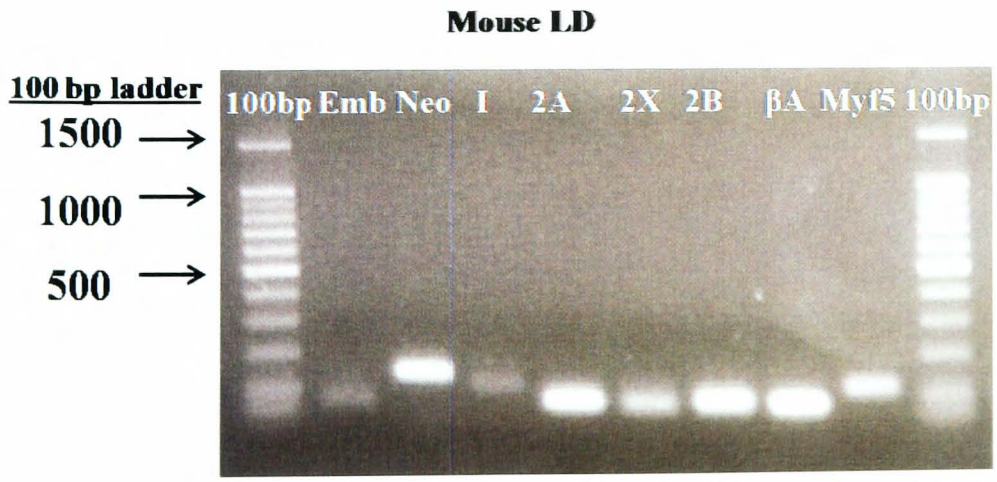


Figure 3.5-4 Expression of MHC in Mouse LD muscle, verified using primers designed by da Costa *et al.* (2007). PCR products electrophoresed in 1.5% agarose gel, 10µl of product synthesised from 5µl cDNA template (equivalent to 0.125µg total RNA) loaded with 2µl dye, ladder is 100bp with major band sizes indicated.

The basic PCR produced from C2C12 cell cDNA are shown in figure 3.5-5. From this image it can be seen that the C2C12 cells expressed a larger range of the MHC genes, although the band produced with the neonatal MHC primers was the most predominant, bands of varying intensity can be seen for all the MHC isoforms. The expression of the fast isoforms IIA/2A, IIX/2X and IIB/2B appears to increase as the cells differentiate, whereas the MHC I/β isoform seems to be most expressed during the process of differentiation at days 2 and 4.

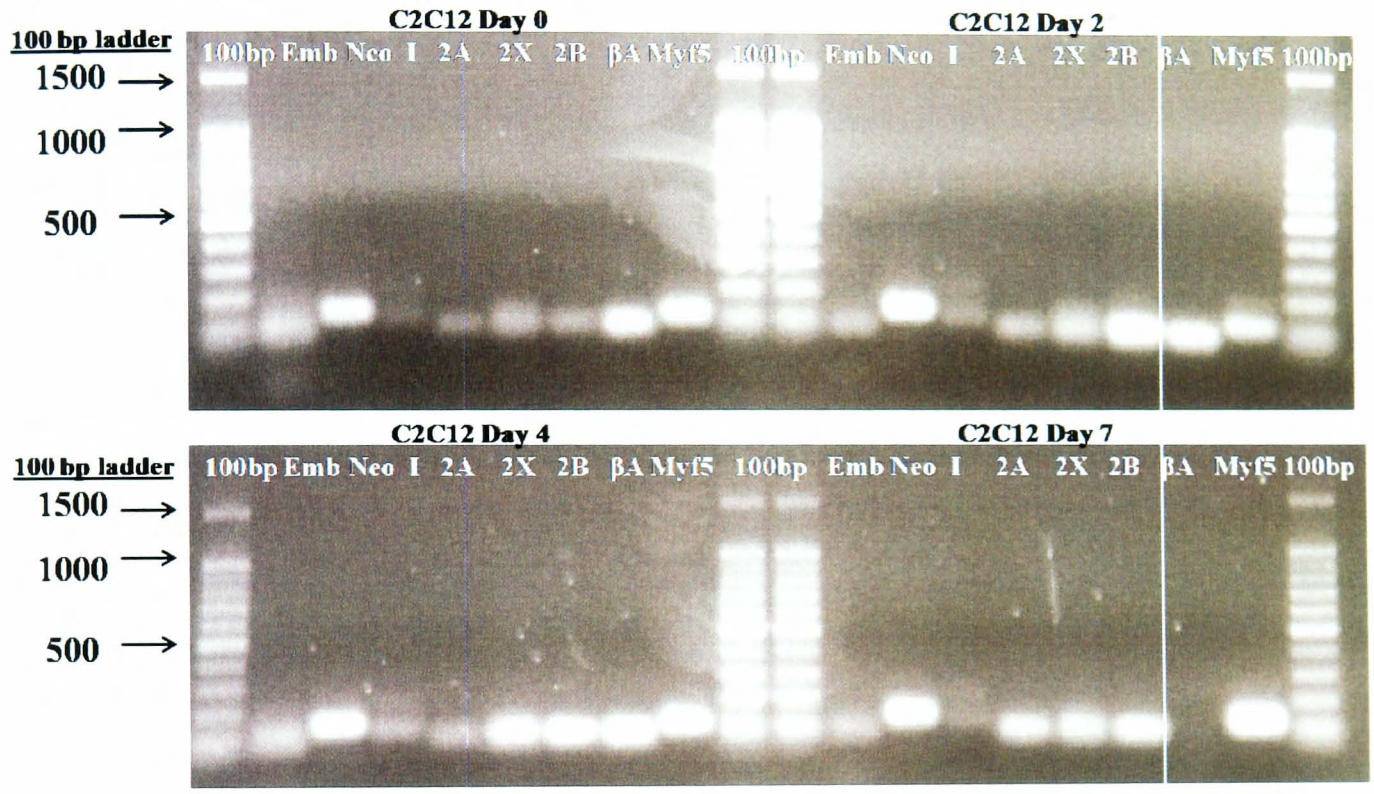


Figure 3.5-5 Expression of MHC in the C2C12 muscle cell line at four different ages post differentiation (day 0 is day media is substituted to cell fusion media). PCR products electrophoresed in 1.5% agarose gel, 10µl of product synthesised from 5µl cDNA template (equivalent to 0.125µg total RNA) loaded with 2µl dye, ladder is 100bp with major band sizes indicated.

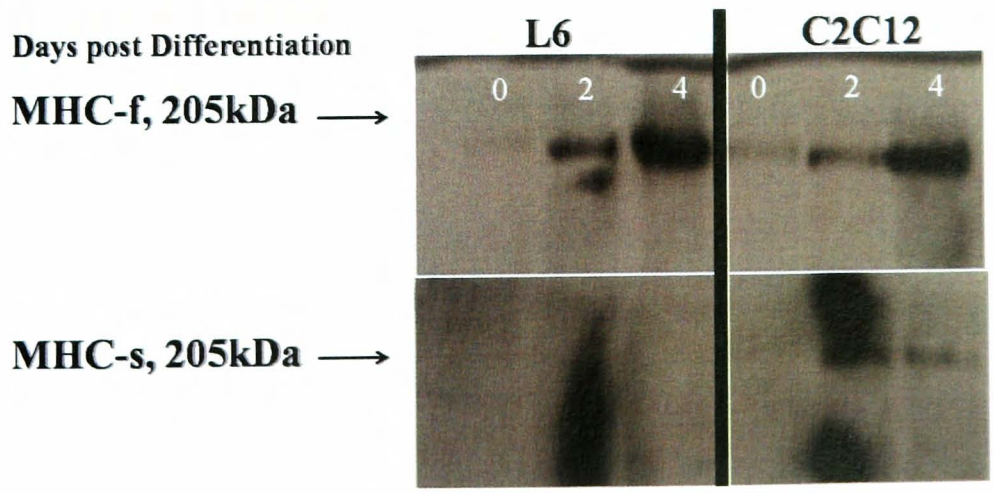


Figure 3.5-6 Expression of MHC fast (MHC-f) and slow (MHC-s) in L6 and C2C12 cell lines at three stages post differentiation, where day 0 is time of substitution of media to cell fusion media., labelled 0, 2 and 4 days post differentiation in diagram. Volume 30µl cell protein extracts (confluent cells extracted directly into 500µl SDS-mix) separated by SDS-PAGE using 8% acrylamide gel followed by western blotting, probed for MHC fast (MHC-f) and slow (MHC-s) (antibody information in Appendix D).

Following isotyping the cells at a gene level, the expression of the fast and slow MHC isoforms was verified at the protein level using antibodies developed to either MHC fast, an antibody which detects all fast isoforms of the MHC, or MHC slow, both antibodies were optimized and verified by Sazili (2003). The resulting western blot produced is shown in figure 3.5-6, which demonstrates that there is an increasing expression of fast MHC at the protein level across differentiation in both the L6 Aston and C2C12 cells with age. There is a similar effect seen in the expression of slow myosin at the protein level in the C2C12 cells whereas the MHC slow could not be detected in the L6 Aston cells at quantities of protein loaded.

3.5.4 Results and Discussion: Characterisation of skeletal muscle cells for culture work

The primary muscle cultures prepared from rat pups expressed MHC genes of a more immature fibre type, predominantly expressing the developmental embryonic and neonatal MHC genes and MHC IIX when myoblasts were induced to differentiate, but expression of MHC IIA increased as cells fused into myotubes. The L6 Aston rat derived cell line expressed a relatively "fast" phenotype, with MHC IIA and IIX predominantly expressed during the myoblast phase and the expression of embryonic and IIB increasing as myotubes developed. The C2C12 mouse derived cell line was the only muscle cell type to express MHC I/β and expressed the largest range of adult MHC throughout the differentiation process. The assessment of the MHC expression was verified at the protein level for the L6 Aston and C2C12 cell lines, where the C2C12 cells were the only cell line to express MHC slow. The analysis of cell lines for their MHC expression at the mRNA level has not been reported in the L6 Aston cell line although a study by de Arcangelis *et al.* (2005) reported the L6 derivative cell line L6-C5 expressed a similar expression of the MHC proteins using SDS-PAGE and western blotting techniques. The L6 cell line investigated in de Arcangelis *et al.*'s study was similar to the L6 Aston cells used in this thesis, with protein levels of MHC consisting predominantly of embryonic MHC, followed by neonatal MHC and MHC IIB, although their study only reported the expression after 6 days of differentiation.

Despite the rat primary skeletal muscle cells being the model for the microarray, it was decided not to pursue the next experiments using these cells as they showed a high level of embryonic and neonatal isoforms and were only beginning to express MHC I/β at very low levels after 7 days of differentiation. The L6 Aston cells did express the more mature fast isoforms of MHC but there was no expression of MHC I/β at all and these cells were not viable for the subsequent experiment.

The real-time qPCR primer designed to the mouse MHC genes had been used in study reported by da Costa *et al.* (2008) who reported that the MHC profile of C2C12 cells was similar to the findings of the C2C12 reported in this chapter. The resolution observed with the primers used to evaluate MHC

gene expression in the mouse cells is quite low as the primers were designed for real time PCR using a probe for detection and so the products are small and are not highly visible, however, it can be concluded from these images that C2C12 cells have the widest expression of the MHC genes, and do express the MHC I/β which was of importance to the experimental hypothesis. Therefore the C2C12 cells were used to examine the effects of 1α(OH)D analogue on muscle cells in culture.

3.6 Treatment of C2C12 cells with vitamin D analogue 1α(OH)D.

Following selection of the C2C12 skeletal muscle cell line, the subsequent experiments were performed to investigate the effects of an active analogue of vitamin D on the differentiation of muscle cells. Cells were treated with the vitamin D analogue at a range of time points across differentiation so that vitamin D's effects on proliferation, the differentiation process and on MHC isoform expression in mature muscle cells could all be investigated. The aim of this study was to investigate whether a constitutively active vitamin D analogue, 1α-OH vitamin D (alfacalcidiol), could influence the expression of the MRFs and whether the time of the treatment across the differentiation process was a factor in the response of the cells to the vitamin D. If 1α(OH)D was to affect the process of differentiation then it was likely that the levels of expression of MRFs such as Myf5 and myogenin would be altered. The process of myogenesis is regulated by the temporal expression of the MRFs and so measuring the changes in their expression would be an indicator of the progression of the cells through the myogenic programme. Myf5 is expressed in the early days of myogenesis and is associated with the proliferation of myoblasts before they leave the cell cycle and begin to differentiate into myotubes. The initiation of differentiation into myotubes is then regulated by myogenin, which is expressed after Myf5 in the myogenic programme and regulates the differentiation of the immature myoblasts into mature muscle cells. In their study Endo *et al.* (2003) reported that treatment of C2C12 cells with $1,25(OH)_2D_3$ when myoblasts were 80% confluent (at onset of differentiation) resulted in a decrease in Myf5 and myogenin mRNA expression and decreased the level of expression of the MHC neonatal isoform. In addition to the changes in expression of the MRFs, the extent of differentiation can be evaluated by the presence of skeletal muscle proteins which are expressed in differentiated myotubes. Desmin is an early marker of muscle cell differentiation, and interacts with the sarcomeres laterally between each myofibril structure (see diagram 1.6-2) and therefore the expression of this protein at the mRNA level was measured. The second hypothesis of this investigation was that 1α(OH)D could alter the MHC isoform expression profile of muscle cells, and could induce cells to alter their MHC isoform expression. In order to assess these effects, the expression of the MHC was measured using real time RT-PCR. In addition, the effects of 1α(OH)D on expression of transcripts that had been

identified as responding to $1,25(OH)_2D_3$ by the microarray experiment (described in section 3.4) was examined, these targets are summarised in table 3.4-9.

Prior to treatment with 1α(OH)D, it was important to be familiar with the cells in regards to their normal growth pattern and it was found that the cells were fully differentiated into multinucleated myotubes by 7 days after supplementation with cell fusion media. Figure 3.6-1 shows images of the C2C12 cells growing from the day before media was changed from cell growth media to cell fusion media, denoted day -1, up to 9 days after differentiation was induced. It was observed that myoblasts were beginning to elongate after 1-2 days of incubation in cell fusion media. Myotubes were apparent after 3 days of differentiation and by 7 days of differentiation the cells were fully differentiated. There was some infrequent spontaneous twitching of the cells observed at the very latest age of 9 days post differentiation.

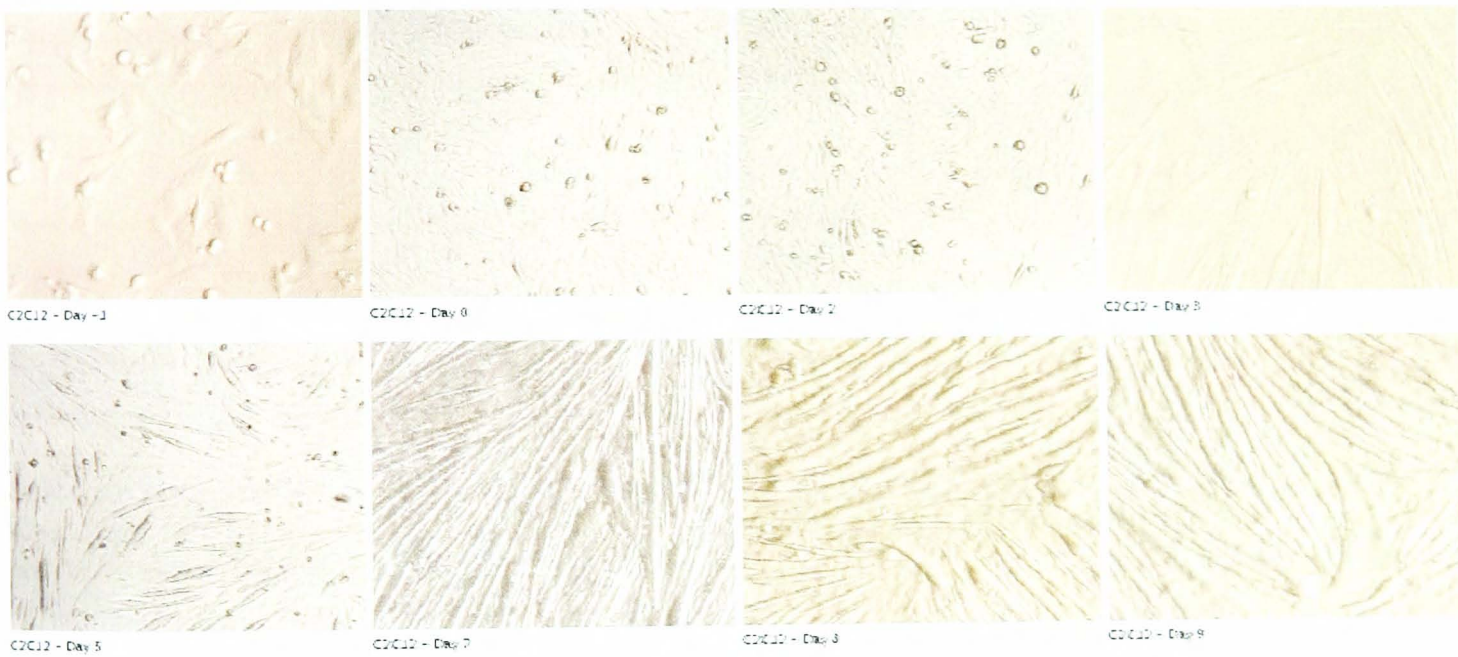


Figure 3.6-1 Phase contrast microscopy (First image is magnification x5, all others magnification x10) of C2C12 skeletal muscle cells, seeded at a density of 3 x 10^4 cells per well, images show cells throughout the process of differentiation from 80% confluence (day -1), fusion media added on day 0 and refreshed every 48 hrs for 9 days.

From these observations it was decided to treat cells at four different time points, the first being the addition of 1α(OH)D directly to the proliferating cells at 75% confluence in cell growth media, before adding 1α(OH)D to the cell fusion media when cells were around 90% confluent (day 0), after 3 days of differentiation when myotubes were beginning to appear and after 7 days of later by which point mature myotubes were apparent.

At every time point, 1α(OH)D was supplemented at four decreasing concentrations; 10^{-5}M, 10^{-7}M, 10^{-9}M and 10^{-11}M directly within the media and cells were incubated for 48 hours before protein and mRNA samples were harvested. A fifth set of cells designated as controls were treated with 0.1% DMSO within the media. The period of 48 hours for 1α(OH)D treatment was chosen in consideration of the effects observed in Endo *et al.'s* (2003) study, 48 hours was the shortest treatment period with which they saw a response, and was also the longest period in which media could be left without need for change. A schematic timeline of the cell experiment is shown in figure 3.6-2.

<table>
<tr><th></th><th>Experiment 1</th><th>Experiment 2</th><th>Experiment 3</th><th>Experiment 4</th></tr>
<tr><td></td><td colspan="4">Myoblasts plated into 6-well plates in Growth Media</td></tr>
<tr><td>Days Differentiation</td><td>Grow Cells until 75% Confluent</td><td colspan="3">Grow Cells until 90% Confluent</td></tr>
<tr><td>-1</td><td rowspan="3">Vit D in GM
48Hrs
Harvest</td><td rowspan="3">Cell Fusion Media
+ Vit D
48 Hrs</td><td colspan="2">Add Cell Fusion Media</td></tr>
<tr><td>0</td><td rowspan="2"></td><td rowspan="4"></td></tr>
<tr><td>1</td></tr>
<tr><td>2</td><td rowspan="3"></td><td rowspan="3">Cell Fusion Media
+ Vit D
48 Hrs</td></tr>
<tr><td>3</td></tr>
<tr><td>4</td><td rowspan="3"></td></tr>
<tr><td>5</td><td rowspan="3"></td><td rowspan="3"></td></tr>
<tr><td>6</td></tr>
<tr><td>7</td><td rowspan="3"></td><td rowspan="3"></td><td rowspan="3">Cell Fusion Media
+ Vit D
48 Hrs</td></tr>
<tr><td>8</td></tr>
<tr><td>9</td></tr>
</table>

Figure 3.6-2 Schematic time line of 1α(OH)D treatments directly on C2C12 muscle cells. Growth media in which cells were proliferating is depicted in grey, blue colour represents growth media treated with 1α(OH)D. Maroon colour represents cell fusion media, red represents cell fusion media treated with 1α(OH)D.

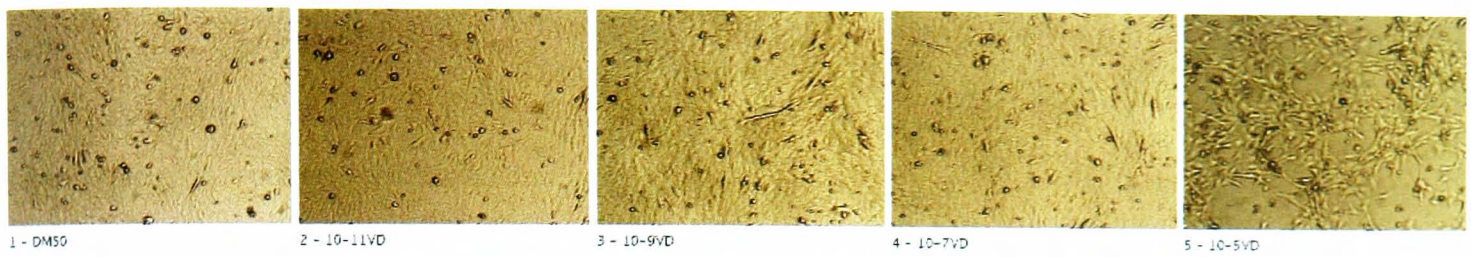
3.6.1 Results: Effects of 1α(OH)D on mouse C2C12 muscle cell line.

The morphology of the C2C12 cells during the experiment was imaged and the resulting photographs are shown in figure 3.6-3. The photographs illustrate that the cells were able to fully differentiate during the experiment as had been expected. However it became apparent that the cells did not tolerate the highest dose of 1α(OH)D especially at the younger stages of development when myoblasts were still present. In the first two time points, 1α(OH)D at a concentration of 10^{-5}M appeared to cause the cells to detach from the plate and prevented further proliferation, demonstrated by large voids across the growth surface. However, at the later stages of development, when myotubes had begun to appear, it seemed the cells tolerated the higher dose

and fewer cells detached. Following preparation of mRNA and protein there were no statistically significant differences found in the yield of either sample between the doses, and the mRNA produced from the cells is shown in Appendix H. The toxic effects of the highest dose became more apparent during qRT-PCR analysis when expression of certain genes were consistently low with the highest dose and frequently too low to measure with any confidence. As a result of these observations it was concluded that the highest dose was causing apoptosis to a certain extent and so it was decided to remove the 10^{-5}M samples from the analysis of gene expression.

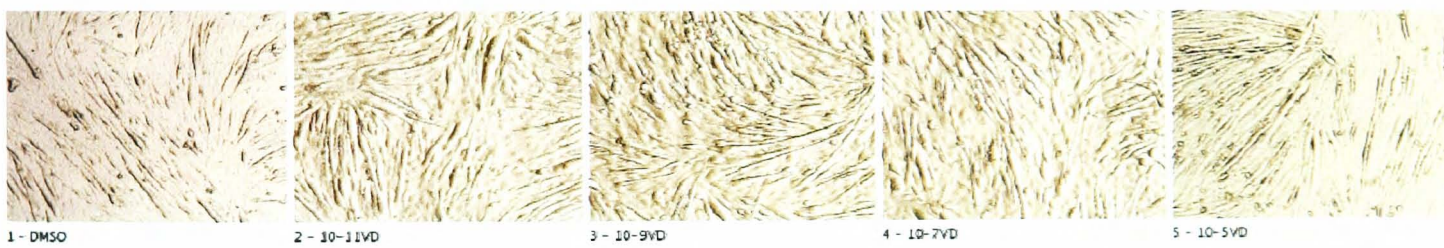
The samples produced from the experiment essentially formed two separate experiments, the first investigating the effects of 1α(OH)D alone in cells which were still proliferating and the second was the effects of 1α(OH)D when treated with cell fusion media over the course of differentiation from myoblast to myotube. This course of treatment essentially produced two separate experiments, the first was to investigate the effects of 1α(OH)D on the onset of differentiation without the influence of the cell fusion media. The second experiment was designed to investigate 1α(OH)D's influence on the myoblasts as they grow over the full course of differentiation and in this experiment 1α(OH)D was administered within the cell fusion media. As a result the data collected from the two experiments was analysed separately, the first 1α(OH)D/cell growth media experiment was analysed using ANOVA while the 1α(OH)D/cell fusion media time course experiment was analysed using two-way ANOVA.

Pre-confluence Myoblasts - Growth Media + Vitamin D



Day 0 Myoblasts - Differentiation Media + Vitamin D

Montage of Day 3 Myoblasts - Differentiation Media + Vitamin D



Day 7 Myoblasts - Differentiation Media + Vitamin D

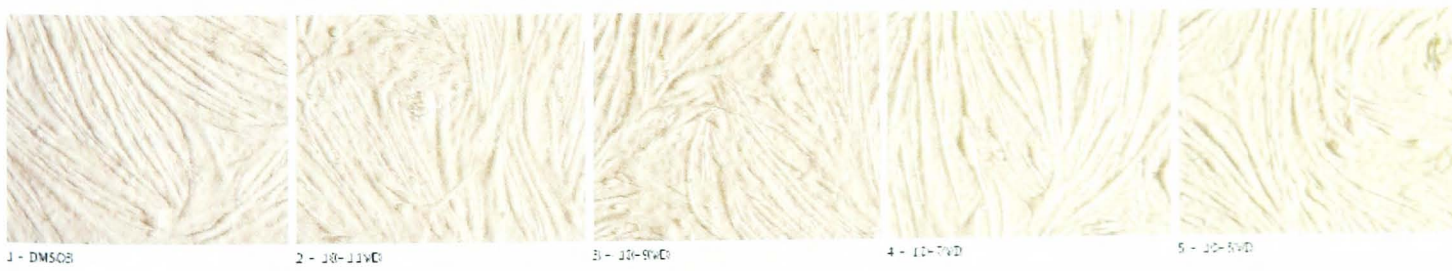


Figure 3.6-3 Morphology of C2C12 cells following 48 hour treatment with 1α(OH)D. Myoblasts were imaged following 48 hour incubation in respective treatment media at X10 magnification immediately prior to mRNA and protein harvest.

3.6.2 Results: Effects of 1α(OH)D on mRNA expression of housekeeping genes during proliferation and differentiation of C2C12 myoblasts.

Total RNA for qRT-PCR analysis was quantified using a Nanodrop Spectrometer, diluted to constant concentration and the integrity evaluated using agarose gel electrophoresis (described in sections 2.7.3-2.7.4), the images of the electrophoresed RNA samples are shown in appendix H. The expression of a number of mRNA transcripts were measured using qRT-PCR and it was important to correct any variation in sample preparation by the measurement of a housekeeping gene which was expected to be unchanged by vitamin D_3 treatment. β-actin had been used successfully as a housekeeping gene in the earlier work of this study performed in sheep where it had been unaffected by dietary vitamin D_3 treatment, in addition, two other commonly used control genes, cyclophilin A and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were also assessed for this purpose. As described in section 2.13.7, two standard curves were used to evaluate changes in expression of transcripts. A standard curve was created from cDNA produced from the LD of a mature 3 month old mouse, and was used to quantify genes that were not expressed at a high level in the myoblast C2C12 cells, such as the adult MHC isoforms. In addition a second standard curve was created from cDNA produced from some C2C12 myoblasts that had been grown for 10 days in cell fusion media which could be used to analyse development genes such as embryonic MHC. Both standard curves were included in each gene expression assay for all experimental samples, and so each gene could be analysed using the most appropriate standard curve. As described in section 2.13.8, it is necessary that the qRT-PCR reaction follows a linear reaction across varying concentrations to ensure accuracy of measurements and to achieve this, the standard curve must produce a linear response against which the unknown samples are compared. This meant that transcripts were analysed using the most suitable standard curve and all three housekeeping genes were analysed using both the mouse LD and the C2C12 standard curves, which provided evidence that the two standard curve approach produced similar data with similar responses. All comparisons were made within a gene and there were no comparisons made across genes, therefore the use of different standard curves did not affect the interpretation of results and simply provided a means to accurately evaluate relative changes within a gene caused by treatment.

3.6.2.1 Housekeeping in genes in proliferating myoblasts

All samples had been normalized for total RNA concentration and had been checked for integrity using agarose electrophoresis, following these steps to ensure all total RNA samples were consistent; the RT step was performed at the same time to ensure variation between preparations of cDNA samples was minimal. It was found that mRNA expression of β-actin was significantly altered by 1α(OH)D treatment when proliferating C2C12 cells were treated with 1α(OH)D directly into their growth media. The effect of 1α(OH)D on the expression of β-actin was to significantly increase expression in cells treated with vitamin D at 10^{-11}M compared to expression of cells treated with 10^{-7}M doses (table 3.6-1; $P<0.05$). Cyclophilin A and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were also found to be significantly affected by vitamin D treatment ($P<0.001$ and $P<0.004$ respectively, table 3.6-1) with both genes showing a decreased expression with vitamin D doses 10^{-9} and 10^{-7}M in comparison to expression in the DMSO treated controls. In addition it can be seen in the data shown in table 3.6-1 where the relative expression of the genes in comparison to either the LD or C2C12 day 10 standard curves is shown, that both standard curves generate the same relative changes and levels of significance are similar, independent of the standard curve.

Table 3.6-1 Expression of housekeeping genes in myoblasts treated with 1α(OH)D in growth media analysed using q-RT PCR.

Day-1 Myoblasts: 48 hrs Vitamin D In Growth Media						ANOVA P value
Gene (LD std curve)	DMSO	10-7	10-9	10-11	S.E.D.	Treatment
Beta Actin	0.303^{a}	$0.2503^{ab\dagger}$	0.2966^{a}	$0.3574^{ac\dagger}$	0.01907	0.027
Cyclophilin A	0.154^{a}	0.0226^{b*}	0.0443^{b*}	0.1169^{a}	0.02109	<.001
GAPDH	0.01419^{a}	0.00363^{b*}	0.00658^{b*}	0.01185^{a}	0.002127	0.004

Day-1 Myoblasts: 48 hrs Vitamin D In Growth Media						ANOVA P value
Gene (C2C12 day 10 std curve)	DMSO	10-7	10-9	10-11	S.E.D.	Treatment
Beta Actin	0.2147^{a}	$0.1824^{ab\dagger}$	0.2107^{a}	$0.2476^{ac\dagger}$	0.01649	0.027
Cyclophilin A	0.0996^{a}	0.0131^{b*}	0.0261^{b*}	0.0740^{a}	0.01339	<.001
GAPDH	0.0494^{a}	0.0075^{b*}	0.0168^{b*}	0.0384^{a}	0.00745	0.002

Total RNA was diluted to constant concentration prior to cDNA synthesis, unknown samples were compared to standard curve generated using cDNA from either mouse LD or C2C12 cells 10 days post differentiation. ANOVA P value calculated with Genstat is shown in far right column. Values with the same superscript are not statistically different; † denotes Dunnet's test significance of $P<0.05$, * denotes Dunnet's test significance of $P<0.01$. Values are arbitrary values produced in comparison to relative dilution of standard curves, n=3.

3.6.2.2 Housekeeping in genes across differentiation

Three time points were used to investigate 1α(OH)D 's effects on differentiating myoblasts at different stages of differentiation, the identified time points were day 0 (first day of cell fusion media, predominantly myoblasts), day 3 (three days post induction of differentiation, myotubes beginning to form) and day 7 (7 days post addition of cell fusion media, fully differentiated myotubes). The same three genes were assessed for their possible use as housekeeping genes and their expression was measured across three ages of differentiation with each treatment and analysed as a two-way ANOVA. It was found that all three genes were significantly changed by either treatment or the stage of differentiation. The P values from the ANOVA are shown in the legends in figure 3.6-4, showing the level of significance for the effect of age (either day 2, 5 or 9 post differentiation), treatment and the interaction. β-actin was significantly changed by both age and treatment where expression was significantly decreased with growth post-induction of differentiation and the expression within the time points was affected by the treatment of vitamin D, demonstrated in figure 3.6-4-A. Cyclophilin A expression was not affected significantly by vitamin D treatment ($P<0.302$), however, the expression was affected by different stages of differentiation ($P<0.002$), shown in figure 3.6-4-B. GAPDH was the final gene investigated for its use as a housekeeping gene and was found to be affected by the time post differentiation ($P<0.05$) where its expression was higher during the appearance of myoblasts around days 3 to 5 after addition of cell fusion media, shown in figure 3.6-4-C.

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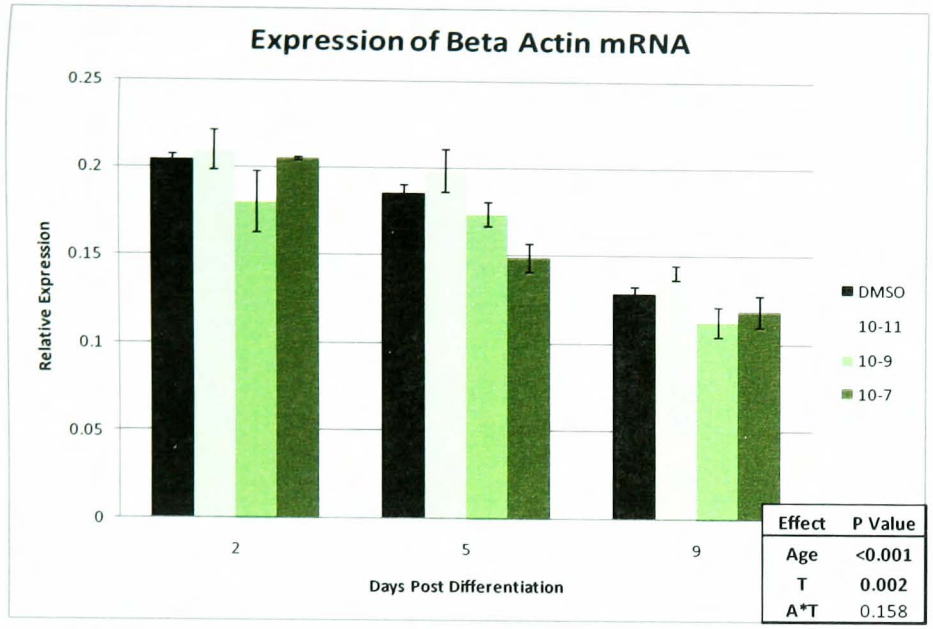

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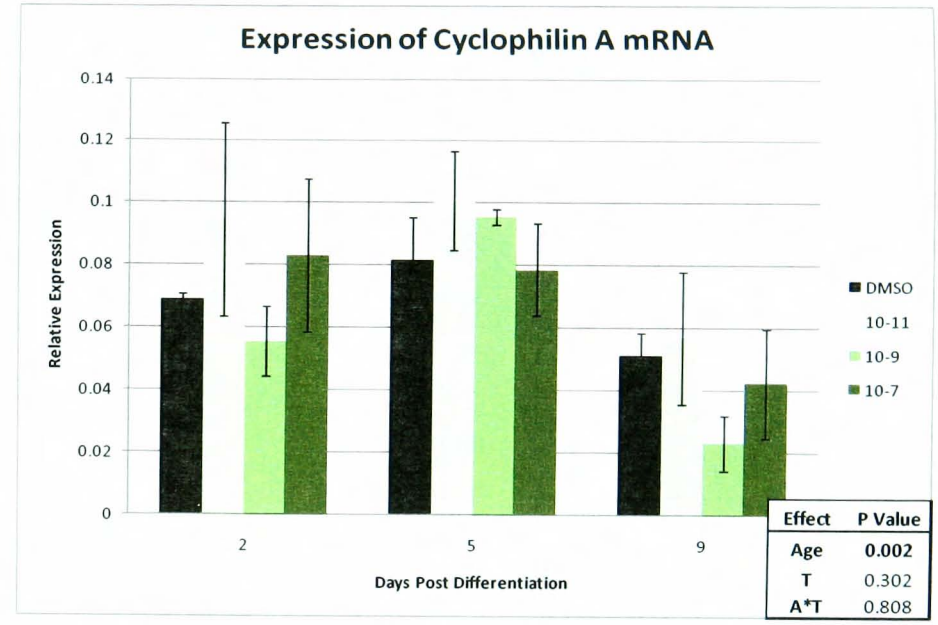

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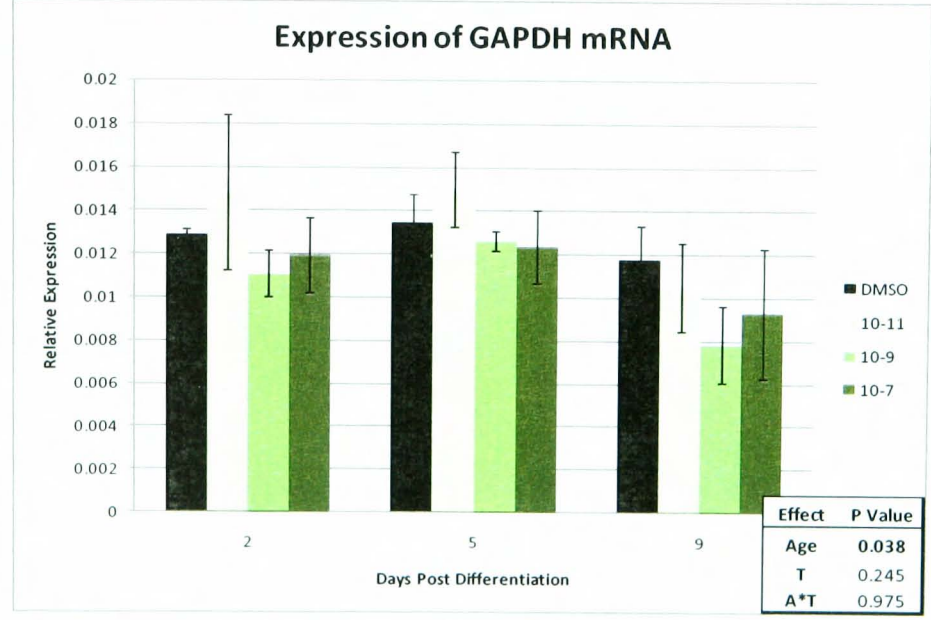


Figure 3.6-4 Relative expression of housekeeping genes quantified by real-time RT-PCR, relative mouse LD standard curve, n=3 A) β-actin, B) cyclophilin A and C) GAPHD, all at three stages post induction of differentiation with treatment of DMSO or 1α(OH)D at three concentrations. Error bars are SEM.

3.6.2.3 Average values of housekeeping genes

The changes in expression of the candidate housekeeping genes were not surprising given that the cells were undergoing large changes over the course of differentiation and it would be difficult to find a gene which retained the same level of expression at all stages of myotube development with additional treatment. All three housekeeping genes were affected by treatment upon individual analysis in the pre-confluence myoblast experiment, while all three were affected by age but only beta actin was affected by treatment in the time course experiment. In an effort to find a value that was unchanged by treatment, an average of all three housekeeping gene values was taken and analysed for treatment and age effects. Additionally, as cyclophilin A and GAPDH were unaffected by treatment in the time course assay, an average of these two alone was also investigated for its potential as a normalisation value. It was found that both average values were still significantly affected by treatment in the pre-confluence myoblast experiment, shown in table 3.6-2.

Table 3.6-2. Average values of housekeeping genes in myoblasts treated with 1α(OH)D in growth media analysed using q-RT PCR.

Day -1 Myoblasts: 48 hrs Vitamin D in Growth Media						ANOVA P Value
	DMSO	10-7	10-9	10-11	S.E.D.	Treatment
Average of all three Housekeeping genes						
LD Standard Curve	0.1571	0.0922	0.1158	0.1621	0.0112	<.001
Day 10 Standard Curve	0.1212	0.0677	0.0845	0.12	0.00849	<.001
Average GAPDH and Cyclophilin A						
LD Standard Curve	0.0841	0.0131	0.0254	0.0644	0.01146	<.001
Day 10 Standard Curve	0.0745	0.0103	0.0214	0.0562	0.00988	<.001

The same analysis for the time course experiment was performed, and average values of all three housekeeping genes and the average of cyclophilin A and GAPDH are shown in Appendix K, with the results from the two-way ANOVA analysis. Both the average of all three housekeeping values and the average of cyclophilin A and GAPDH were significantly affected by age ($P<0.001$, $P<0.01$ respectively, Appendix K). The average of all three genes was also approaching a significant affect of treatment ($P=0.058$ and $P=0.083$ for LD and Day standard curves respectively, Appendix K) although the average of GAPDH and cyclophilin A was not affected by 1α(OH)D treatment ($P=0.303$ and $P=0.274$ for LD and Day 10 standard curves respectively, Appendix K).

3.6.3 Summary of Housekeeping Genes

All three housekeeping genes were significantly changed by 1α(OH)D treatment in the first experiment where myoblasts were treated with vitamin D directly in their growth media, results showed that 1α(OH)D at the higher doses of 10^{-9} and 10^{-7}M caused significant reductions in the expression of these genes. These results meant that none of the three potential housekeeping genes could be used to normalise subsequent genes of interest in this experiment. In the second investigation where 1α(OH)D was added to cell fusion media at three stages of differentiation, the treatment was found to have less effect on the expression of cyclophilin A and GAPDH in maturing cells; however β-actin was significantly affected by 1α(OH)D at all three ages. When the individual values for each potential housekeeping gene were averaged the effects of treatment remained in the pre-confluence myoblast experiment, and again when the average of cyclophilin A and GAPDH was analysed. The effects of age and treatment remained when all three of the housekeeping genes were averaged in the time course experiment. There was no effect of treatment on the average of cyclophilin A and GAPDH but there was still a significant effect of age.

The results reported within this section show significant effect of both 1α(OH)D treatment and age on the raw values for each housekeeping gene individually and those values obtained when the average of either all three, or two genes, cyclophilin A and GAPDH, were analysed. Despite efforts to find a value which was unaffected by age and treatment, there was no value which was unaffected, and in light of the nature of sample preparation whereby the variability in each step was kept to a minimum by:

1. the concentration of all samples was checked using the Nanodrop spectrometer
2. all samples were diluted to a constant concentration
3. total RNA samples diluted to a constant concentration were then electrophoresed in agarose gels using a constant load of total RNA and then imaged to check visually for uniformity between samples
4. the RT step on all samples was carried out at the same time using the same method and reagents;

it was concluded that the cDNA produced would contain minimal variation and so subsequent gene expression analysis was performed without normalisation to a housekeeping gene and instead expression values are reported relative to the arbitrary concentration of the appropriate standard curves. To evaluate the variability in the real-time RT-PCR method three replicates of each individual sample were used, with three biological replicates for each age and treatment.

3.6.4 Results: Effects of 1α(OH)D on mRNA expression of markers of differentiation during proliferation and differentiation of C2C12 myoblasts.

The mRNA expression of two MRFs, Myf5 and myogenin were measured to evaluate the stage of differentiation of the C2C12 cells during the experiment. Myf5 is a regulator of the early stages of myogenesis when C2C12 myoblast cells are still proliferating; in comparison myogenin is expressed at later stages of differentiation of muscle cells when myoblasts are maturing into multinucleated myotubes (Dedieu *et al.*, 2002). It was expected that Myf5 expression would decrease when cells began to differentiate and that myogenin expression would become more dominant as myotubes appeared. As another marker of the extent of differentiation, the expression of desmin mRNA was also measured as its appearance would coincide with the formation of the skeletal muscle specific protein structures as myoblasts differentiated.

3.6.4.1 Expression of markers of differentiation in proliferating myoblasts

In the first experiment treating proliferating myoblasts with 1α(OH)D in their growth media (prior to differentiation, shown in figure 3.6-3), it was found that the expression of the myogenic regulatory factors Myf5 and myogenin were significantly affected by treatment, data shown in table 3.6-3. The expression of Myf 5 mRNA was decreased by 10^{-9} and 10^{-7} M doses of 1α(OH)D causing Myf5 expression to be reduced by 46% and 69% respectively. Myogenin expression was decreased significantly by 72% with the 10^{-7}M dose of 1α(OH)D. Desmin was unchanged by 1α(OH)D treatment in proliferating myoblasts (P=0.394).

Table 3.6-3 mRNA expression of markers of differentiation in Myoblasts treated with 1α(OH)D in growth media.

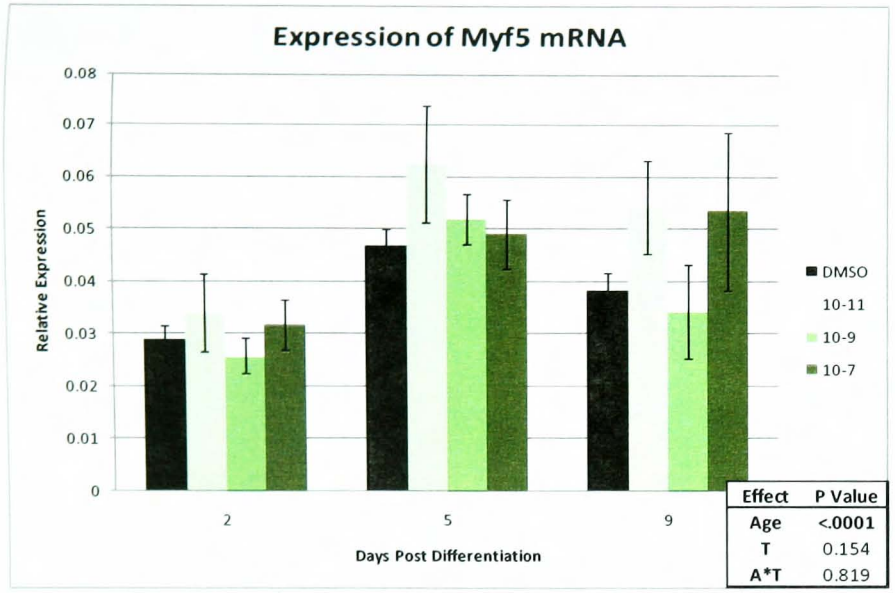
Day-1 Myoblasts: 48 hrs Vitamin D In Growth Media						ANOVA P value
Gene	**DMSO**	**10-11**	**10-9**	**10-7**	**S.E.D.**	**Treatment**
Myf 5	0.1054^{a}	0.1197^{a}	$0.0563^{b\dagger}$	0.032^{b*}	0.01414	**<.001**
Myogenin	0.371^{a}	0.321^{a}	0.163^{ab}	$0.102^{b\dagger}$	0.0803	**0.03**
Desmin	0.218	0.2512	0.2133	0.2256	0.02249	0.394

Values with similar superscript are statistically the same, † denotes Dunnet's test significance of P<0.05, * denotes Dunnet's test significance of P<0.01. Values measured by qRT-PCR are arbitrary values produced in comparison to relative dilution of standard curves, n=3.

3.6.4.2 Expression of markers of differentiation in differentiating muscle cells

In the time course experiment these markers of differentiation were measured at progressive stages of myogenic development and it was found that expression of Myf5 and myogenin were both significantly changed by time during the process of myotube development ($P<0.001$ and $P<0.01$ respectively, significance values shown in legends of figure 3.6-5, raw data shown in appendix J). However, there was no effect of 1α(OH)D treatment on these factors and there was no subsequent interaction between time after the addition of cell fusion media and the treatment given. Myf5 expression was lowest at the earliest age of cells, 2 days post induction of differentiation, then increased to its highest levels at day 5 post onset of differentiation at which point myotubes were appearing, demonstrated in figure 3.6-5-A. Over the whole experiment, Myf5 expression was highest after 5 days post differentiation with 48 hour treatment with 1α(OH)D. By the final stages of myogenesis, after 9 days of differentiation, myotubes were predominant and the expression of Myf5 mRNA was decreased again. The expression of myogenin followed a different spatial pattern and was found to start with the highest level of mRNA expression 2 days post differentiation and this expression was decreased with the progression of the differentiation process, shown in figure 3.6-5-B. Finally, the expression of desmin, which had been proposed as a marker of the extent of mature muscle cell formation, was found to remain at a relatively constant level of expression throughout the process, shown in figure 3.6-5-C, and there was no significant effect of time on the relative mRNA expression values ($P=0.988$). However, the expression of desmin was affected by 1α(OH)D treatment ($P=0.016$) where expression appeared to be lower at days 2 and 9 post differentiation when the cells were treated with 1α(OH)D at 10^{-9}M concentration.

A



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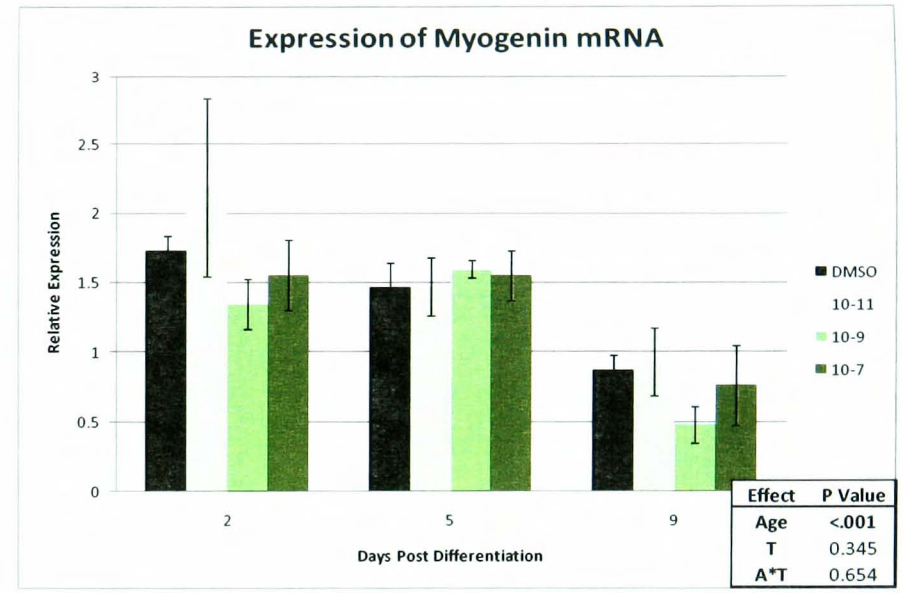

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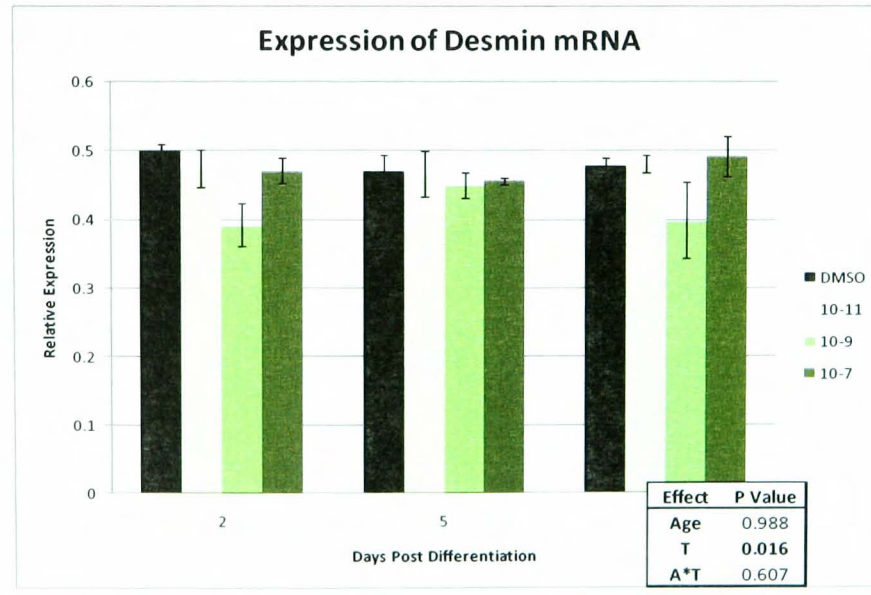

Figure 3.6-5 Relative expression of myogenic regulatory factors Myf5 and myogenin and the marker of differentiation, desmin; at three stages post induction of differentiation quantified using real-time RT-PCR, n=3; A) Myf5 (relative to day 10 standard curve) B) myogenin (relative to mouse LD standard curve) C) desmin (relative to mouse LD standard curve), n=3. Error bars are SEM.

3.6.5 Results: Effects of 1α(OH)D on MHC gene expression.

To investigate 1α(OH)D 's effects on skeletal muscle formation and fibre type of muscle cells, the expression of all 6 MHC genes was measured. Endo *et al.* (2002) had reported a decrease in the mRNA expression of MHC neonatal and proposed $1,25(OH)_2D_3$ treatment could influence the differentiation of muscle cells via altering the normal expression of the MHC genes through development. Further, the possibility for $1,25(OH)_2D_3$'s ability to alter calcium status in tissues could simultaneously, albeit indirectly, modify fibre type of muscle tissue via mechanisms previously described in section 1.3.5.

3.6.5.1 Expression of MHC genes in proliferating myoblasts

All 6 MHC genes were measured for their expression in proliferating myoblasts and the effects of vitamin D alone on the MHC gene expression at this stage were minimal. Expression of neonatal MHC and MHC IIA were too low to be quantified accurately and no data was collected for these genes. Embryonic MHC and I/β were the most predominant MHC genes to be expressed at this stage and was estimated at around 10 fold higher than the expression of the other adult MHCs; MHC IIX and MHC IIB, relative expression values in arbitrary units shown in table 3.6-4. MHC IIB was the only MHC gene to be significantly changed by 1α(OH)D treatment with expression decreasing with increasing dose of 1α(OH)D ($P<0.05$), with expression decreasing 51% and 65% with 10^{-9}M and 10^{-7}M respectively, relative to 0.1% DMSO control. It did not appear that there was any subsequent rise in another MHC gene to compensate for the reduction of MHC IIB.

Table 3.6-4 Expression of MHC genes in C2C12 myoblasts treated with 1α(OH)D in growth media.

Day-1 Myoblasts: 48 hrs Vitamin D In Growth Media						ANOVA P value
Gene	**DMSO**	**10-11**	**10-9**	**10-7**	**S.E.D.**	**Treatment**
Embryonic MHC	0.0311	0.0354	0.0268	0.0301	0.00325	0.142
Neonatal MHC		Not Expressed			N/A	N/A
MHC I/β	0.0295	0.0334	0.0268	0.0306	0.00746	0.847
MHC IIA		Not Expressed			N/A	N/A
MHC IIX	0.0196	0.0198	0.0125	0.0123	0.00356	0.119
MHX IIB	0.001027^{a}	0.001023^{a}	0.000502^{a}	$0.000354^{b\dagger}$	0.0002089	**0.023**

Values with similar superscript are statistically the same, † denotes Dunnet's test significance of P<0.05, * denotes Dunnet's test significance of P<0.01. Values analysed by real-time RT-PCR are arbitrary values produced in comparison to relative dilution of standard curves, n=3.

3.6.5.2 Expression of MHC genes in differentiating muscle cells

All MHC genes were significantly changed throughout the course of differentiation ($P<0.001$, full data shown in Appendix J) with the expression of the embryonic isoform decreasing with age and simultaneously the adult isoforms became more predominant. Again, the expression of neonatal MHC was too low to detect with any certainty and data was not included for this gene. The changes in expression of the MHC genes over the course of differentiation are illustrated in figures 3.6-6 – 3.6-7, where the large decrease in embryonic MHC and the increase in the adult isoforms are apparent. The expression of embryonic MHC was significantly reduced with the onset of differentiation with the relative expression reduced by 12% and 35% after 5 and 9 days of differentiation in the DMSO controls. The response of the C2C12 cells in their embryonic MHC expression seemed to form a dose response, with the cells reacting similarly to each dose of 1α(OH)D at each progressive stage of development. At the earliest stage of development, 2 days post cell fusion media, the lower doses of 10^{-11}M and 10^{-9}M decreased embryonic MHC expression by 5% and 17% respectively, there was little difference in the expression between the DMSO controls and the highest dose of 10^{-7}M 1α(OH)D. This response was observed again at the second age of development, during myotube appearance, where 1α(OH)D at 10^{-11}M and 10^{-9}M reduced embryonic MHC expression by 10% and 7 % respectively and there was little change with the highest 1α(OH)D concentration. Finally, at the latest stage of differentiation, when the myotubes were most predominant, the effect of 1α(OH)D at 10^{-11}M and 10^{-9}M concentrations was to reduce expression by 10% and 20% respectively. This significant effect of 1α(OH)D on the expression of embryonic MHC is reinforced by the lack of interaction between age and treatment (figure 3.6-6) and therefore can be interpreted as a stand-alone effect of 1α(OH)D on muscle cells.

The expression of all four adult MHC isoforms was affected by age in this experiment, but only MHC I/β was affected by vitamin D treatment. Expression of MHC IIA, IIX and IIB increased with time in line with the appearance of multinucleated myotubes, whereas the expression of MHC I/β declined. Expression of MHC I/β in the DMSO controls declined with age, where expression was reduced by 41% by 5 days post differentiation and by 50% after 9 days of differentiation in comparision to MHC I/β expression after 2 days differentiation. Similar to the pattern of embryonic MHC expression, the effect of 1α(OH)D treatment was to significantly reduce the expression of MHC I/β ($P<0.05$, figure 3.6-7-A) in comparison to DMSO controls at day 2 and day 9 post differentiation (compare figures 3.6-6. and 3.6-7-A). 1α(OH)D caused an decrease in the expression of MHC I/β after 48 hours treatment at the onset of differentiation, with the lower doses of 10^{-11}M and 10^{-9}M causing the largest reductions of 15% and 27% respectively. During the transitional stage of myotube formation,

5 days post differentiation, the highest dose of 1α(OH)D at 10^{-7}M caused an increase in the expression of MHC I/β of 48.5%. At the final stage of development, when mature, multinucleated myotubes were predominant, only the lowest dose of 1α(OH)D had an effect on MHC I/β expression, with 1α(OH)D at a concentration of 10^{-11}M causing a reduction in expression by 25.1%. The fast adult isoforms of the MHC genes, IIA, IIX and IIB were not affected by 1α(OH)D treatment (P= 0.275, 0.204 and 0.431 respectively, see figures 3.6-7 B,C and D) although these were increased significantly through the process of myotube formation. The expression of MHC IIA was relatively unchanged after 5 days post differentiation but after 9 days the expression was 6 fold higher than in the early differentiating myoblasts. Expression of MHC IIX mRNA was also increased throughout the course of differentiation, increasing by 18% after 5 days of differentiation and by 99% after 9 days differentiation, meaning expression of MHC IIX doubled in the mature multinucleated myotubes in comparison to differentiating myoblasts. Finally MHC IIB expression rose by 55% after 5 days of differentiation and after 9 days this had increased to 3 times the expression than had been observed in the early differentiating myoblasts. Overall these results show that the expression profile of the MHC isoforms was changed throughout the course of differentiation, with the expression of the embryonic and MHC I/β isoforms decreasing and the expression of the fast adult MHC isoforms rising in parallel. However comparisons between changes in the expression of isoforms cannot be made directly as they have been measured using different primers and probes with differing reaction efficiencies, therefore it cannot be surmised as to whether the rise in fast MHC makes up for the fall in embryonic MHC, and therefore isoform switching cannot be fully evaluated. The expression of embryonic MHC decreased by a third during differentiation and MHC I/β by half, and given that relative mRNA expression of these isoforms was around 10 times that of the MHC fast isoforms, this reduction is the beginning of a transition. The relative expression of MHC IIA, IIX and IIB increased by 6-, 2- and 3 fold between the early myoblast stage through to the presence of fully differentiated myotubes. Overall, while these changes are significant with time, it does not show a large change in isotype of the cells after 9 days of differentiation.

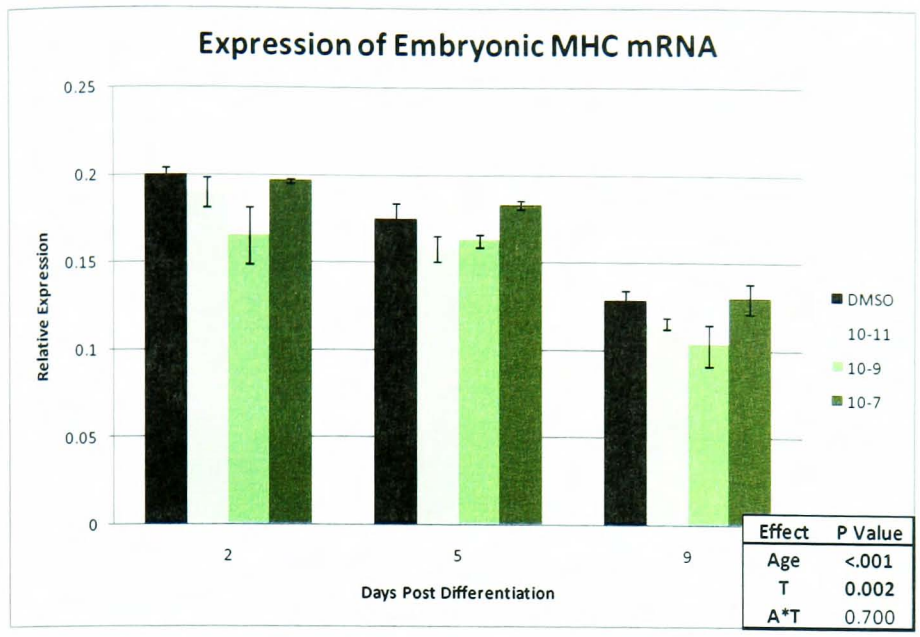

Figure 3.6-6. Relative expression of Embryonic MHC (to day 10 standard Curve) at three stages of differentiation. Error bars are SEM, n=3.

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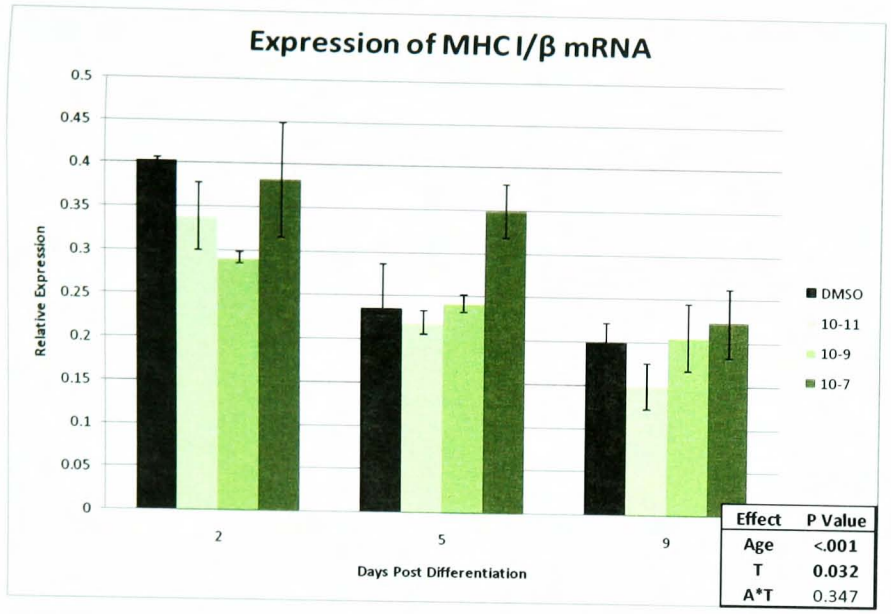

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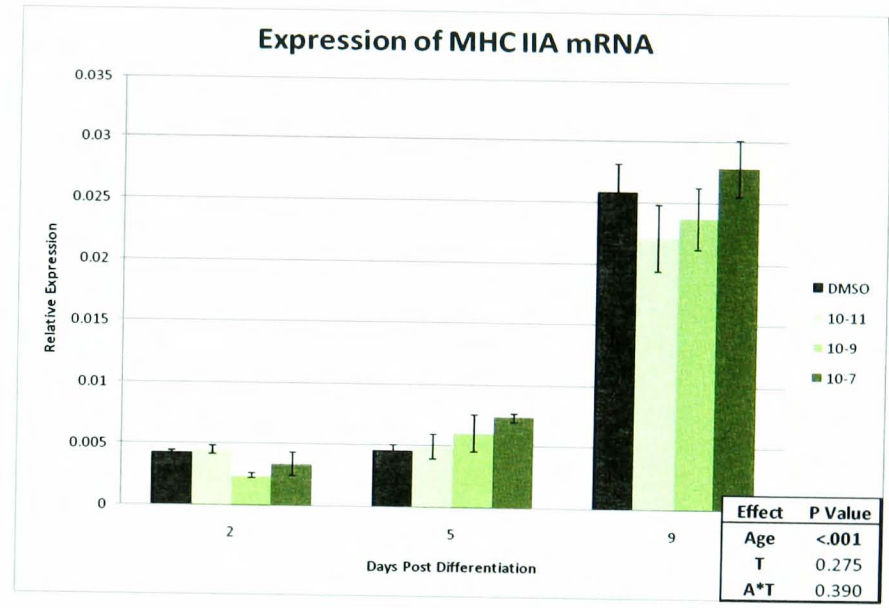

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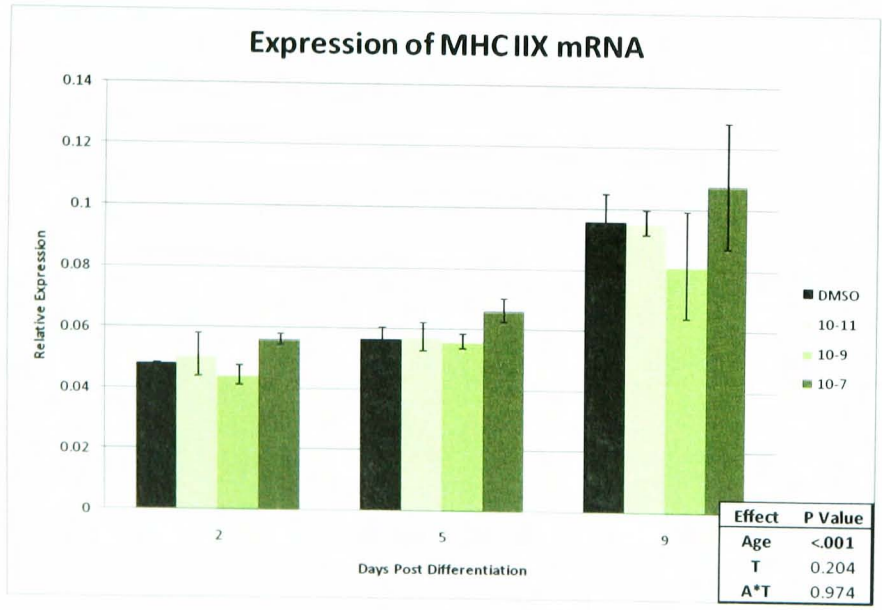

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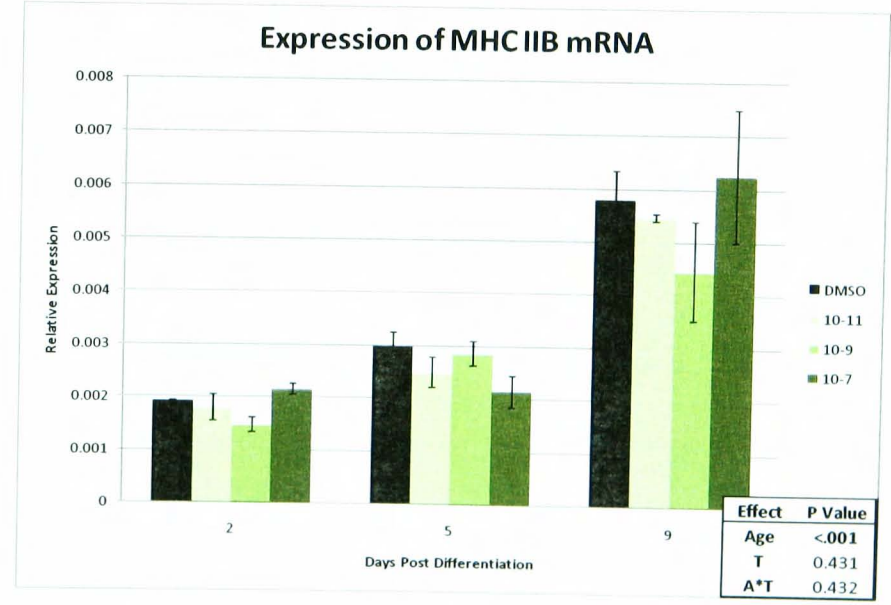


Figure 3.6-7 Relative expression of A) MHC I/β, B) MHC IIA, C) MHC IIX and D) MHC IIB (all relative expression to mouse LD standard Curve) at three stages post induction of differentiation, n=3. Error bars are SEM.

3.6.6 Effect of dietary vitamin D on MHC expression in sheep trials

In light of the treatment effects observed on the MHC genes in the cell culture experiments, the expression of the adult MHC genes were investigated in the dietary sheep trials previously reported in this thesis.

3.6.6.1 Effect of two dietary vitamin D regimes on mRNA expression of adult MHC genes in sheep

There was no effect on the mRNA expression of the adult MHC isoforms in sheep fed a high dose of vitamin D for 4 days immediately prior to slaughter or when sheep were fed vitamin D for 7 days with an extra bolus on the last two days immediately prior to slaughter, data shown in table 3.6-5.

Table 3.6-5 Effect of two vitamin D feeding regimes immediately prior to slaughter on relative expression of mRNA of adult MHC genes

LD mRNA	4 Days Vitamin D			
MHC Genes : Beta Actin Ratio	Control	Vitamin D	S.E.D.	P
MHC I	0.7732	0.9936	0.148	0.160
MHC IIA	0.9522	0.8966	0.144	0.646
MHC IIX/IIB	0.9042	0.8965	0.120	0.954
	7 Days Vitamin D + Ca			
MHC I	0.9760	1.0730	0.172	0.618
MHC IIA	1.1550	1.0480	0.099	0.326
MHC IIX/IIB	1.0720	0.9900	0.119	0.418

Values are expressed as ratio of relative mRNA expression of MHC gene: β-actin, values are arbitrary units produced from real-time RT-PCR analysis, n=18.

3.6.6.2 Effect of 7 days Vitamin D_3 + Ca on protein levels of MHC Genes in ovine LD

The expression of myosin heavy chain at the protein level was measured using two antibodies, one developed to the slow MHC protein the other to all fast varieties of the protein, antibody data presented in Appendix D, verified to cross react with sheep LD by Sazili (2003).

Table 3.6-6. Effect of 7 days vitamin D & Ca on MHC fast and slow protein levels in ovine LD.

7 days vitamin D + Ca	Absorbance/ 3 µg protein			
LD muscle protein	Control	Vit D	S.E.D.	P
MHC Slow	2.631	2.651	0.717	0.981
MHC Fast	7.419	7.457	0.742	0.955

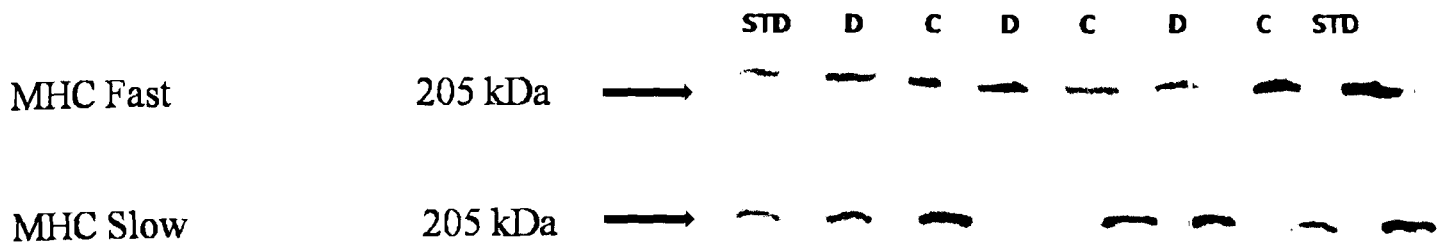


Figure 3.6-8 Representative image of Myosin Heavy Chain fast and slow, visualized at 205kDa following SDS-PAGE of 3µg whole homogenate fraction of LD using 8% acrylamide gel. Further antibody information presented in Appendix D, MHC fast n=13, MHC slow n=8.

3.6.7 Summary of MHC gene expression

The expression of embryonic MHC across the course of differentiation was significantly reduced and the effects of 1α(OH)D on this mRNA was consistent for each time point, with 1α(OH)D decreasing the expression of embryonic MHC when administered at the lower doses of 10^{-11}M and 10^{-9}M. This response is convincing evidence that 1α(OH)D has a direct effect on the embryonic MHC isoform and upon treatment the cells reduce their expression of this isoform, the consequence of which is that the cells progress from an immature MHC isoform expression to a more adult MHC expression profile, which supports the notion that 1α(OH)D promotes muscle cell differentiation. Analysis of the effects of the two sheep trials did not indicate an effect of the dietary supplementation regimes on the expression of the mature MHC isoforms, despite significant changes in the circulating levels of vitamin D in both trials and of serum and muscle calcium in trial 2.

3.6.8 Results: Effect of 1α(OH)D on transcripts up-regulated by $1,25(OH)_2D_3$.

The results of the microarray shown in section 3.4 highlighted a small selection of genes which were up-regulated by the treatment with $1,25(OH)_2D_3$ in a dose response. Three of these genes were retinol binding protein (Rbp4), metallothionein 2A and C/EBPβ, all of which showed significant up-regulation with $1,25(OH)_2D_3$ treatment in comparison to controls, and the extent of the increase in their expression was higher with increased dose of $1,25(OH)_2D_3$, see table 3.4-9. It was decided to measure the expression of these genes in the final 1α(OH)D /cell culture trial to investigate their validity as end-points of vitamin D signalling. The hypothesis was that if these genes were significantly changed by both concentrations of $1,25(OH)_2D_3$ in the microarray then they should respond in the same way to the active analogue 1α(OH)D. To measure these genes primer and probes were purchased from ABI (Applied Biosystems) as "assays on demand" and the product and gene code are shown in Appendix G. It was found that despite large changes in Rbp4 in response to $1,25(OH)_2D_3$ as measured by microarray in rat primary muscle cells, the expression of Rbp4 in the C2C12 was too low to detect with any degree of certainty and no data is shown for this gene.

3.6.8.1 Effect of 1α(OH)D on mRNA expression of $1,25(OH)_2D_3$ targets in proliferating myoblasts

Three of the four $1,25(OH)_2D_3$ targets were found to be significantly changed when proliferating myoblasts were treated with 1α(OH)D directly in cell growth media. C/EBPβ is a transcription factor which was found to show a dose response to $1,25(OH)_2D_3$ as measured by the microarray, where it was up-regulated in comparison to the DMSO control. In this experiment the expression of C/EBPβ was up-regulated significantly by 1α(OH)D at the lowest dose of 10^{-11}M showing a 10 fold increase in relative expression in comparison to DMSO control ($P<0.002$, table 3.6-7), the higher doses of 1α(OH)D did not cause significant changes in the expression of this gene. In addition, metallothionein 2A was another target discovered by microarray which was shown to respond to $1,25(OH)_2D_3$ and data from this experiment suggested a trend for changes in its mRNA expression, whereby metallothionein 2A expression was decreased in the highest dose of 10^{-7}M in comparison to the DMSO control. Parvalbumin has been reported as a $1,25(OH)_2D_3$ responsive gene in a number of tissues (De Viragh *et al.*, 1989; Antrobus *et al.*, 1995); however this experiment showed no effect of 1α(OH)D on the expression of parvalbumin in proliferating myoblasts. Finally c-Myc expression was reported to increase with vitamin D treatment (Drittanti *et al.*, 1989) and was implicated as a target of the IGF-1 and PI3K signalling pathway (Sears *et al.*, 2000) which promotes muscle growth (Latres *et al.*, 2005) and was reported to be a marker of atrophy (Grounds M.D., 2002). It was found

that the expression of c-Myc was significantly reduced in the two higher doses of 1α(OH)D at 10^{-9}M and 10^{-7}M by 53% and 81% respectively.

Table 3.6-7 Effect of 1α(OH)D on expression of $1,25(OH)_2D_3$ targets in Myoblasts treated within growth media.

Day-1 Myoblasts: 48 hrs Vitamin D In Growth Media						ANOVA P value
Gene	DMSO	10-11	10-9	10-7	S.E.D.	Treatment
C/EBPβ	0.064^{a}	0.698^{b}*	0.088^{a}	0.0310^{a}	0.1259	**0.002**
Metallothionein 2A	0.0658	0.0508	0.0734	0.0122	0.02156	**0.084**
Parvalbumin	0.0118	0.0077	0.0079	0.012	0.0041	0.595
c-Myc	0.0663^{a}	0.0569^{a}	$0.0309^{b\dagger}$	0.0148^{b}*	0.01181	**0.009**

Values with the same superscript are not statistically different, where superscript letters are different † denotes Dunnet's test significance of P<0.05, * denotes Dunnet's test significance of P<0.01. Values analysed by real-time RT-PCR are arbitrary values produced in comparison to relative dilution of standard curves, n=3.

3.6.8.2 Effect of 1α(OH)D on mRNA expression of $1,25(OH)_2D_3$ targets in differentiating muscle cells

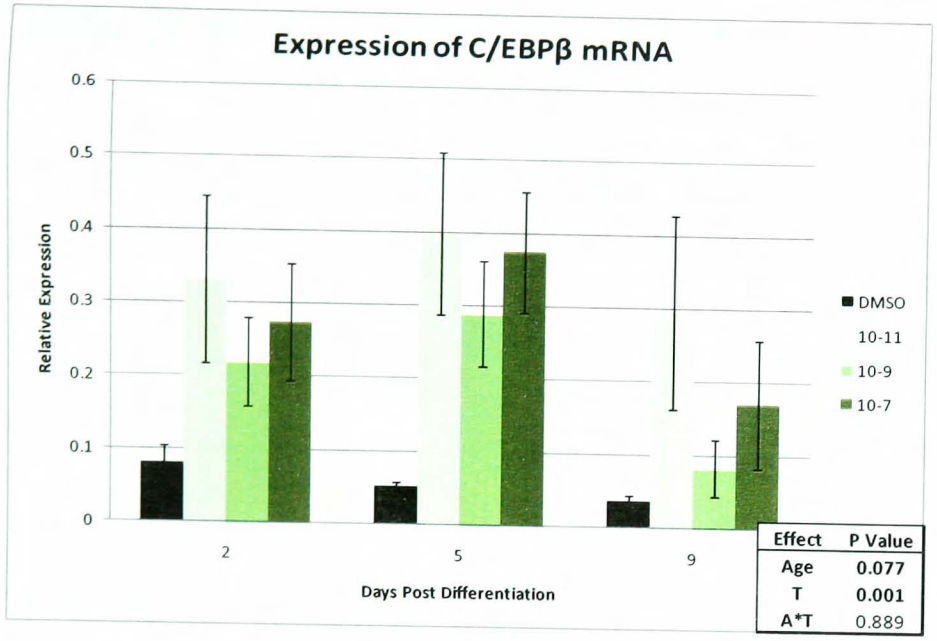
All four targets of $1,25(OH)_2D_3$ signalling were found to be significantly changed by treatment with 1α(OH)D during the process of myotube formation in comparison to DMSO controls, P-values from two-way ANOVA analysis shown in legends of figure 3.6-9, complete data set is shown in Appendix J. Both targets of $1,25(OH)_2D_3$ highlighted in the microarray performed in rat primary muscle cells in this study were found to again be affected by 1α(OH)D treatment in C2C12 cells when compared with expression in DMSO controls. C/EBPβ was significantly increased by 1α(OH)D at all three time points, shown in figure 3.6-9-A, by all three concentrations of 1α(OH)D.

The fold changes in the expression of C/EBPβ were consistently high. At each time point in the development process, the largest changes in expression of C/EBPβ were caused by the lowest concentration of vitamin D 10^{-11}M, giving 4.1, 7.8 and 8.4 fold increases in the expression of C/EBPβ at days 2, 5 and 9 post differentiation respectively, shown in table 3.6-8.

Table 3.6-8 Fold changes in C/EBPβ expression affected by 1α(OH)D treatment relative to DMSO controls across the differentiation process.

Fold Changes in C/EBPβ expression in Time Course Experiment	Vitamin D Concentration (M)		
Days Post Differentiation	10-11	10-9	10-7
2	4.1	2.7	3.4
5	7.8	5.7	7.3
9	8.4	2.3	4.9

A



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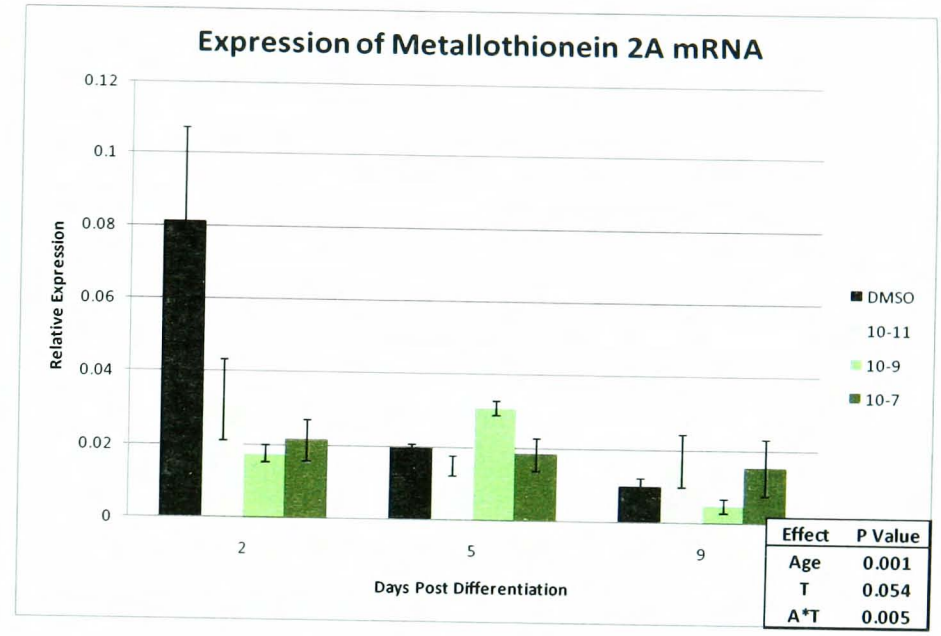

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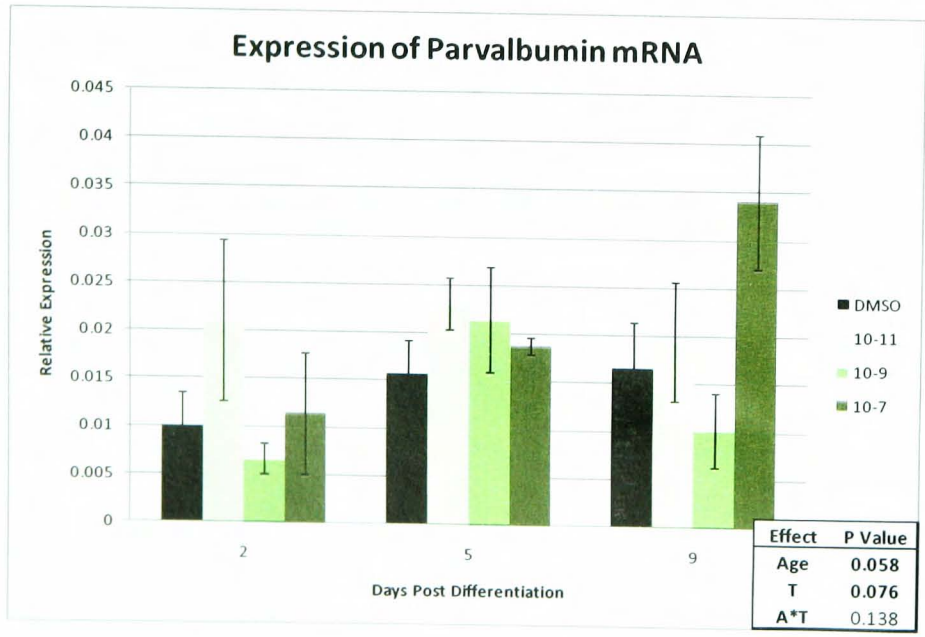

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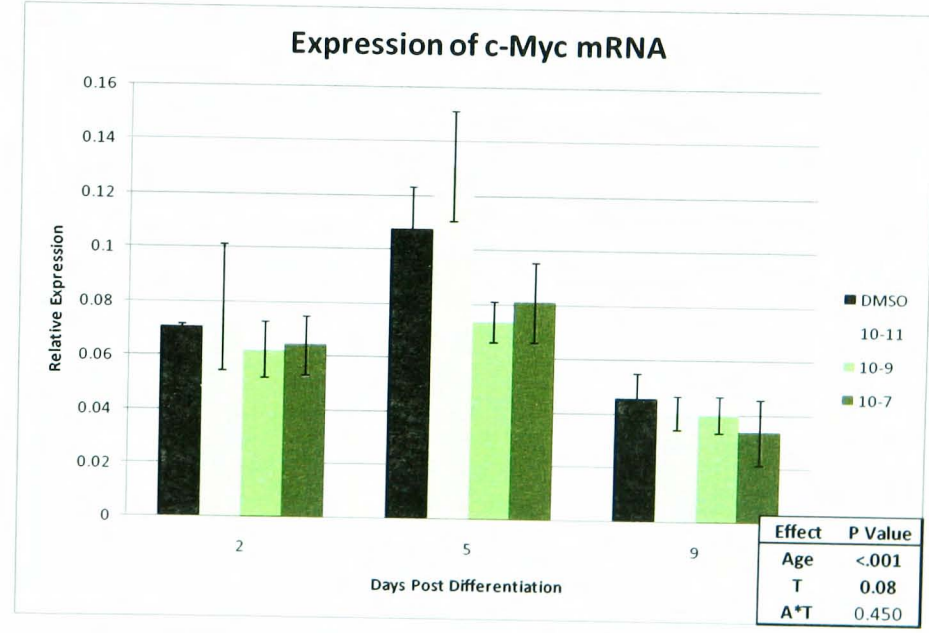


Figure 3.6-9 Relative expression of A) C/EBPβ, B) metallothionein 2A, C) parvalbumin and D) c-Myc (all relative expression to day 10 standard Curve) at three stages post induction of differentiation, n=3. Error bars are SEM.

Expression of metallothionein 2A mRNA was similarly affected by both 1α(OH)D treatment and time, and showed an interaction between the time and dose of 1α(OH)D (Figure 3.6-9-B). Expression of metallothionein 2A was high in the early stages of myogenesis in the DMSO controls before expression was significantly reduced by 75% after the appearance of myotubes. Treatment of the myoblasts with 1α(OH)D at the onset of differentiation caused a significant decrease in the metallothionein 2A expression, with each increasing dose of vitamin D 10^{-11}M, 10^{-9}M and 10^{-7}M reducing it to 60, 78 and 73% of the expression observed in the DMSO control. This decrease in metallothionein 2A mRNA expression with 1α(OH)D treatment was enough to reduce expression to the same levels observed after 5 days differentiation in the DMSO control cells and this response may be due to 1α(OH)D inducing differentiation earlier than was observed in the DMSO controls. The response of the cells to 1α(OH)D at days 5 and 9 post differentiation was different again, with 1α(OH)D at the intermediate dose causing an increase in metallothionein 2A in comparison to DMSO controls of cells at 5 days post differentiation, and at the same dose causing a decrease in the expression at 9 days post differentiation. The highest and lowest concentrations of 10^{-7}M and 10^{-11}M did not produce responses largely different to the DMSO control cells. There was a trend for parvalbumin expression in C2C12 cells to be affected by age (P=0.058) and treatment (P=0.076) but there was no interaction between the two. Expression of parvalbumin increased with appearance of myotubes (as observed in the DMSO controls), rising by 56% from 2 to 5 days post differentiation, from which point expression rose slightly again by 5% between days 5 and 9 post differentiation, see figure 3.6-9-C. The effect of treatment with 1α(OH)D on expression of parvalbumin mRNA in C2C12 cells at 5 days post differentiation was to raise mRNA levels 1.2, 1.3 and 1.4 fold with decreasing 1α(OH)D concentration; 10^{-7}M to 10^{-11}M respectively. The effects of the three doses of 1α(OH)D at either the early myoblast stage, 2 days post differentiation, and after 9 days of differentiation when myotubes were predominant, on parvalbumin expression was to slightly reduce expression with the intermediate dose of 10^{-9}M but to raise expression with the highest concentration, 10^{-7}M. This effect was greatest in the differentiated myotubes after 9 days of differentiation where the highest dose of 1α(OH)D, 10^{-7}M, caused a 2-fold increase in parvalbumin expression in comparison to control cells at the same stage of development. The final target gene of $1,25(OH)_2D_3$ signalling was c-Myc which was significantly changed with age (P<0.001) and showed a trend to be affected by 1α(OH)D treatment (P=0.08), and there was no interaction between age and treatment (Figure 3.6-9-D). The expression of c-Myc was highest after 5 days of differentiation when the myoblasts were fusing and myotubes were beginning to appear, see figure 3.6-9-D. C-Myc expression was 1.5 fold higher at this stage than after 2 days post differentiation. The expression of c-Myc was lowest after 9 days of differentiation where expression was 56% lower than at day 2 post differentiation. The effect of 1α(OH)D was

unclear at the earlier and later stages but there was a clear effect at the intermediate stage of differentiation where vitamin D at 10^{-9}M and 10^{-7}M cause a decrease in c-Myc expression by 31% and 21% respectively.

3.6.9 Summary 1α(OH)D effects on transcripts which responded to $1,25(OH)_2D_3$

The transcription factor C/EBPβ was significantly increased 10-fold by 10^{-11}M dose administered within cell growth media. C-Myc was significantly decreased 53% and 77% by the 10^{-9} and 10^{-7} M doses of 1α(OH)D respectively. Binding proteins metallothionein 2A and parvalbumin were not changed significantly by 1α(OH)D treatment, although there was a trend in metallothionein 2A to be decreased by 1α(OH)D treatment ($P<0.1$).The data in table 3.6-7 shows that 1α(OH)D at the higher concentrations of 10^{-9} and 10^{-7}M appeared to cause down-regulation of c-Myc mRNA expression in proliferating myoblasts.

In the time course experiment all four targets of 1α(OH)D were significantly affected by age and treatment. 1α(OH)D treatment was most potent in increasing the expression of C/EBPβ mRNA in a dose response at every time point. Additionally, 1α(OH)D decreased expression of matellothionein 2A with a significant interaction of age and time ($P=0.005$), where the effect of 1α(OH)D at the earlier time of 2 days post differentiation was to reduce expression of metallothionein 2A with each dose.

3.6.10 Results: Effects of 1α(OH)D on protein signalling.

The effect of 1α(OH)D treatment of the extent of phosphorylation of two proteins related to cell growth and protein synthesis was evaluated, these were the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 1/2 (ERK 1/2), and the enzyme, eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), respectively.

3.6.10.1 Effects of 1α(OH)D on protein targets in proliferating C2C12 myoblasts

The level of phosphorylation of the MAPK, ERK 1/2, was assessed using antibodies for both total and phosphorylated versions of the protein before the level of phosphorylation was calculated using a ratio of the levels of phosphorylated protein to the level of total protein; representative images of the protein blots are shown in figure 3.6-10, values are shown in table 3.6-9. There was a significant effect of 1α(OH)D on the extent of phosphorylation of ERK1/2 ($P<0.002$), with 1α(OH)D at 10^{-9}M dose significantly decreasing the level of phosphorylation of this enzyme by 36% ($P<0.01$) and by 21% with the 10^{-7}M dose ($P<0.05$). There was no effect of 1α(OH)D treatment on level of phosphorylation of 4EBP1 in proliferating myoblasts, data shown in table 3.6-9.

Table 3.6-9. Effect of 1α(OH)D treatment on level of phosphorylation on two protein targets in proliferating myoblasts

Phosphorylation of Protein	DMSO	10-11	10-9	10-7	S.E.D.	P
Ratio ERK 1/2	0.906^{a}	$0.714^{b\dagger}$	0.571^{b*}	0.854^{a}	0.0585	**0.002**
Ratio 4EBP1	0.906	0.885	1.003	0.960	0.0591	0.259

Values with similar superscript are statistically the same, † denotes Dunnet's test significance of $P<0.05$, * denotes Dunnet's test significance of $P<0.01$. Values are ratio of phosphorylated protein to level of total protein, n=3.

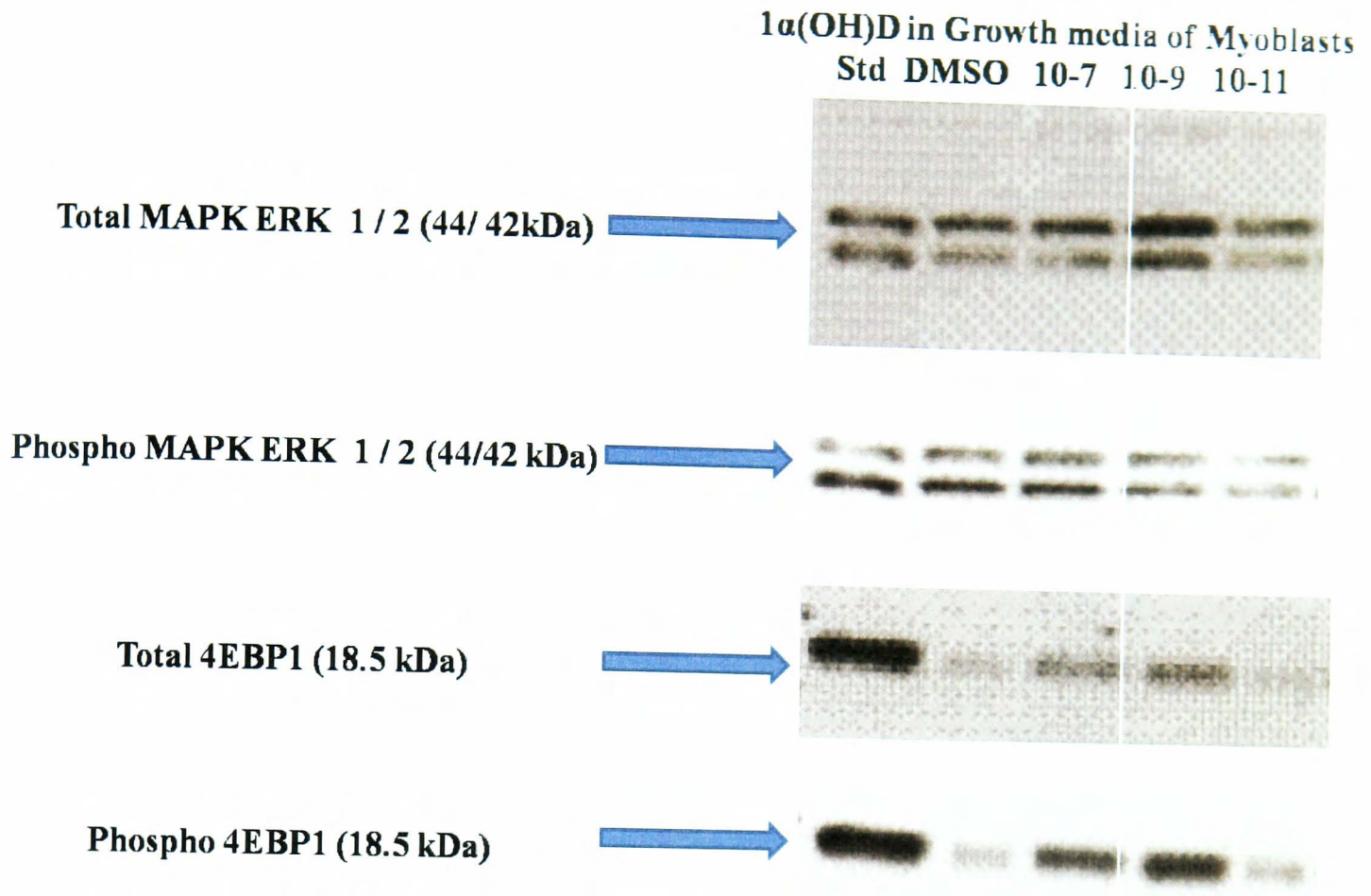


Figure 3.6-10 Representative blot of C2C12 proteins analysed by SDS-PAGE followed with western blotting using antibodies for total protein before stripping the membrane and re-probing with the antibody to the phosphorylated protein fraction. Total MAPK ERK 1 /2 and Phosphorylated MAPK ERK 1 /2: 5µg cell C2C12 protein separated by SDS-PAGE using 10% acrylamide gel; Total 4EBP1 and phosphorylated 4EBP1: 5µg cell C2C12 protein separated by SDS-PAGE using 15% acrylamide gel. Further antibody information presented in Appendix D, n=3.

3.6.10.2 Effects of 1α(OH)D on protein targets in differentiating C2C12 muscle cells

The effect of 1α(OH)D treatment on the level of phosphorylation of both ERK1/2and 4EBP1 was measured in muscle cell protein extracts taken from muscle cells across the course of differentiation following 48 hours treatment with the analogue, representative protein blots are shown in figure 3.6-11, raw data is shown in Appendix L and P-values of two-way ANOVA shown in legends of figure 3.6-12. The phosphorylation of ERK1/2 was found to be significantly affected by the age of the cells (P=0.016), with phosphorylation of ERK1/2 being highest during the transition of the cells from myoblasts to multinucleated myotubes, and phosphorylation of ERK1/2 remained high after 7 days of differentiation in comparison to the level of phosphorylation when cells were predominantly myoblasts, shown in figure 3.6-12-A. Treatment of the cells with 1α(OH)D was found to decrease the extent of phosphorylation (P<0.001) at all age points, and there was interaction between the age of the cells and dose of vitamin D (P=0.007). The decrease in extent of phosphorylation was greater when cells were treated at 3 days post induction of differentiation, where the 1α(OH)D treatment at 10^{-11} and 10^{-9}M reduced the extent of phosphorylation by 56% and 62% respectively. This response was similar again when cells were treated 7 days post induction of differentiation, where treatment of muscle cells which were predominantly multinucleated myotubes with 1α(OH)D again caused a decrease in the extent of phosphorylation of ERK 1/2 by 42% with both 10^{-11}M and 10^{-9}M doses and by 50% with 10^{-7}M.

The was a significant effect of differentiation on the level of phosphorylation of 4EBP1 (P=0.019, raw data in Appendix L, P-value of two-way ANOVA shown in legend of figure 3.6-11-B) where phosphorylation of 4EBP1 was high during the early stages of differentiation when myoblasts were still present, and decreased after 7 days of differentiation, shown in figure 3.6-12-B. There was no effect 1α(OH)D in the level of phosphorylation of 4EBP1 (P=0.852).

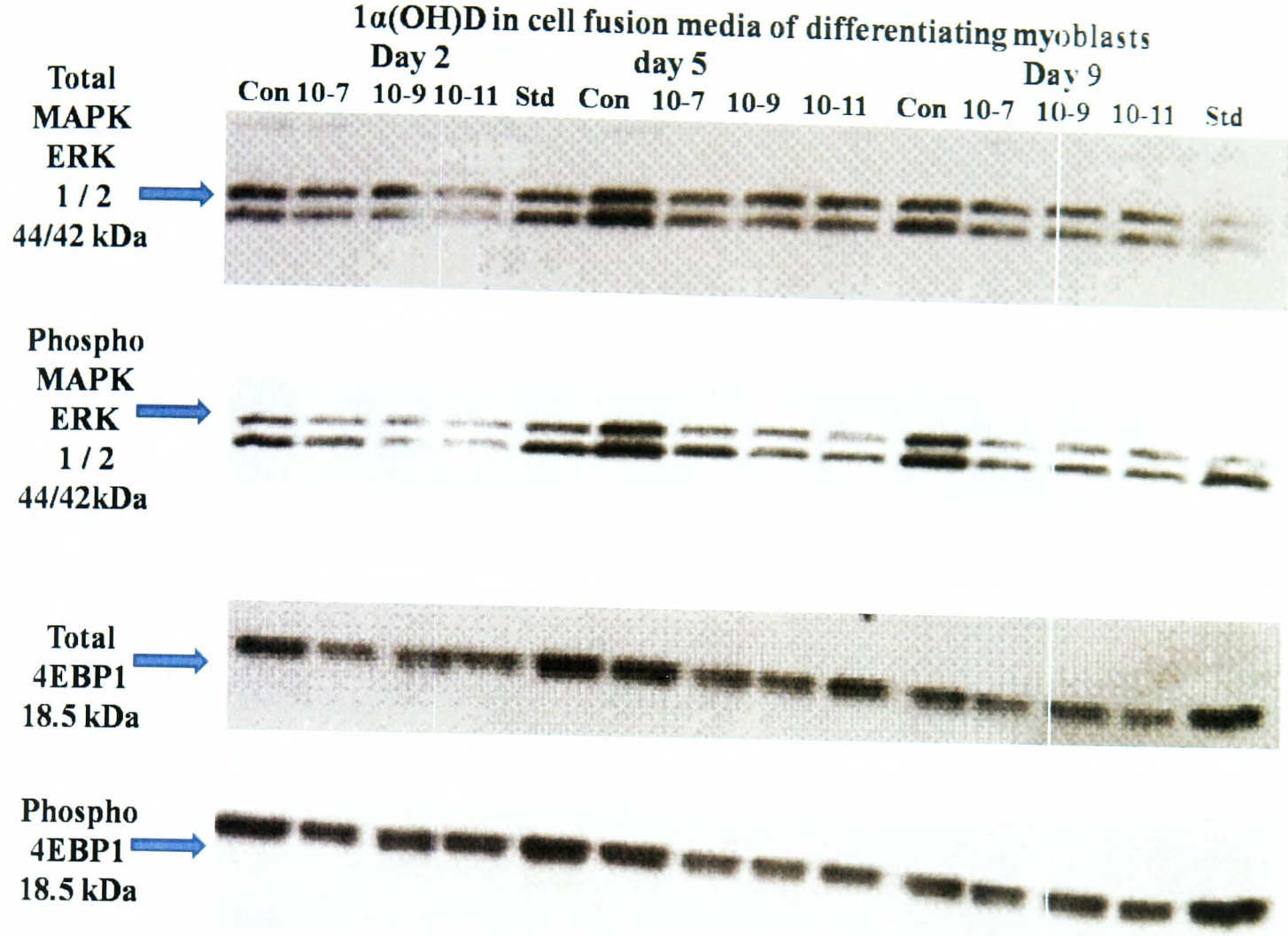
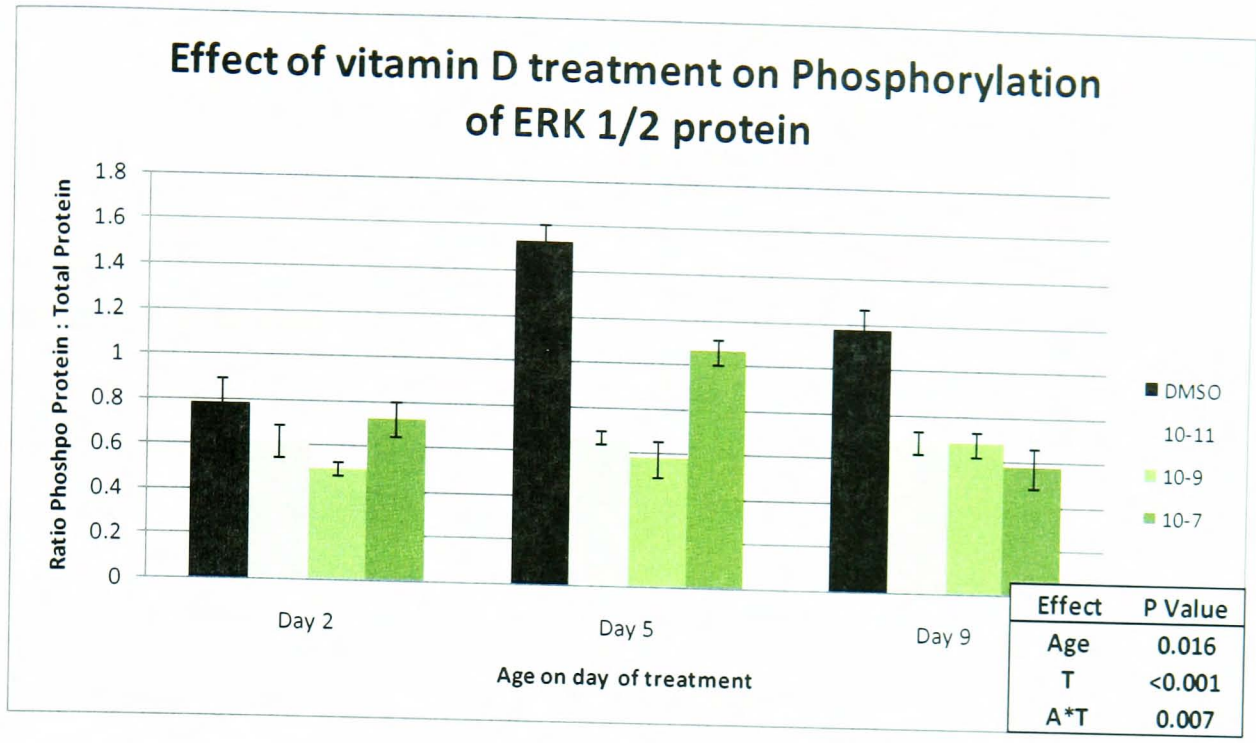


Figure 3.6-11 Representative blot of C2C12 proteins analysed by SDS-PAGE followed with western blotting using antibodies for total protein before stripping the membrane and re-probing with the antibody to the phosphorylated protein fraction. Samples were loaded as described above bands, Con = DMSO control treated proteins, Std = standard sample created from pool of samples loaded repeatedly across gel to account for variation in electrophoresis and blotting. Total MAPK ERK 1 /2 and Phosphorylated MAPK ERK 1 /2: 5µg cell C2C12 protein separated by SDS-PAGE using 10% acrylamide gel; Total 4EBP1 and phosphorylated 4EBP1: 5µg cell C2C12 protein separated by SDS-PAGE using 15% acrylamide gel. Further antibody information presented in Appendix D, n=3.

A



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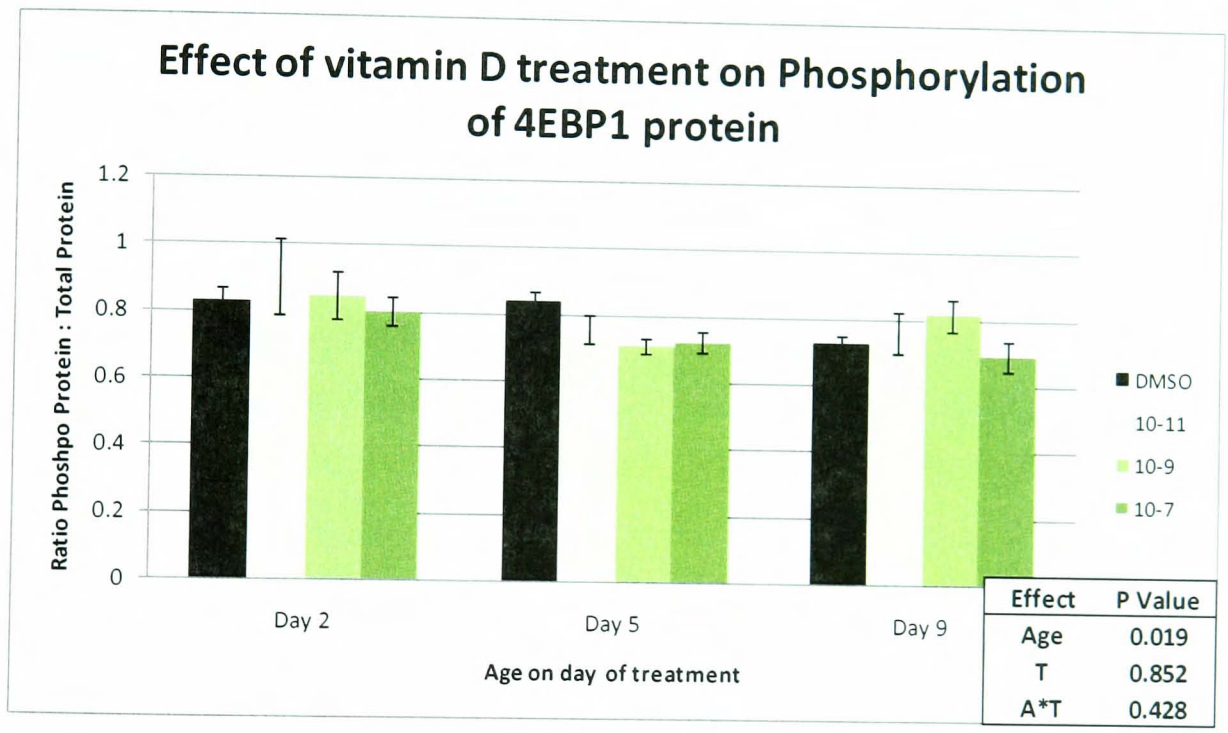


Figure 3.6-12. Effect of level of phosphorylation of two protein targets in C2C12 myoblasts after 48 hours treatment with 1α(OH)D at three different stages of differentiation, A) ERK 1/2 B) 4EBP1, n=3.

Discussion

The aim of the experiments performed in this thesis was to investigate the effects of vitamin D and its active analogues on signalling events in skeletal muscle in terms of meat quality, and factors that influence muscle function which might subsequently influence animal production. The hypothesis of the first trials was that vitamin D supplementation immediately prior to slaughter could improve the tenderisation of meat post mortem via increased activity of the calpain enzymes. However, the results indicated vitamin D's involvement in regulating the fundamental processes of muscle tissue gene expression which is likely to have implications for animal production in terms of muscle growth and development. Therefore the subsequent experiments focused on identifying novel targets of biologically active vitamin D in muscle tissue by microarray analysis and assessing its potential influence on the development of growing muscle cells. Targets identified by microarray analysis were verified in the final experiments of this thesis, investigating the effects of a biologically active form of vitamin D on muscle cells at varying stages of development.

The main novel findings reported within this thesis are:

- Assessment of dietary vitamin D regimes on altering 25(OH)-D_3 levels, calcium concentration in both serum and LD muscle of sheep.
    - 4 days vitamin D: increased 25(OH)-D_3 10-fold but had no effect on serum or LD calcium.
    - 7 days vitamin D + calcium: increased 25(OH)-D_3 25-fold, increased serum and LD calcium 10 fold.
- The assessment of dietary vitamin D's effects on mRNA and protein expression of LD calpain system genes and kidney calbindin in sheep.
    - 4 days vitamin D: increased mRNA of calpain I 3.7% and calpain II 10%, no effect on mRNA of calpastatin, no effects at protein level and no effect on calbindin mRNA or protein in the kidney. There was a trend (P=0.103) for vitamin D receptor protein levels to be decreased with dietary vitamin D.
    - 7 days vitamin D + calcium: decreased the mRNA of calpain I by 5.2%, but no effects on calpain II or calpastatin at the mRNA level. At the protein level calpain II protein was increased 16% and calpastatin 17% but there was no effect on calpain I protein. Calbindin mRNA in the kidney was increased 8.8% but there was no effect on calbindin protein levels. VDR levels in the kidney were significantly increased by 100%.

- The first two trials of this thesis provided additional evidence to that in the literature that dietary vitamin D supplemented immediately prior to slaughter does not improve the shear force of lamb LD chops even though there were changes in calcium status.
- The microarray performed in neonatal rat myoblasts is the first reported microarray performed using active $1,25(OH)_2D_3$ in muscle cells and provided a large list of novel targets which are responsive to vitamin D in muscle. Of these targets, metallothionein I and 2A and CCAAT/enhancer binding protein β and δ have been previously implicated to mediate muscle's responses to IGF-1 during hypertrophy and to glucocorticoids during atrophy (Latres *et al.*, 2005), and these findings strongly suggested that $1,25(OH)_2D_3$-mediated effects are associated with the control of muscle growth.
- The comparative evaluation of three cell lines for their myosin heavy chain gene expression has not been reported before. The experiments demonstrated that skeletal muscle cells differentially express MHC genes at progressive stages of differentiation *in vitro*. Thereby representing differing *in vitro* models for different fibre types or muscle development stages.
    - The primary muscle cultures prepared from neonatal rat pups predominantly expressed embryonic and neonatal MHC genes and MHC 2X, with expression of MHC 2A increasing during cell fusion.
    - The L6 Aston rat-derived cell line was almost exclusively a "fast" phenotype, expressing MHC 2A and 2X in myoblasts, and expression of embryonic and 2B increased as myotubes formed.
    - The C2C12 mouse derived cell line was the only muscle cell type to express MHC I/β, and expressed the largest range of MHC genes throughout the differentiation process.
    - The assessment of the MHC expression was verified at the protein level for the L6 Aston and C2C12 cell lines, the results agreeing with the semi-quantitative PCR whereby both cell lines expressed fast MHC at the protein level but the C2C12 cells were the only cell line to express MHC slow.
- The final cell experiments were the first of their kind to be reported, treating with $1\alpha(OH)D_3$ (constitutively active vitamin D) at a range of time points across proliferation and differentiation in C2C12 cells. C/EBPβ was responsive to $1\alpha(OH)D_3$ in muscle cells. Additionally the results showed that intracellular signalling of $1\alpha(OH)D_3$ was mediated by the MAPK ERK 1/2 signal cascade. These results provide continuing evidence that activated vitamin D metabolites influence signalling pathways that are able to contribute to control of myogenesis.

4.1 Effects of dietary Vitamin D on meat quality in Sheep trials

The hypothesis of the sheep trials reported in this thesis was that feed supplemented with vitamin D immediately prior to slaughter could promote tenderisation of meat post mortem by increasing the calcium levels and thereby stimulate the expression of the calpain system; the calcium-activated proteolytic enzymes responsible for post-mortem proteolysis. The first trial hypothesised that a short but high dose of feed supplemented with vitamin D immediately prior to slaughter (4 days) would instigate an increase in calcium absorption before the vitamin D autoregulatory axis could attenuate circulating levels of the active hormone. As a result of high levels of calcium before slaughter, post-mortem there would be an increase in calcium-mediated tenderisation. The second trial investigated the hypothesis that a high intake of vitamin D supplemented in the feed for 7 days immediately prior to slaughter combined with an added calcium bolus on the final 2 days could achieve greater increases in calcium levels and subsequent increase in calcium mediated tenderisation than supplementing vitamin D in the feed alone.

Both trials significantly increased the circulating levels of vitamin D, as measured by circulating levels of $25(OH)D_3$ in plasma of treated animals, by a factor of 10- and 25- fold respectively ($P<0.001$). Previous published investigations using vitamin D supplementation to manipulate meat quality in sheep (Wiegand *et al.*, 2001; Boleman *et al.*, 2004) did not measure the levels of circulating vitamin D metabolites and the two trials described in this thesis appear to be the first to do so in sheep. Studies in beef steers (Montgomery *et al.*, 2000; Foote *et al.*, 2004; Montogmery *et al.*, 2004), cull dairy cows (Cho *et al.*, 2006) and pigs (Wilborn *et al.*, 2004) reported changes in $25(OH)D_3$ levels in the range of 3-13 fold increases, which were in general agreement with the findings reported in this thesis. Despite the significant increase in circulating levels of $25(OH)D_3$ in the first trial of this study, there was no effect on the calcium status in the circulation nor in the LD after four days of vitamin D supplementation (Table 3.1-2). However, the longer vitamin D supplementation with an additional calcium bolus in trial 2 did result in a 25 fold increase in plasma vitamin D status which was accompanied by significant 10% increase in serum calcium ($P<0.001$). Previous studies in sheep have attempted to investigate optimal dietary vitamin D supplementation in order to achieve significant increases in calcium status; Wiegand *et al.* (2001) reported a slight, non-significant 2.5% rise in serum calcium following two days supplementation with 2.0×10^6 IU/day, which rose to a significant 14.9% increase after 5 days ($P<0.05$), which was a greater response than that seen in trial 2 of this thesis using 7 days supplementation with an additional calcium bolus. Boleman *et al.*, (2004) used lower doses of vitamin D supplementation, ranging from 0.25×10^6 to 0.75×10^6 IU/day, which were still at least 1100-fold higher than the normal recommended vitamin D intake for lambs (McDonald

et al., 2002), and did not observe any significant increases in serum calcium; however they reported a trend for higher serum calcium when lambs were supplemented with the higher dose for 5 days. Two previous experiments performed in this laboratory have investigated the effects of two additional dietary regimes, the first study investigated the efficacy of 7 days vitamin D alone in improving meat quality (Sazili, 2003) while a second study investigated the effects of a short term vitamin D supplementation for 4 days with an additional calcium bolus on the final day (Smith 2003, unpublished data). Both these studies reported significant increases in circulating calcium levels ($P<0.001$) with the 7 days vitamin D alone providing a 15% increase in serum calcium and the short 4 days plus calcium bolus increasing serum calcium by 6.9%. It is likely that 4 days of vitamin D alone at 2.0×10^6 IU/day is not sufficient to increase circulating levels of calcium and that the significant increase observed in this laboratory (Smith, 2003) was due to the additional calcium bolus.

It was hypothesised that the vitamin D supplementation regime would increase calcium levels in the muscle tissue and that this, in turn, would promote activation of the calpain system post mortem. Despite significant increases in plasma vitamin D, there was no effect on serum or muscle calcium in the first trial (vitamin D for 4 days alone), however, in the second trial (vitamin D for 7 days with calcium bolus), there were significant increases in plasma vitamin D, and a 10% increase in both serum and LD calcium levels. The previous trials performed in this laboratory did not report any increases in muscle calcium, in agreement with Wiegand *et al.* (2001) who saw no changes in muscle calcium following vitamin D supplementation at 2×10^6 IU/day for 5 days, whereas Boleman *et al.* (2004) did not measure muscle calcium levels in their study. It seems that the second trial in this thesis is the first experiment in sheep to report significant increases in muscle calcium levels following vitamin D supplementation; however it did involve a calcium bolus.

The 4 day supplementation of vitamin D was not successful in increasing the calcium status of the animals and might explain the lack of effect on the tenderness of the LD chops ($P=0.351$, table 4.1-1). In the second trial the effects of dietary vitamin D or calcium cannot be segregated from one another, however, it can be concluded that this feeding regime is sufficient to raise calcium levels of sheep. The results of the studies described above are summarised in table 4.1-1., and together these data demonstrate that there is an increase in effects on calcium status with increasing length of vitamin D dose. Numerous studies in beef cattle have reported positive effects of vitamin D supplementation on calcium levels in the plasma (Montgomery *et* al., 2000; Karges *et al.*, 2001; Scanga *et al.*, 2001; Montgomery *et* al., 2002; Foote *et al.*, 2004) whilst other studies have reported the effects in LD (Swanek *et al.*, 1999; Montgomery *et al.*, 2002; Foote *et al.*, 2004; Montgomery *et al.*, 2004), the data of these trials is summarised in table 4.1-2. Swanek *et al.* (1999), supplemented

dietary vitamin D at 5 x 10^6 IU/day for 7 days prior to slaughter and reported significantly raised calcium levels in the LD by 43% ($P<0.05$), and in the same publication, they report a second experiment supplementing 7.5 x 10^6 IU/day for 10 days where muscle calcium levels were raised 50% ($P<0.02$). Montgomery *et al.* (2000), supplemented beef cattle with vitamin D at doses of 5 and 7.5 x 10^6 IU/day for a period of 9 days prior to slaughter, and reported that both doses of vitamin D significantly raised calcium levels in the blood ($P<0.05$) and the higher dose caused a 30-35% increase in serum calcium after 8 days. Both these studies in cattle used much higher doses of vitamin D for longer periods than have been used in sheep studies which might explain the differences in the calcium responses observed between the species, although this is not taking into account the large differences between the bodyweights.

There have been studies in cattle investigating shorter (Karges *et al.*, 2001) and lower (Montgomery *et al.*, 2002) vitamin D supplementation regimes. Karges *et al.*, 2001 investigated using a high dose of 6 x 10^6 IU/day for 4 and 6 days prior to slaughter and reported that both regimes significantly increase blood calcium levels by 14% and 22% respectively ($P<0.001$) but that the more chronic dose did increase calcium levels to a greater extent ($P<0.05$), however there was no measurement of muscle calcium in this experiment. Shorter vitamin D feeding periods did not improve tenderness of the LD chops in Karges *et al.*'s study, whereas supplementing with vitamin D for two more days did significantly reduce shear force of the LD ($P<0.05$) after 7 and 14 days of conditioning. Although the level of vitamin D supplementation in Karges *et al.*'s trial was three times that which has been used in sheep, it does agree with the results presented in this thesis whereby the longer supplementation time period does cause significantly greater responses in calcium levels, but suggests that 4 days of vitamin D supplementation alone is sufficient to increase blood calcium levels in cattle. Montgomery *et al.* (2002), investigated the effects of lower doses of vitamin D in cattle (0.5 x10^6, 1.0 x10^6, 2.5 x10^6, 5.0 x10^6 and 7.5 x10^6 IU/day) for a period of 9 days prior to slaughter and reported that plasma calcium increased linearly in response to vitamin D dose, with increases of 5.7, 8.0, 13.8, 16.0 and 19.5% respectively at 10 days post supplementation. However the effects on muscle calcium reported by Montgomery *et al.* (2002) found larger responses in the LD than was seen in the blood, where muscle calcium content was increased by 17, 60, 34, 34 and 57% with each increasing dose of vitamin D. In retrospect these values equate to an overly large quantity of calcium, and it is probable an increase in muscle calcium content by 50% in cattle would just not be feasible. In trial 2 of this study, the effects of vitamin D on serum and muscle calcium in sheep were lower (a 10% increase in both) than the effects reported in cattle (Swanek *et al.*, 1999; Montgomery *et al.*, 2002; Montgomery *et al.*, 2004). A potential explanation is that cattle respond to the large increase in circulating calcium by actively sequestering the calcium into muscle resulting in larger increases in

that tissue, although no other study has provided an explanation for the differential responses between serum and muscle calcium changes observed in cattle.

Previous studies have reported that infusion of ovine carcasses with calcium chloride solution improve meat tenderisation (Koohmaraie *et al.*, 1989; Clare *et al.*, 1997; Duckett *et al.*, 1998; Polidori *et al.*, 2000). Therefore it has been hypothesised that vitamin D-induced increases in body calcium levels will have a similar effect of improving meat quality. However few vitamin D/meat quality studies in sheep have reported clear evidence for shear force improvements nor investigated the levels of calpain system activity, which are believed to mediate the improved tenderisation. While the first trial in this thesis observed no effects on shear force values of LD chops, the second trial reported significant increases in the shear force of LD chops from lambs fed vitamin D plus calcium immediately prior to slaughter ($P<0.002$), and shear force values of LD chops were higher at all time points. In both of the previous trials performed in this laboratory, shear force of LD chops was performed after 14 days of conditioning, with 7 days of vitamin D supplementation alone (Sazili, 2003) having no effect on shear force values, whereas when animals were fed the 4 days plus calcium diet regime (Smith, 2003) the shear force of LD chops were numerically lower, but this effect was not significant. Overall the data collected from the numerous trials performed by this laboratory do not support the hypothesis that feeding vitamin D prior to slaughter results in improvements in the tenderness of meat, and more importantly, only trial 2 of this thesis observed increases in muscle calcium and this led to an unexpected decrease in tenderness of LD chops. The available data in the literature on the effects of feed supplemented vitamin D on lamb meat quality is shown in table 4.1-1. Boleman *et al.* (2004) reported no effect of 0.75×10^6 IU/day for 14 days on shear force of *semitendinosus* and *semimembranosus* muscles but did report a significant increase in the shear force of LD muscles ($P<0.05$) in agreement with trial 2 of this thesis. In contrast Wiegand *et al.* (2001) reported numerically lower values for the shear force of LD chops at three aging time points (days 1, 14 and 21 days) and significantly lower values by 20% at 7 days aging ($P<0.05$), which was accompanied with an increase in muscle calcium concentration, but there was little analysis of the activities of the calpain system.

Table 4.1-1 Effects of vitamin D supplementation in sheep

Study	Vit D x 10^6 IU/ day	Serum Ca^{++}	Muscle Ca^{++}	LD SF 14 days	0hr Calpain System Activity		
					Calpain I	Calpain II	CAST
Trial 1 this thesis	2.0 / 4 days	↔	↔	↔	Measured mRNA and protein expression		
					mRNA ↑3.7% (P=0.099)	↑10% (P<0.05)	↔
					Protein ↔	↔	↔
Trial 2 this thesis	2.0 / 7 days + Ca	↑ 10% (P<0.001)	↑ 10% (P<0.05)	↑ 7.0% (P=0.133)	Measured mRNA and protein expression		
					mRNA ↓5.2% (P<0.05)	↔	↔
					Protein ↔	↑16% (P<0.05)	↑17% (P=0.087)
Smith, 2003	2.0 / 4 days + Ca	↑ 6.9% (P<0.001)	not measured	↓ 5.4% (P>0.05)	not measured		measured protein expression ↓ 9.4% (P>0.05)
Sazili 2003	2.0 / 7days	↑ 15% (P<0.001)	↓ 39% (P<0.05)	↔	↔	↔	↑ 0.4% (P=0.055)
Wiegand *et al*., 2001	2.0 / 7 days	↑ 14.9% (after 5 days)	↑ 32% (P=0.28)	↓ 35% (P>0.05)	Measured Troponin T degradation no effect		
Boleman *et al*., 2004	0.75 / 14 days	↔	not measured	↑ 11.4% (P<0.05)	not measured		

Table 4.1-2. Effects of vitamin D supplementation in beef steers

Study	Vit D x 10^6 IU/ day	Serum Ca^{++}	Muscle Ca^{++}	LD SF 14 days	24hr Calpain System Activity		
					Calpain I	Calpain II	CAST
Swanek *et al*., 1999	5.0 / 7 days		↑ 43.2%	NS			
	7.5 / 10 days		↑ 50%	↓ 13% (P=0.07)	↓66% (P=0.05)	↓37% (P=0.07)	↓15% (P=0.04)
Montgomery *et al*., 2000	5.0 / 9 days	↑ 16%		↓ 13% (P=0.0015)	measured Troponin T degradation; increased degradation after 14 days aging with 5.0 dose but not 7.5 dose		
	7.5 / 9 days	↑ 22%		↓ 14% (P=0.0015)			
Karges *et al*., 2001	6.0 / 4 days	↑ 14%		↓ 8% (P=0.0.04)			
	6.0 / 6 days	↑ 22%		↓ 13% (P=0.04)			
Scanga *et al*., 2001	1.0, 2.0, 3.0, 4.0, 5.0 / 2, 4, 6 or 8 days	↑ (P<0.05) (12 to 17%)		↔			
	2.0 / 2, 4, 6 or 8 days plus $CaCo_3$	↔		↔			
	4.0 / 2, 4, 6 or 8 days plus $CaCo_3$	↔		↔			
Montgomery *et al*., 2002	0.5 / 9 days	↑ 5.7%	↑ 17%	↔			
	1.0 / 9 days	↑ 8.0%	↑ 60%	↔			
	2.5 / 9 days	↑ 13.8%	↑ 34%	↔	no significant effect		
	5.0 / 9 days	↑ 16.0%	↑ 34%	↔			
	7.5 / 9 days	↑ 19.5%	↑ 57%	↔			
Reiling & Johnson, 2003	5.0 / 7	not measured		↔			
Foote *et al*., 2004	5.0 / 9 (slaughter day 11)	↑ 20.9%	↔	Trend↓ (P<0.1)			
	25(OH) 4 day prior to slaughter	↔	↔	↔			
	$1,25(OH)_2D$ 3 day prior to slaughter	↑ 24.5%	↔	↔			
Montgomery *et al*., 2004	0.5 / 8 days		↑ 22%	↓19% (P<0.05)	↓54% (P<0.05)	↔	
	1.0 / 8 days		↑ 27.7%	↓ 20% (P<0.05)	↓59% (P<0.05)	↔	
	5.0 / 8 days		↑ 23.5%	↓16 (P<0.05)	↓55% (P<0.05)	↔	

Table 4.1-2 summarises the data on the effects of feed supplemented vitamin D on meat quality in cattle. When the results of these studies are considered as a whole there is little evidence which clearly supports vitamin D supplementation consistently improving shear force of meat. However, the calpain system is an essential part of the mechanism and has only been investigated in a smaller number of studies. The calpain system needs investigating to fully elucidate the mechanism of vitamin D's effects on muscle proteolysis during the post mortem period. The results of the first chronological eight studies to be performed in beef steers are summarised in table 4.1-2, and demonstrates the lack of consistency in the measurement of all aspects of the hypothesis (serum and muscle calcium, shear force and calpain system analysis) which could explain why the inconsistencies exist in the reported conclusions of the general vitamin D supplementation regimes on meat quality. Of the trials summarised in table 4.1-2, there are two experiments which have reported convincing evidence for the vitamin D dietary regimes to be able to promote tenderisation of meat via the calpain system, these are Swanek *et al.* (1999) and Montgomery *et al.* (2004). The activity of the calpain enzymes were measured in Swanek *et al.*'s (1999) and Montgomery *et al.*'s (2004) studies and both reported a decrease in the activity of calpain I at 24 hours post mortem in the LD and additionally Swanek *et al.* reported a decrease in calpastatin activity with vitamin D supplementation; both were associated with an increase in tenderness of the LD. Their work was in agreement with that performed in the calcium chloride carcass marination where calpain enzymes were reported to be activated early postmortem resulting in chops requiring less time to reach acceptable tenderness (Koohmaraie *et al.* 1989). Studies performed in sheep have not thoroughly investigated the effects of vitamin D supplementation on the calpain system, summarized in table 4.1-1, and of the published trials, only Wiegand *et al.* (2001) measured troponin T degradation as an indicator of calpain enzyme activity (Huff-Lonergan *et al.*, 1996) and found no effect. Of the trials performed in this laboratory, Sazili (2003) investigated the activities of the calpain system and found no effect on calpain enzyme activity although calpastatin activity was marginally increased (0.04% increase, P=0.055), and there was no effect of diet on shear force of LD chops in that study.

Cell culture work performed by Ravid *et al.* (1994) demonstrated that calpain I mRNA expression was upregulated with $1,25(OH)_2D_3$ treatment in a cancerous kidney cell line, and it was further shown that $1,25(OH)_2D_3$ treatment of leukaemia cells caused an increase in calpain I at protein level (Berry & Meckling-Gill, 1999). Therefore given these apparent direct effects on the calpain system the experimental investigations of this thesis sought to clarify whether dietary vitamin D influenced calpain system expression at both mRNA and protein levels, and whether any changes could provide an explanation of the negative shear force effects observed in the second trial of this thesis.

The effect of dietary vitamin D given 4 days immediately prior to slaughter on the mRNA expression in sheep LD and kidney was a significant increase in LD mRNA expression of both calpains I and II by 3.7 and 10% respectively. There was no change in the calcium concentrations in the serum or muscle observed in this trial suggesting that the changes were a result of vitamin D's direct signalling via the nuclear vitamin D receptor. The results of trial 1 of this study directly agree with a study performed in cull dairy cows by Cho *et al.* (2006), which investigated the effects of treating cull dairy cows with 25(OH)-vitamin D (equivalent to a single dose of 5×10^6 IU vitamin D) 6 days prior to slaughter. In Cho *et al.*'s study (2006) there was no effect of vitamin D treatment on the concentration of calcium in the blood, on muscle calcium levels or shear force values, despite significant increases in plasma 25(OH)D and $1,25(OH)_2D$ of 4.3 and 2.5 fold respectively ($P<0.0001$). However, there was a significant effect on the calpain enzymes at mRNA level, with LD calpain I and calpain II expression increasing by 3.97 and 4.97 fold respectively, whilst calpastatin mRNA expression was decreased by 86%. There was also a significant increase in calpain I at the protein level by 20% but no effect on the protein levels of calpain II and calpastatin. Both the study by Cho *et al.* (2006) and trial 1 of this thesis support the hypothesis that vitamin D directly up-regulates the transcription of the calpain enzyme genes in muscle. Four days of supplementation may not have been long enough to allow for translation of the mRNA transcripts into protein and therefore could explain why there were no changes in the calpain enzymes at the protein level.

Trial 2 of this thesis did not agree with the two experiments reported above as mRNA expression of calpain I was significantly reduced by 5% ($P<0.05$) and calpain II and calpastatin mRNA expression was unchanged. However, the protein levels of calpain II and calpastatin were significantly higher in trial 2. A possible explanation is that changes in mRNA expression had already been translated into changes at the protein level and therefore mRNA that was transcribed has already degraded. The increase in calpastatin and the unchanged amount of calpain I at the protein level agree with the results of the 7 days vitamin D-alone supplementation experiment carried out in our laboratory (Sazili, 2003), which reported an increase in calpastatin activity at 24 hours post mortem ($P=0.055$) and numerical but not significant increases in calpastatin protein levels ($P>0.05$). Both trial 2 of this thesis and the study performed by Sazili (2003) found significant changes in serum calcium whereas trial 1 and Cho *et al.* (2006) did not. Therefore the differences between these studies could be explained by interactive mechanisms between vitamin D and calcium signalling. When vitamin D was supplemented but did not result in changes in serum calcium, as in trial 1 and Cho *et al.*'s (2006) study, the response observed is to upregulate the expression of the calpain genes at the mRNA level, a result of vitamin D signalling via the VDRnuc. Conversely, when there is an increase in the calcium status of the animals, as reported in trial 2 of this thesis and Sazili (2003), the protein level of both

calpain II and calpastatin increased, whilst calpain I mRNA decreased. Despite the second trial of this thesis up-regulating the protein levels of calpain II, it was accompanied with increased calpastatin protein levels and this is likely to be responsible for the increased shear force of the meat. Although calpastatin protein activity in Sazili's study (2003) increased, shear force of the meat was not affected, in contrast, the 17% increase in calpastatin protein levels in trial 2 of this thesis was associated with reduced tenderness. There is a strong relationship between shear force and protein levels of calpastatin (Goll *et al.*, 1998) and so this effect may account for the shear force results of trial 2 of this thesis. A possible explanation for the increased calpastatin levels observed in trial 2 could be that the animals were undergoing increased muscle hypertrophy in a response to the raised calcium levels observed in the vitamin D/calcium-treated animals' muscles. It is known that intracellular calcium activates the signalling pathways which mediate hypertrophic responses in muscle. Intraceullar calcium levels regulate calcineurin activity which is a crucial modulator of hypertrophy in skeletal muscle *in vivo* (Michel *et al.*, 2004). Calcineurin activity is modulated by calcium influxes induced during exercise (Meissner *et al.*, 2006) and by hormone treatments such as growth hormone (Sensky *et al.*, 2006), both of which are known to promote muscle hypertrophy. Additionally, Sensky *et al.* (2006) hypothesised that β-adrenergic agonists (BAA) could promote hypertrophy through protein kinase A/calcium ion signalling, and found BAA treatment significantly raised calpastatin expression levels by 50% in pigs. It is therefore possible that the increased calpastatin levels were indicative of hypertrophy, which is possibly mediated by increased intramuscular calcium levels, although further investigations would need to be performed to validate this hypothesis.

A final investigation into dietary vitamin D's signalling effects at the nuclear level investigated the treatment's affects on calbindin D-28K in the kidney and on the MHC genes in the LD. Calbindin is a calcium binding protein which is responsible for binding calcium ions and assisting their transport through cells of the intestinal lining (Lee *et al.*, 2007) and in the kidney (Roth *et al.*, 1989) and is a known genomic target of vitamin D in cells (Norman & Collins, 2001). In trial 1, the short 4 day supplementation of sheep with vitamin D resulted in no changes in the level of calbindin D-28K at both the mRNA and protein level, which was an unexpected result in light of the effects of the same vitamin D supplementation regime on calpain I mRNA levels. There was however, a numerical decrease in the level of vitamin D receptor at the protein level measured in the whole homogenate fraction of the kidney protein extract ($P=0.103$) observed in the kidneys of treated sheep. In contrast there were significant effects of the 7 day vitamin D plus calcium diet, with expression of calbindin D-28K mRNA increasing in the kidneys of the treated animals by 8.8% ($P<0.05$, table 3.2-6) although there was no detected effect on the protein levels of calbindin D-28k ($P=0.147$), demonstrating the

vitamin D/calcium diet was able to affect expression of genes involved in calcium homeostasis. Again, the lack of effect observed at the protein level could be a result of the time required to observe changes in the circulating vitamin D levels before resulting changes in mRNA transcription and subsequent translation at protein level, although the same length of time was sufficient to observe changes in protein level of calpain II in the LD. A possible explanation for this could be that circulating vitamin D directly stimulated mRNA expression of calpain II within a few days from onset of dietary treatment, as it was shown in trial 1 that 4 days of vitamin D alone was sufficient to induce calpain mRNA changes, whereas the mRNA expression of calbindin D-28K was not induced until the calcium status of the animals had changed, which could have been a factor of either 7 days of vitamin D alone (Sazili's experiment was sufficient to incur increases in serum calcium) or the additional calcium bolus at the end of the treatment period. The expression of the VDR at protein level in the kidney was significantly increased by the vitamin D/calcium bolus treatment with protein levels being increased just over 2-fold. There is no published data on the expected vitamin D receptor changes in vitamin D supplemented sheep, although it is known that vitamin D binds to its receptor and causes a translocation to the nucleus to attenuate its own gene expression. It could be that the decrease in VDR levels in the first trial is the result of VDR translocation into the nucleus, whereas the increase in VDR protein levels observed in the second trial could be due to an increase in transcription of the VDR gene itself as vitamin D is known to upregulate VDR mRNA expression (Norman & Collins, 2001), however, the level of VDR mRNA was not measured in this study, such data would provide further explanation for these results.

As reported in section 1.1.11 of this thesis, there is a large body of evidence to suggest vitamin D deficiency is related to a loss of fast fibre types and muscle strength in the elderly and that re-alimentation with vitamin D supplements can reverse the effects. In addition, the study reported by Endo *et al.* (2003) reported abnormal MHC gene expression in VDR (-/-) mice and that changes in MHC gene expression could be inferred with $1,25(OH)_2D_3$ treatment directly onto growing muscle cells. However, in the trials in this thesis there was no effect of dietary vitamin D on either mRNA or protein levels of the MHC genes.

4.1.1 Conclusion of effects of dietary Vitamin D on meat quality in Sheep trials

Neither trial reported in this thesis achieved an improvement in shear force of LD chops, but the results provided evidence for vitamin D's ability to alter the expression of the calpain system at both mRNA and protein levels. Changes in calcium status in sheep were accompanied with reduced tenderness of meat. This effect was attributed to an increase in calpastatin protein levels possibly as a result of hypertrophy of the muscle via calcium mediated signalling, although this hypothesis needs to be investigated further. Thus the hypothesis of increasing calcium levels ante-mortem may be flawed and so the addition of calcium into the muscle post mortem by mechanisms such as marination, injection or infusion in calcium chloride solutions might be the most effective strategy to decrease post mortem aging times. These trials did provide evidence for vitamin D's direct signalling effects within muscle, an example being the increase in calpain I and II mRNA observed in trial 1 where there was no change in calcium status of the animals but an increase in the circulating levels of $25(OH)D_3$, suggesting a direct effect of vitamin D on gene expression in the muscle vitamin D supplemented animals. There is a growing body of evidence to support possible beneficial roles for vitamin D supplementation in affecting muscle function, as introduced in section 1.1.11 of this thesis and, investigated further by the final experiments of this thesis.

4.2 Effects of direct Vitamin D treatment on muscle cells

The hypothesis of the final experiments of this thesis was that active vitamin D administered directly to muscle cells could mediate a response in growth of those cells, where effects on muscle cell proliferation, differentiation and fibre type would be of relevance to the meat industry in terms of production and meat quality. The exact mechanism of vitamin D's action within muscle cells is still unknown but there are numerous studies providing partial insights into the signalling pathways involved. The purpose of the second experiment of this study was to identify novel targets of $1,25(OH)_2D_3$ in muscle cells by microarray analysis which were subsequently validated in a second muscle cell experiment using an active vitamin D metabolite $1\alpha(OH)D_3$.

3.6.10.3 Circulating levels of vitamin D metabolites in plasma

It is worth noting at this point that the concentrations of active vitamin D metabolites used in both cell studies were similar to the concentrations achieved in the plasma of the sheep fed vitamin D during the first two trials of this thesis. The molecular weights of the vitamin D metabolites used in the cell culture experiments were 416 and 400 for $1,25(OH)_2D_3$ and $1\alpha(OH)D_3$ respectively. Using a molecular weight of 400 to convert the results of the vitamin D assays in sheep plasma, presented in

units of ng/ml in tables 3.1-2 and 3.2-2, the relative molar concentration of circulating vitamin D ($25OHD_3$) in the plasma of sheep were in the regions of 7×10^{-11}M, 8×10^{-10}M and 3×10^{-9}M in the control animals, 4 days vitamin D treated sheep and 7 days vitamin D plus calcium treated sheep respectively. Therefore it is reasonable to assume that the concentrations used in the cell experiment could be achieved in vivo, although the plasma assay measured level of $25(OH)D_3$ as an indicator of total vitamin D status of the animals and levels of active metabolites are likely to be lower due to the tight regulation by the kidney.

4.2.1 Effect of $1,25(OH)_2D_3$ on muscle gene expression assessed by microarray analysis

There were marked differences in the effects observed between the doses of 10^{-5}M and 10^{-7}M even before the microarray was analysed. Cells treated with the 10^{-5}M $1,25(OH)_2D_3$ yielded lower quantities of total RNA compared to those treated with 10^{-9}M (table 3.4-2, $P<0.05$). This was considered to be the result of reduced growth and probably cell death in cells treated with the higher dose of $1,25(OH)_2D_3$. There was a much larger response in gene changes with myoblasts treated with the highest dose of vitamin D for 24 hours, where, out of a total of 475 significantly changed genes, 318 genes were down-regulated at least 1.5 fold, (67% of the significantly changed genes detected). With the 10^{-9}M $1,25(OH)_2D_3$ treatment, only 43 genes changed at least 1.5 fold, of which 16 genes showed decreased expression amounting to 37% of the overall significantly changed genes detected. On the whole, the number of changed genes was small in respect of the 31,099 genes analysed, where 1.53% and 0.14% of genes changed with the 10^{-5}M and 10^{-9}M doses respectively. The number of changed genes reported in the microarray of this thesis was similar with the number of genes changed in Latres *et al.*'s (2005) microarray, where IGF-1 treatment in C2C12 cells caused significant 2-fold changes in 242 genes out of 22,000 genes analysed, equivalent to 1.1%.

Further analysis of the gene lists using the pathway analysis software showed that the higher dose of $1,25(OH)_2D_3$ was associated with cell death and cancer signaling, where three of the five pathways associated with the genes were related to cancer and cell death, and in figure 3.4-5 there were a number of changed genes directly associated with the caspases and cytochrome C, known mediators of apoptosis (Cooper & Hausman, 2007). At the time it was considered that the higher $1,25(OH)_2D_3$ dose was causing toxicity in the myoblast cells and that the treatment was too high to observe growth responses in the muscle cells. The analysis of the genes changed when myoblasts were treated with 10^{-9}M $1,25(OH)_2D_3$ showed similar associations with cell death, with the last of the three pathways being related to DNA damage and repair. There is much evidence for vitamin D's role in up-regulating apoptosis and cell death in a variety of conditions including cancer, although

the response is not unanimous across cell types (DeHaes *et al.*, 2004). Figure 3.4-5 shows that the higher dose of $1,25(OH)_2D_3$ upregulated some of the upstream regulators of apoptosis mediated by caspase and cytochrome c and this contributed to the conclusion that the highest dose of $1,25(OH)_2D_3$ was causing cell death in this experiment. The transcription factors C/EBPβ and δ were both responsive to $1,25(OH)_2D_3$ in the microarray experiment of this thesis and both transcripts exhibited a dose response. Interestingly both C/EBP-β and-δ have been shown to respond to glucocorticoid treatment in skeletal muscle (Yang *et al.*, 2005; Penner *et al.*, 2002) and it was hypothesised that C/EBPβ would attenuate expression of calpain I in the wasting response in light of the C/EBP response element in the calpain genes (Penner *et al.*, 2002). Hence C/EBPβ was chosen as another potential target for vitamin D signalling to be verified by the final experiments of this thesis. Additionally the mRNA expression of retinol binding protein was also highly up-regulated and was chosen as another target to be verified in the subsequent experiments.

The final targets identified by the results of the microarray were retinol binding protein, C/EBPβ, metallothionein 2A, and additionally c-Myc and parvalbumin were chosen as targets at the gene transcript level, along with ERK 1/2 and 4EBP1 as targets for events in protein phosphorylation. The final experiments of this thesis then were designed to assess the effects of an active form of vitamin D, $1\alpha(OH)D_3$, on muscle cells undergoing proliferation through to fully differentiated myotubes.

4.2.2 Effect of $1\alpha(OH)D_3$ on muscle gene expression in proliferating C2C12 myoblasts

The active analogue of vitamin D, $1\alpha(OH)D_3$ or alfacalcidiol, was used in the final experiments of this thesis. Alfacalcidiol is biologically active and is used in the treatment of musculoskeletal disorders such as osteoporosis because it is already 1α-hydroxylated and therefore bypasses the regulatory step in the kidney (see figure 1.2-2) (Ringe *et al.*, 2005). The effect of the $1\alpha(OH)D_3$ treatment was to significantly decrease the mRNA expression of a number of genes including the MRFs which are responsible for the control of myogenesis, the proliferation controlling c-Myc gene which is a known oncogene, the MHC isoform IIB and the enzyme GAPDH both of which are related to fast glycolytic metabolism (Table 4.2-1). The only gene to be upregulated was that of the transcription factor C/EBPβ, a protein which is related to increasing the expression of cell-type specific genes (Locker, 2001). The consequences of these expression changes will be discussed in terms of cell proliferation events within this section.

Table 4.2-1 Effect of $1\alpha(OH)D_3$ on muscle gene expression in proliferating C2C12 myoblasts. Direction of mRNA expression change indicated by colour, yellow indicates no treatment effect, red indicates $1\alpha(OH)D_3$ caused decreased expression, green indicates $1\alpha(OH)D_3$ caused increased expression.

		Effect in comparison to DMSO			ANOVA P Value
Gene	**Function**	10^{-11}M	10^{-9}M	10^{-7}M	Treatment
Beta Actin	Cytoskeletal protein	↔	↓	↓	**<0.05**
Cyclophilin A	Basic Functional enzyme	↔	↓	↓	**<0.001**
GAPDH	Metabolic enzyme	↔	↓	↓	**<0.01**
Myf5	Early myogenic factor	↔	↓	↓	**<0.001**
Myogenin	Differentiation Factor	↔	↔	↓	**<0.05**
Desmin	Cytoskeletal protein	↔	↔	↔	0.394
C/EBPβ	Transcription Factor -cell specific genes	↑	↔	↔	**<0.01**
c-Myc	Proliferation - oncogene	↔	↓	↓	**<0.01**
Metallothionein 2A	Metal Ion binding	↓	↓	↓	**<0.1**
Parvalbumin	Calcium binding, $1,25(OH)_2D_3$ responsive	↔	↔	↔	0.595
Embryonic MHC	Developmental MHC	↔	↔	↔	0.142
Neonatal MHC	Developmental MHC	Not Expressed			N/A
MHC I/β	Slow Oxidative MHC	↔	↔	↔	0.847
MHC IIA	Fast Oxidative MHC	Not Expressed			N/A
MHC IIX	Fast Intermediate MHC	↔	↔	↔	0.119
MHC IIB	Fast Glycolytic MHC	↔	↔	↓	**<0.05**

Treating C2C12 myoblasts with $1\alpha(OH)D_3$ significantly decreased mRNA expression of both MRFs, where Myf5 was decreased by the two higher doses of $1\alpha(OH)D_3$ by 46% and 69% (10^{-9}M and 10^{-7}M respectively, $P<0.001$) and myogenin was decreased by 72% when myoblasts were treated with the highest dose of $1\alpha(OH)D_3$. This is evidence to show $1\alpha(OH)D_3$ is able to cause changes in the expression of the myogenic regulatory factors which control muscle cell development. Endo *et al.* (2003) performed a similar experiment treating C2C12 cells with 10^{-8}M $1,25(OH)_2D_3$ for 48 hours in the presence of fusion media and similarly reported significant decreases in Myf5 and myogenin. Myf5 is thought to promote myoblast proliferation (Buckingham *et al.* 2003, section 1.3 of this thesis), being one of the earliest MRFs to be expressed in myogenesis, and the effect of down regulating its expression would reduce the extent of proliferation of myoblasts present, the outcome being a reduced number of muscle fibres in the adult muscle. Myogenin expression was reported to increase in the untreated C2C12 cells in Endo *et al.*'s (2003) study whereas $1,25(OH)_2D_3$ treatment caused a reduction in myogenin mRNA expression, as did $1\alpha(OH)D_3$ treatment in this study. The likely outcome of reducing myogenin expression could be a reduction in the differentiation of the muscle cells. Myogenin expression is usually temporally up-regulated to follow Myf5 and MyoD during normal myogenesis and is associated with the later phases of myotube differentiation and this has been observed during C2C12 muscle cell development (Dedeiu *et al.*, 2002) where an increase in myogenin expression was associated with the fusion of cells at around 6 days after induction of differentiation. The reduction of myogenin expression in response to $1\alpha(OH)D_3$ in this

thesis and by $1,25(OH)_2D_3$ in Endo *et al.*'s study provides evidence that vitamin D and its metabolites are likely to affect the process of differentiation and this is likely to have a negative effect on the development of myotubes. However there are two other MRFs which also play a role in myogenesis and their expression was not investigated in the studies of this thesis. Expression of MyoD was not reported to change significantly over the course of C2C12 differentiation in Dedeiu *et al.*'s (2002) study and there was no expression of MRF4, and in Endo *et al.*'s experiment MyoD and MRF4 expression was not analysed, although they had reported that despite Myf5 and Myogenin deregulation in VDR (-/-) mice there were no changes in the expression of MyoD and MRF4 between the knockouts and the wildtype mice. Both Dedeiu *et al.*'s and Endo *et al.*'s studies do not impart much relevance to the expression of MyoD and MRF4 on the process of differentiation In C2C12 cells, but it is possible for the MRFs to show redundancies (section 1.3.2 of this thesis) and thus the changes in Myf5 and myogenin affected by vitamin D treatment could be compensated for by MyoD and possibly MRF4 to allow normal myotube formation to occur, therefore analysis of the expression of MyoD and MRF4 would provide further explanation of the effect of $1\alpha(OH)D_3$ treatment on the process of myoblast differentiation. In relationship to the MRFs, the expression of embryonic MHC, MHC I/β and MHC IIX were not affected by the treatment, with the exception of MHC IIB which was significantly decreased by 10^{-7}M $1\alpha(OH)D_3$ treatment and numerically decreased by the 10^{-9}M treatment (table 3.6-5, P=0.023), suggesting that the changes in MRFs examined did not have a large effect on the MHC expression profile.

Of the mRNA transcripts selected for expression analysis (C/EBPβ, parvalbumin, metallothionein 2A and c-Myc) only parvalbumin expression was unaffected by $1\alpha(OH)D_3$ treatment. Expression of C/EBPβ was upregulated 10-fold by the lowest dose of $1\alpha(OH)D_3$, but unaffected by the higher doses, and this is the first evidence to show that C/EBPβ responds to $1\alpha(OH)D_3$ in muscle cells, the likely effects of its elevated expression will be discussed in the latter parts of this chapter. The expression of c-Myc, a well-known oncogene responsible for increasing proliferation of cells, was investigated owing to its known responsiveness to $1,25(OH)_2D_3$ (Simspon *et al.*, 1987; Simpson *et al.*, 1989). These studies reported that treatment of leukemia cells with $1,25(OH)_2D_3$ leads to a reduction in expression of c-Myc, thus reducing tumour proliferation and induction of differentiation (Simpson *et al.*, 1987, Simpson *et al.*, 1989). The mRNA expression of c-Myc was significantly down-regulated in myoblasts treated with $1\alpha(OH)D_3$ at the 10^{-9}M and 10^{-7}M ($P<0.009$), and this is evidence to support that treatment with $1\alpha(OH)D_3$ could be acting to reduce proliferation and promote differentiation in myoblasts in this experiment. However there was a significant reduction of the phosphorylation of ERK 1/2 when treated with $1\alpha(OH)D_3$ at 10^{-11}M and 10^{-9}M concentrations (table 3.6-12, $P<0.05$). ERK 1/2 is a MAP Kinase enzyme which is activated in a large variety of cell

responses and the outcome of its activation is to promote cell growth and differentiation (section 1.1.5 of this thesis). In contrast to the data obtained for c-Myc expression the down regulation of ERK 1/2 in this experiment is indicative of a lack of growth and differentiation caused by $1\alpha(OH)D_3$. However no direct activity measurements of this kinase or downstream factors were measured to confirm this effect.

Overall these findings do not fully elucidate the effect that $1\alpha(OH)D_3$ is eliciting on the proliferating myoblasts, but it is possible that proliferation is being reduced, as shown by the decrease in the expression of factors which would be associated with cell proliferation such as Myf5 and c-Myc, and the possible outcome of this would be an induction of differentiation and a reduced number of muscle cells leading to reduced fibre number in the mature muscle tissue. However, other factors that would have been expected to increase with the onset of muscle cell growth and differentiation were unchanged; these were desmin, the MHC genes and 4EBP1, although myogenin expression was decreased in line with a lack of differentiation. Further work needs to be performed to understand the role of $1\alpha(OH)D_3$ at this stage of differentiation such as investigating the expression of the remaining MRFs, MyoD and MRF4, in addition to assessing muscle cell growth using measurements such as DNA/protein ratio.

4.2.3 Effect of $1\alpha(OH)D_3$ on muscle gene expression in differentiating C2C12 myoblasts

The aim of the final experiment of this thesis was to investigate the effects of treating differentiating muscle cells with the active analogue $1\alpha(OH)D_3$ at different time points across the differentiation process, covering induction of differentiation, formation of myotubes and hypertrophy of mature myotubes. The analysis of Myf5 and myogenin expression showed that these myogenic factors were not affected by the $1\alpha(OH)D_3$ treatment. This was unexpected following the reduced expression observed after the initial myoblast experiment and the findings of Endo *et al.* (2003), who reported that 48 hours treatment with 10^{-8}M $1,25(OH)_2D_3$ caused a decrease in the expression of Myf5 and myogenin in C2C12 cells during the early phases (up to 3 days) of differentiation. There was a possible effect of 48 hours treatment with $1\alpha(OH)D_3$ on the expression of myogenin during the first two days of differentiation which was not detected in the time course analysis. Desmin expression remained unchanged during the differentiation process in the control cells, but expression was decreased with $1\alpha(OH)D_3$ treatment relative to the control cells at days 2 and 9, this again supports the idea that $1\alpha(OH)D_3$ is affecting the differentiation of the muscle fibres, although the outcome of reduced desmin in terms of muscle growth would be reduced hypertrophy.

The expression of each MHC gene changed significantly over the course of differentiation and provided an insight into the development of fibre type over the myogenic process. Immature C2C12 myoblasts expressed MHC embryonic and I/β almost exclusively, which was succeeded by the expression of the fast MHC genes whose mRNA levels increased as myotubes developed, suggesting a switch to a faster fibre type. $1\alpha(OH)D_3$ treatment significantly reduced the expression of embryonic and MHC I/β, suggesting that $1\alpha(OH)D_3$ was advancing the muscle development process as there was also significant changes in the mRNA expression of some of the fast MHC genes at differing time points across the differentiation process. After 5 days of differentiation, when myotubes were forming, the expression of MHC IIA appeared to be increased by $1\alpha(OH)D_3$ treatment. At the same time point and at the latest stage of development when myotubes were fully formed, the expression of MHC IIB appeared to be decreased by the treatment with $1\alpha(OH)D_3$, and this decrease in expression of the glycolytic MHC IIB was mirrored by the decrease in the expression of GAPDH. Taken together the effects of $1\alpha(OH)D_3$ on mRNA expression appears to promote a fast-oxidative fibre type and decreases the glycolytic potential of the muscle fibres.

The overall effects of $1\alpha(OH)D_3$ on expression of the developmental MHC genes agrees with the findings of Endo *et al.* (2003), who reported that VDR (-/-) mice had adversely high levels of embryonic and neonatal MHC, although the fast MHC genes were unaffected in this phenotype. In Endo *et al.*'s study the result of treating C2C12 cells with $1,25(OH)_2D_3$ was also a decrease in neonatal MHC expression. These findings support the model that vitamin D and its active metabolites are affecting the process of muscle development, especially during the early phases when myoblasts are present and that vitamin D's role is in the regulation of cell proliferation and regulating the time of onset of differentiation. Taken together, both the effects on the developmental MHC and the effects of MHC I/β and IIA against IIB indicate that there are two possible roles for active vitamin D metabolites in muscle tissue, the first being to reduce proliferation and induce differentiation, whilst the second effect is to reduce glycolytic potential and increase oxidative capacity via expression of MHC I/β and IIA.

Finally, all four selected novel targets of $1\alpha(OH)D_3$ in muscle were found to be changed by both age and treatment without an interaction between the two. The expression of these four proteins changed over the course of differentiation, C/EBPβ and metallothionein 2A expression both decreased over the differentiation process, whereas parvalbumin increased as myotubes formed. The expression of c-Myc was highest during the transition from myoblasts to myotubes and indicates that it is regulating cell proliferation until the myoblasts are fully differentiated, after which the myotubes do not divide and c-Myc expression decreases to coincide with this. The effect of

$1\alpha(OH)D_3$ was to decrease c-Myc's expression at these time points suggests that $1\alpha(OH)D_3$ is preventing cells from proliferating, indicating $1\alpha(OH)D_3$ encourages the formation of myotubes. This agrees with work performed by Simpson *et al.* (1989) who showed that $1,25(OH)_2D_3$ decreases expression of c-Myc via induction of the protein kinase C intracellular signalling pathway in leukaemia cells. The idea that $1\alpha(OH)D_3$ downregulates proliferation and induces differentiation in muscle cells is supported by $1\alpha(OH)D_3$'s effect on metallothionein, where treatment causes a profound decrease in expression after 48 hours during the early myoblast phase. Apostolova *et al.* (1999) reported that metallothionein levels in muscle cells during the early phases of differentiation were necessary to prevent apoptosis, and as the muscle cells differentiated the expression of metallothioneins decreased. Parvalbumin was shown to respond to $1\alpha(OH)D_3$ but effects were unclear, especially in the myotubes where expression decreased with the 10^{-9}M dose but increased with the 10^{-7}M dose. The hypothesis in this thesis was that parvalbumin would bind calcium in a protective mechanism to prevent calcium induced apoptosis, in a similar mechanism to calbindin in kidney cells as hypothesised by Christakos *et al.* (2003). However without measuring the effect of $1\alpha(OH)D_3$ on calcium and apoptosis levels, it was not possible to confirm this.

Like in the treatment of rat primary muscle cells with $1,25(OH)_2D_3$, the most consistent and clear responses to $1\alpha(OH)D_3$ in the C2C12 myogenic process were observed in the mRNA expression of C/EBPβ and the phosphorylation of ERK 1/2 , where expression of C/EBPβ was consistently upregulated by all doses of $1\alpha(OH)D_3$ at all ages, and ERK 1/2 phosphorylation was consistently down-regulated. The effects on these two targets point towards a mechanism for $1\alpha(OH)D_3$'s effects at both the gene expression level and in the intracellular signalling pathways of muscle, and whether the changes in mRNA expression observed are an effect of the VDRnuc or VDRmem is unclear. This information does agree with much of the literature (Buitrago & Boland, 2008; Buitrago *et al.*, 2006; Morelli *et al.*, 2001) in that vitamin D can mediate signals in the MAPK signalling pathways and this is the first evidence to show that the active vitamin D metabolite $1\alpha(OH)D_3$ signals via this mechanism also. The outcome of the effects reported in this thesis show that vitamin D and its active metabolites do perform a role in muscle tissue and the final section of this discussion aims to propose the signalling pathways involved and the physical effects these signalling mechanisms have on muscle. It is likely that vitamin D will not have an isolated effect on one outcome but will in fact regulate a number of mechanisms within the muscle, in particular interest to meat quality are those effects on the growth of the muscle cells and the fibre type.

4.3 General discussion of direct Vitamin D treatment on muscle cells

3.6.10.4 MRF expression

Dedieu *et al.* (2000) report that MyoD and Myf5 both mediate early myogenesis and their expression is followed by myogenin and then MRF4, which have higher mRNA expression in the latter phases of differentiation in C2C12 cells. The C2C12 results in this thesis do not agree with this as myogenin was relatively high all the way through, but Myf5 expression did show a similar expression pattern to that reported by Dedeiu *et al.* (2000). An explanation for the differences in the expression of myogenin could be that our C2C12 cells were too confluent and had already begun spontaneous differentiation. Performing this experiment with less confluent cells might provide the best insight into the effects of $1\alpha(OH)D_3$ on proliferation. In addition it would be useful to measure all four MRFs as it is reported in Dedieu *et al.*'s (2000) study that MyoD is expressed at a similar level to Myf5 during early development and that MRF4 levels are expressed at a similar time to myogenin, thus this will help to clarify the developmental stage of the C2C12s throughout our experiment.

Effects of proliferation, growth and differentiation

The $1\alpha(OH)D_3$ treatment caused a decrease in ERK 1/2 phosphorylation, an enzyme which plays a number of possible roles in muscle tissue. Opinion of ERK 1/2 's role in the regulation of the myogenic programme is divided with some work reporting that activation of ERK 1/2 leads to reduced myogenesis in muscle cells (Coolican *et al.*, 1997) and others reporting a positive action of ERK 1/2 activity in promoting myogenesis (Gredinger *et al.*, 1998). However, Wu *et al.* (2000) proposed that ERK 1/2 activation served a dual purpose in the process, dependant on the extent of differentiation of the muscle cells. In myoblasts ERK 1/2 activation inhibited the differentiation process and thereby maintained the cells in the proliferation stage. After the myogenic programme was initiated, ERK 1/2 activation promoted the muscle growth process. In our study the effect of $1\alpha(OH)D_3$ treatment was to reduce activity of ERK 1/2 at all stages of the differentiation process. During the myoblast stage the effects of $1\alpha(OH)D_3$ seemed to be to reduce proliferation and thus the reduced activity or ERK 1/2 could be in agreement with the Wu *et al.*'s hypothesis. It seems that the effect of $1\alpha(OH)D_3$ in myoblasts which are proliferating is to reduce the extent of cell division, and possibly promote differentiation. During the later stages of myoblasts to myotube formation, the action of $1\alpha(OH)D_3$ was still to reduce the ERK 1/2 activity which would reduce the differentiation process according to this same theory (Wu *et al.*, 2000) but this did not seem to be the case as $1\alpha(OH)D_3$ seemed to be promoting the differentiation based on the expression of the MHC genes

and MRFs, where $1\alpha(OH)D_3$ treatment advanced their expression towards what was observed at latter stages of differentiation.

3.6.10.5 MAPK activity

The effect of $1,25(OH)_2D_3$ is frequently reported to be an involvement in proliferation, and the results reported in this thesis support the conclusions that $1\alpha(OH)D_3$ may also regulate cell proliferation. The activity of this metabolite was to reduce ERK 1/2 activity which is opposite to the multiple published reports of $1,25(OH)_2D_3$ in muscle cells (Ronda *et al.*, 2007; Buitrago *et al.*, 2006; Buitrago *et al.*, 2008 & Morelli *et al.*, 2001). All these studies report up-regulation of the ERK 1/2 MAPK by action of $1,25(OH)_2D_3$ in muscle cells, along with simultaneous activation of the MAPK P38, a target which was not investigated in the studies of this thesis. To further understand the effect of $1\alpha(OH)D_3$ in these experiments it would be useful to investigate the level of P38 MAPK activity as this would evaluate further the effects of $1\alpha(OH)D_3$ on muscle cell differentiation. The signalling process by which $1,25(OH)_2D_3$ activates both P38 and ERK 1/2 has been elucidated to be membrane-receptor-mediated tyrosine activation via protein kinase C, which activates c-Src to phosphorylate both the MAPKKs MKK 3/6 and MEK 1/2, the upstream activators of P38 and ERK 1/2 respectively (Ronda *et al.*, 2007; Buitrago *et al.*, 2006; Buitrago *et al.*, 2008 & Morelli *et al.*, 2001), therefore it is probable that the effect of $1\alpha(OH)D_3$ to reduce activity of ERK 1/2 as observed in this thesis would be likely to reduce activity of P38 simultaneously.

The outcome of the effects of $1,25(OH)_2D_3$ on MAPK activity does not appear to have been fully investigated but studies have reported that inhibition of P38 MAPK results in no differentiation of myoblasts into myotubes and a much reduced expression of MHC and myogenin as a result of this lack of myotube formation (Cabane, 2003). The result of P38 MAPK and ERK 1/2 signalling in C2C12 cells was investigated by Wu *et al.* (2000) who reported that P38 enhanced the transcription of MEF2C and 2A, two transcription factors known to promote MRF signalling (see section 1.3.1 of this thesis) and therefore P38 activity is involved in promoting the muscle differentiation process. The role of ERK 1/2 in the process was not so clear and Wu *et al.* (2000) found the activity of this enzyme to be inhibitory of myotube development when muscle cells were undifferentiated myoblasts, where ERK 1/2 activity reduced the transcription of myogenic genes such as MyoD, MHC and myogenin and prevented formation of myotubes. However the effect of ERK 1/2 activation was different once the differentiation process had been induced and at this stage in myogenesis ERK 1/2 activation was complimentary to P38 activity and worked to promote myotubes formation. Taken together, the results of the effects of P38 and ERK 1/2 on C2C12 differentiation and their activation by $1,25(OH)_2D_3$ in muscle cells leads to the conclusion that $1,25(OH)_2D_3$ will generally promote the

process of myotube formation and muscle growth. In light of the inhibitory effect of $1\alpha(OH)D_3$ on ERK 1/2 in the experiments of this thesis and assuming that the myogenic process had already begun in the C2C12 cells, it seems reasonable to conclude that the analogue was inhibiting muscle growth in this experiment.

The results of the mRNA expression of Myf5 and myogenin in this experiment do not agree with this hypothesis as there was no clear down-regulation of these myogenic transcription factors reported in this study. In addition, the effect of $1\alpha(OH)D_3$ to effectively advance the process of differentiation could be interpreted by its effects on the mRNA expression of β-Actin, Myf5, myogenin, embryonic MHC, MHC 2A, metallothioenein 2A and c-Myc. The expression of these genes decreased towards the end of the differentiation process in the DMSO control cells, and the effect of $1\alpha(OH)D_3$ at varying time points across differentiation was to reduce their expression and seemingly induce the gene expression profile to that observed in the fully differentiated muscle cells. Further work to support this hypothesis would need to investigate whether $1\alpha(OH)D_3$ did inhibit P38 activity, and to further investigate the expression of the other MRFs, MyoD and MRF4. In addition the rate of growth of the cells could be evaluated by measuring the ratio of protein to DNA which would indicate hypertrophy in the latter phases of differentiation.

Effects on Fibre Type

Other than the effects of $1\alpha(OH)D_3$ on muscle differentiation, there is another possible role for this compound to have been regulating the fibre type of the muscle cells as indicated by myosin heavy chain expression. C/EBPβ mRNA was significantly increased by $1\alpha(OH)D_3$. This transcription factor is expressed in most cell types and its function appears to be associated with regulating expression of genes that are associated with cell specific responses. An example of C/EBPβ's control of cell type-specific genes is it's up-regulation of peroxisome proliferator-activated receptor gamma- coactivator 1 alpha (PGC1α) in brown adipose tissue (Karamanlidis *et al.*, 2007), where PGC1α expression is associated with increased oxidative capacity of cells via increases in mitochondria population. Similarly, PGC1α expression in muscle drives conversion of muscle into a slow oxidative phenotype by up-regulating MHC I/β, MHC IIA and mitochondria production (Lin *et al.*, 2002). In parallel to this, ERK 1/2 activation was shown to be responsible for promoting maintenance of the fast twitch fibre MHC genes, particularly MHC IIB, and inhibiting the activity of ERK 1/2 led to expression of a slow oxidative fibre type, particularly leading to an increased in the expression of both MHC I/β and MHC IIA. In these cell based experiments it appears that the inhibitory effect of $1\alpha(OH)D_3$ on ERK 1/2 activity and the increase in C/EBPβ expression leads to a general decrease in MHC I/β and embryonic MHC and increases in MHC IIA, particularly at day 5 post differentiation, and a general decrease in

MHC IIB. The net result of this may be a metabolic change toward an oxidative fibre type, expressing MHC IIA. *In vivo*, this change would perhaps be characterised as a movement towards an oxidative-glycolytic fibre type, characterised as type IIA by traditional myosin ATPase histochemistry.

Control of fibre type was described in section 1.3.6 of this thesis, and the mechanism by which the slow oxidative fibre type is controlled, is likely to be associated with $1,25(OH)_2D_3$ in consideration of its classical role in controlling calcium influxes in muscle cells. It is known that $1,25(OH)_2D_3$ signals at the membrane by production of the second messenger signals inositol triphosphate (IP_3) and diacylglycerol (DAG) which communicate the signal intracellularly via protein kinase C (PKC) activation and release of calcium from the SR respectively (Morelli *et al.*, 1993). It has been reported that calcineurin, activated by calcium influxes in C2C12 myotubes, activates NFAT which acts with other myogenic transcription factors MyoD, MEF 2-C and –D and p300 to promote transcription of MHC I/β expression (Meissner *et al.*, 2006). Similarly da Costa *et al.* (2007) reported that calcineurin upregulated MHC IIA expression via this calcineurin-NFAT pathway and simultaneously down-regulated MHC IIX and IIB expression. It is likely that this calcium-calcineurin pathway is activated by $1,25(OH)_2D_3$ signalling in muscle cells and therefore the increase in MHC I/β and IIA observed with $1\alpha(OH)D_3$ treatment at 5 days post differentiation in the highest dose could have been mediated by this calcium-calcineurin-mediated pathway. Interestingly the same group also reported a distinct signalling mechanism responsible for the up-regulation of the intermediate MHC IIX gene via the P38 MAPK signalling pathway (Meissner *et al.*, 2007), which again was mediated by MEF-2C and also CREB-binding protein. ERK 1/2 activity has also been implicated in fibre type changes, where work done by Shi *et al.* (2008) reported that over-expression of ERK 1/2 promoted fast type MHC protein levels and that blocking the ERK 1/2 activity resulted in a promotion of the slow oxidative MHC I/β and IIA proteins. This observation does agree with the final experiment of this thesis in that ERK 1/2 activity was reduced with $1\alpha(OH)D_3$ and that there was an increase in MHCs I/β and IIA at day 5 post differentiation in highest doses. Care must be taken in this interpretation as the change in ERK activity was assessed indirectly through the assessment of phosphorylation ratios and not by direct measurement of activity and more importantly the activation of factors down–stream of this signalling intermediate were not assessed. Indeed treatment of muscle with $1,25(OH)_2D_3$ has repeatedly been shown to up-regulate ERK 1/2 activity, and would therefore, based on the observations reported in the literature, promote a fast twitch phenotype.

The strongest observations of this thesis, were in experiments using both $1\alpha(OH)D_3$ and $1,25(OH)_2D_3$ on both primary muscle cells and muscle cell lines, was the up-regulation of the transcription factor C/EBPβ. This is a novel observation and the possible outcome in muscle cells in unclear. This

transcription factor is known to be involved in controlling specific patterns of gene expression. The transcription factor C/EBPβ has been implicated in a number of physiological processes but is probably best characterised in its role in adipocyte development. Karamanlidis *et al.* (2007) reported that the action of C/EBPβ in white adipocytes was to promote differentiation into brown adipocytes, a phenotype expressing high mitochondrial activity via C/EBPβ's effect to promote PGC-1α expression. The effect of increased PGC-1α expression in muscle tissue has been reported and its action is to drive conversion of muscle into a slow oxidative phenotype by up-regulating mitochondria production and associated oxidative metabolism accompanied with associated changes in MHC I/β and MHC IIA (Lin *et al.*, 2002). Thyroid hormone has also be shown to increase the expression of C/EBPβ in skeletal muscle (Clément *et al.*, 2002) and it is well established that this hormone has a tendency to increase type IIA muscle fibres but decrease type I substantially, as determined by histochemical methods (Larsson *et al.*, 1995). Observations in C/EBPβ knock-out mice suggested that this transcription factor has a fundamental role in regulating genes associated with fat metabolism (Millward *et al.* 2007). Therefore a possible outcome for the $1\alpha(OH)D_3$ mediated transcription of C/EBPβ is an increase in PGC-1α expression which could mediate a change to a more oxidative muscle phenotype and, as indicated above, a potential shift toward a oxidative-glycolytic IIA fibre type.

4.3.1 Conclusions of effects of $1\alpha(OH)D_3$ in developing myoblasts

What can be concluded from these experiments is that the biologically active form of vitamin D is able to initiate intracellular signalling via cascades usually associated with membrane receptors and the response is to down-regulate the activity of ERK 1/2 enzyme. The likely effects on the muscle cells in response to reduced activity of ERK 1/2 are fibre type changes and changes in growth and differentiation. Likewise the effect on an increase in C/EBPβ will be to promote the differentiation of myotubes and modulating toward an oxidative / type IIA fibre type. The hypothesised route of the biologically active form of vitamin D signalling within the muscle cell is summarised in a schematic diagram of intracellular pathways in figure 4.3-1. Further analysis within this scheme of work to evaluate this hypothesis would need to investigate the genes which are thought to be upregulated by C/EBPβ, particularly PGC1α, and to evaluate potential effects of this transcription factor on additional factors influencing fibre type as well as the effects on the calpain system, in particular calpain I as the expression of these genes was shown to respond to vitamin D in muscle tissue in trial 1 of this thesis.

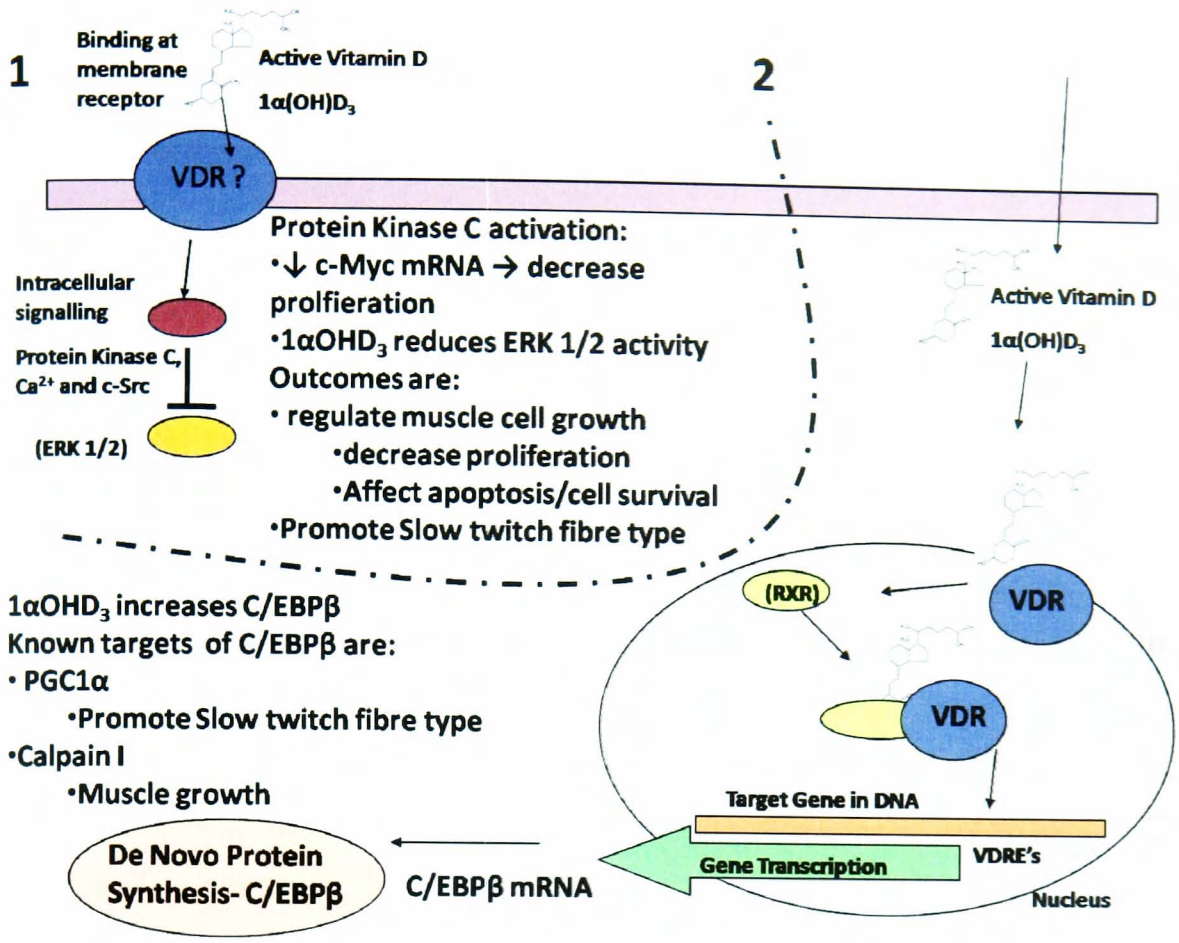


Figure 4.3-1 Hypothesised routes of the biologically active form of vitamin D signalling within the muscle cell at 1) the membrane receptor VDRmem, and 2) the nuclear receptor VDRnuc.

4.4 Conclusion

The first question addressed in this thesis was whether dietary vitamin D supplementation immediately prior to slaughter improves post-mortem tenderisation of meat in the sheep species. The answer provided by the results of the two dietary feeding trials was that there was no positive effect on tenderness of the LD and that increasing the calcium status of meat ante-mortem is likely to be detrimental to tenderisation. The model proposed was that calcium influxes within the muscle could be upregulating hypertrophy via calcium signalling mechanisms which could mediate a reduction in protein degradation via an increase in calpastatin levels. Further work to prove this theory could begin with directly assessing the activity levels of the calpain system in the two trials, thereby comparing between the studies where an increase in calcium status was observed in the second trial but not the first.

The second question to be addressed by the experiments of this thesis was whether the active metabolites of vitamin D were able to exert direct changes in gene expression in cultured rodent muscle cells. The result of the microarray and the subsequent cell culture experiments show that there were numerous genes which were responsive to the vitamin D metabolites in rodent-derived skeletal muscle cells, and thereby present a role for active vitamin D metabolites to influence muscle

function. Further work would need to clarify which effects are directly related to events induced via calcium signalling and which are vitamin D's direct signalling effects. This could be achieved by comparing active vitamin D metabolites against calcium ionophores in cultured muscle cells.

The final question to be answered by this thesis was whether active vitamin D could influence the growth of cultured muscle cells in the early myogenic stages through to mature myotubes. The results in this thesis provided evidence that dietary vitamin D fed to sheep for four days prior to slaughter was sufficient to cause an increase in the mRNA expression of calpain I, evidence for vitamin D's signalling effects in mature muscle *in vivo*. Following this, the treatment of muscle cells *in vitro* with both $1,25(OH)_2D_3$ and $1\alpha(OH)D_3$ did result in gene changes in proliferating myoblasts through to the development of myotubes. The implications of these changes are considered to be important in terms of muscle growth and development, both of which are relevant to production systems in their ability to affect muscle size and fibre number as controlled by the myogenic process, the resulting fibre type of the muscle and meat quality via levels of proteolytic enzymes such as calpain I. It is important to note that observations in this thesis were made using both tissues removed from vitamin D-supplemented sheep and in skeletal muscle cell cultures and it would be useful to verify all the proposed vitamin D signalling effects in one model, such as in the muscle cell cultures *in vitro*.

Finally, the results of this thesis implicate a potential role for vitamin D metabolites which has not yet been fully investigated in skeletal muscle cell development, and this is vitamin D's potential to alter fibre type development. The published work around $1,25(OH)_2D_3$ signalling in muscle cell growth reports the effects in terms of proliferation and apoptosis and resulting myotube development, however the targets known to be stimulated by $1,25(OH)_2D_3$ in these cells are also known to regulate the fibre type of muscle cells. The effect of vitamin D on muscle fibre type in elderly patients was summarised in section 1.3.6 of this thesis and in particular it is notable that $1\alpha(OH)D_3$ supplementation in patients resulted in an increase in fast oxidative fibres and a decrease in fast glycolytic fibres (Sorenson *et al.*, 1979). The results of the final experiments in this thesis showed that the overall fibre type of the C2C12 cells was not greatly changed within the 48 hour treatment of the experiment but there were still effects on the expression of some MHC genes. Expression of embryonic MHC and IIB was decreased with $1\alpha(OH)D_3$ treatment, while MHC I/β expression was increased. These results are important to the meat industry as slow oxidative fibre type, associated with expression of MHC I/β and IIA, is linked to tender meat whereas fast glycolytic, associated with MHC IIB, is linked to tougher meat, as observed in hypertrophy of animals administered with β-adrenergic agonists which leads to increased MHC IIB levels and type IIB fibres.

4.5 Further Work

There is much further work needed to fully evaluate vitamin D's affects on these systems and it is possible that different active metabolites and analogues of vitamin D could promote different effects, as was discussed within the preceding sections of this chapter. Further work to evaluate differences between these vitamin D analogues could easily be performed using the C2C12 cell line using some of the targets considered within this thesis and would exhibit any differences and similarities between vitamin D metabolites. Additional mRNA targets which should be considered in such an experiment would be the calpains I and II and calpastatin, all four MRFs, the MHC genes, the transcription factors C/EBPβ and –δ and factors which control fibre type such as PGC1-α and PGC1-β and the downstream factors of the PPAR isoforms. Additionally future work should further elucidate the effects of metabolites in intracellular signalling pathways such as the MAPK P38, ERK 1/2 and JNK, as all have the potential to be affected by vitamin D signalling. Such work would involve the evaluation of changes in ERK 1/2 activity and activation of proteins downstream of this kinase signalling pathway.

The extent of each metabolites' effect on myoblast proliferation and on myotube development could be further investigated using measurements to assess cell growth and hypertrophy, for example DNA:protein ratio could be a simple measure of hypertophic responses. Additionally work to evaluate whether vitamin D metabolites control proliferation by inducing apoptosis could be performed, for example, work reported by DeHaes *et al.* (1994) found that treatment of skin cells with $1,25(OH)_2D_3$ for 24 hours resulted in increased levels of the anti-apoptosis protein Bcl-2 and decreases in the pro-apoptosis proteins Bax and Bad, a response which was mediated by increased phosphorylation of ERK 1/2 by Akt. Again this implies that $1\alpha(OH)D_3$ decreased phosphorylation of ERK 1/2 could therefore regulate apoptosis and cell proliferation as seen in the final experiment of this thesis but analysis of the apoptotic protein levels Bcl-2, Bax and Bad would provide further insight.

A longer study using active metabolites of vitamin D throughout the course of differentiation in muscle cells over a longer period could provide further insights into the effects of $1\alpha(OH)D_3$ from proliferation through to formed myotubes. Incubating cells in $1\alpha(OH)D_3$ treated media for longer than 48 hours could elucidate whether there is any inhibitory effect on proliferation through the ERK 1/2 inhibition as proposed by Wu *et al.* (2000).

Additional work investigating more of the known vitamin D signalling pathways could elucidate $1\alpha(OH)D_3$'s effects on muscle cells in terms of positive or negative growth responses, $1,25(OH)_2D_3$

has been reported to signal using a number of intracellular pathways including the phospholipids PIP_2 and IP_3, protein kinase C, cAMP and calcium itself.

It would be useful to elucidate which pathways are up-regulated by $1\alpha(OH)D_3$ and $1,25(OH)_2D_3$ in a direct experiment comparing both metabolites. Further there is much evidence to show that $1,25(OH)_2D_3$ also activates the MAPK P38 and analysis of the activation of this protein could elucidate whether the $1\alpha(OH)D_3$ again serves to decrease P38 activation, similar to its effects on ERK 1/2. These experiments would need to assess a number of different signalling pathways to evaluate the effects of these metabolites on muscle growth and fibre type; these would need to include measurements of both ERK 1/2 and P38 activity and effects on calcium influx and calcineurin activity. Downstream targets of these pathways would additionally need to be assessed and these would be NFAT activation, MEF- 2C and -2D, MyoD, and p300 to assess effects on MHC I/β and IIA via the calcineurin pathway; and PGC-1α and mitochondrial specific gene expression to assess effects of C/EBPβ on MHC I/β, MHC IIA and mitochondria in promotion of the slow oxidative phenotype. It is likely that these effects will require longer than 48 hours to be observed .The hypothesised effects in muscle tissue of these two metabolites are summarised in figures 4.5-1 and 4.5-2 below, taking into account results accumulated in this thesis and published data.

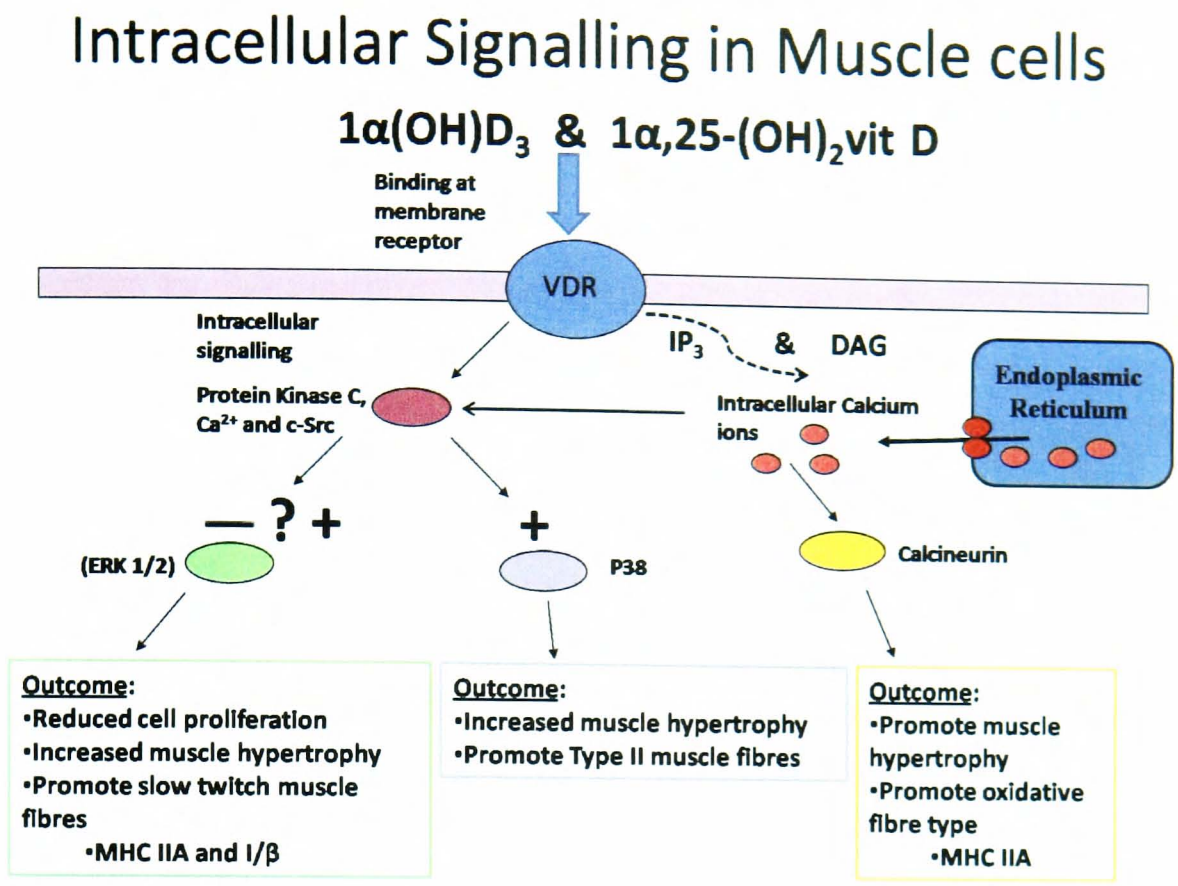


Figure 4.5-1. Proposed mechanism and outcomes of intracellular signalling of biologically active vitamin D isoforms within muscle cells.

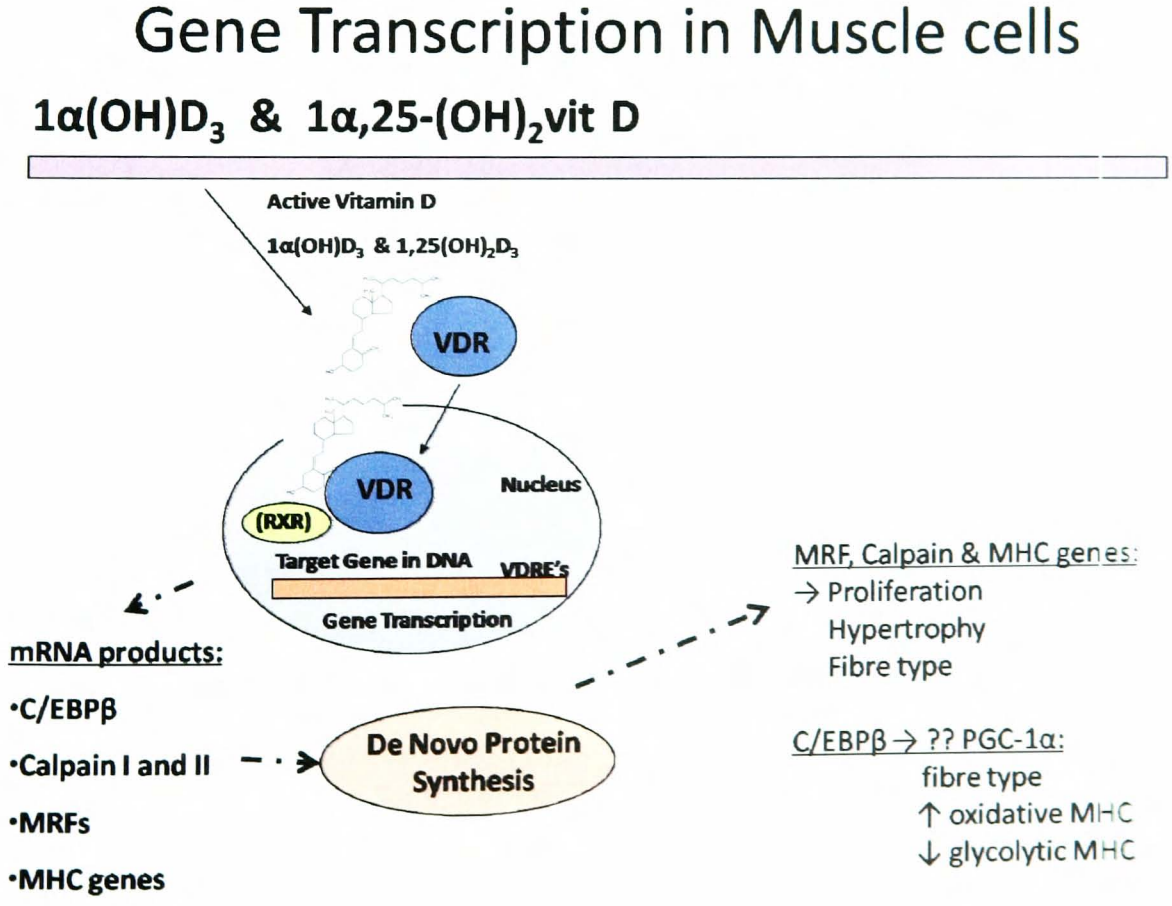
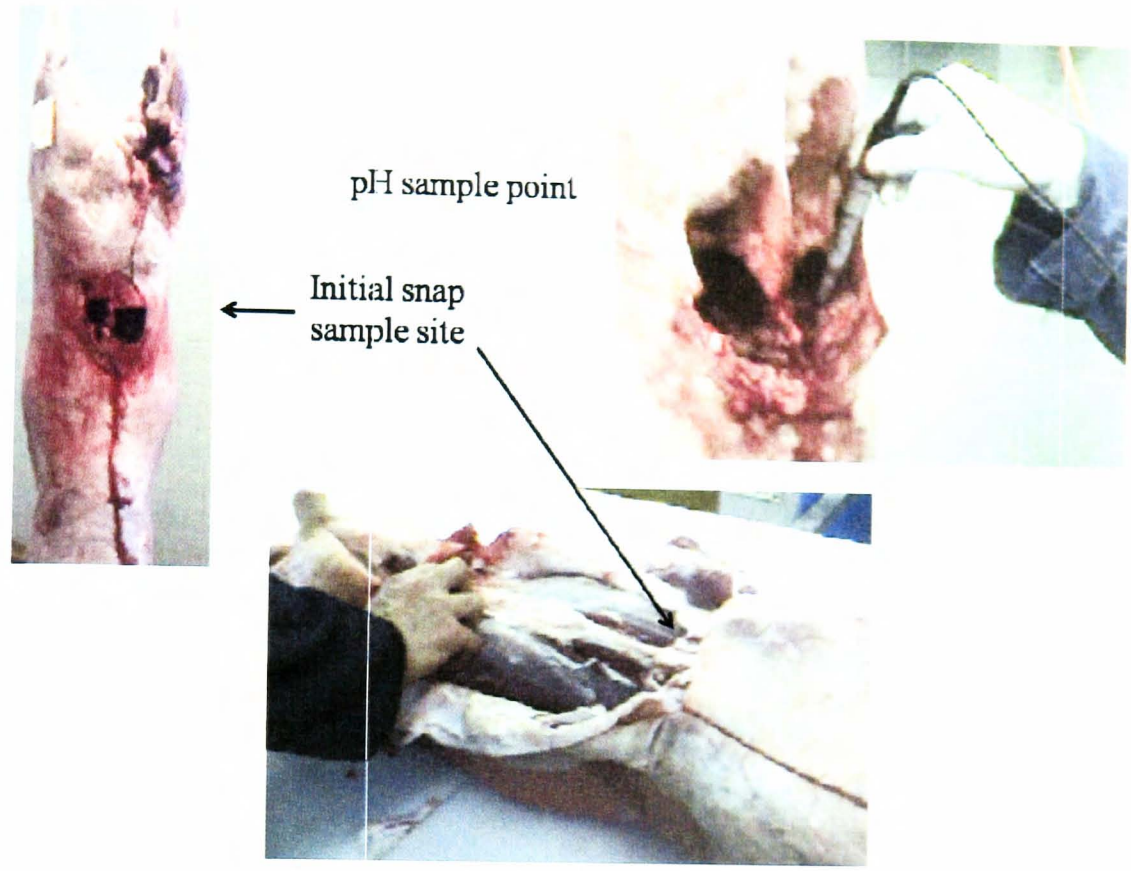


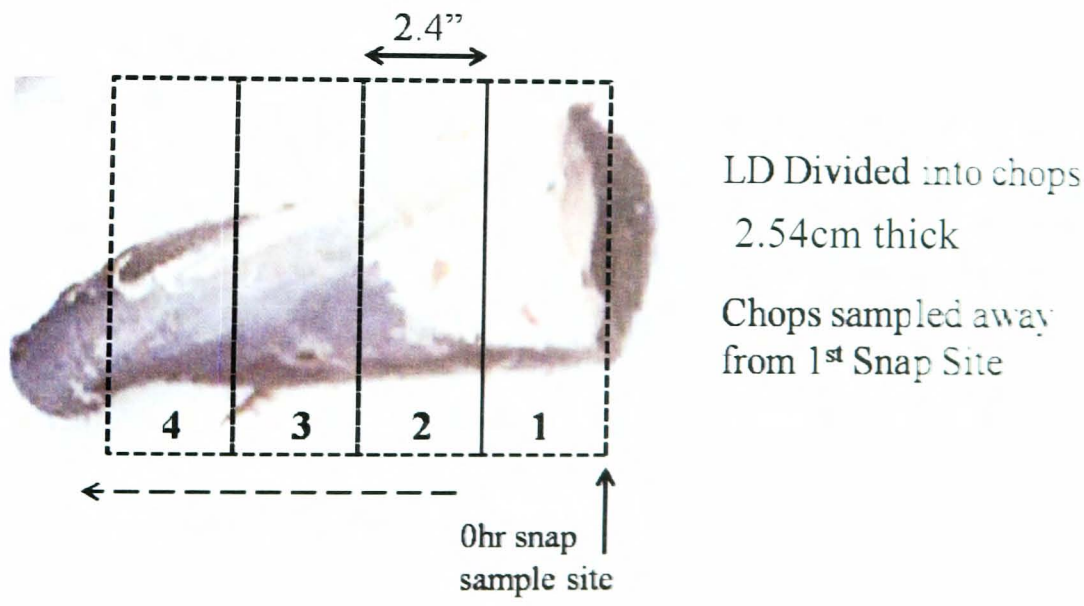
Figure 4.5-2 Schematic diagram of the possible novel gene targets of $1\alpha(OH)D_3$ and $1,25(OH)_2D_3$ discovered in this thesis, considered to be affected through the classical VDR nuclear receptor mechanism, and the hypothesized outcomes in muscle tissue.

Appendix A

WBSF Sample Procedure

Sample procedure for snap LD sample, pH of carcass and removal of LD for shear force chops is demonstrated in diagrams below.





Both the left and right LD muscles were sampled in this way and numbered as set out in diagram above. Left and right chops were vacuum packed separately but were frozen after the same period of aging, allowing two replicate chops per time point; left and right.

Latin Square Arrangement for Shear Force LD Chop Samples

WBSF Sample Procedure; 4 days Vitamin D_3 Trial 1

		Position number of sample			
	Animal Pair	Day 3	Day 7	Day 14	Day 21
Group 1	373/208	3	1	2	4
	219/467	2	3	1	4
	333/209	1	2	3	4
	123/178	3	1	2	4
	293/297	1	2	3	4
	330/466	3	2	1	4
Group 2	181/148	2	3	1	4
	251/254	1	2	3	4
	364/361	3	1	2	4
	310/263	3	1	2	4
	332/169	1	2	3	4
	377/152	2	3	1	4
Group 3	464/462	3	1	2	4
	475/276	1	2	3	4
	421/182	2	3	1	4
	474/258	3	1	2	4
	168/171	1	2	3	4
	386/380	2	3	1	4

1= anterior, 4= posterior.

Note: Some LD samples were too small to recover 4 chops and so data set for chops aged 21 days is smaller.

WBSF Sample Procedure; 7 days Vitamin D_3 = Calcium Trial 2

		Position number of sample			
	Animal Pair	Day 1	Day 3	Day 7	Day 14
Group 1	511/246	2	3	4	1
	308/518	4	2	3	1
	158/234	3	4	2	1
	281/535	3	2	4	1
	455/478	2	4	3	1
	145/378	4	3	2	1
Group 2	296/180	3	2	4	1
	228/450	4	3	2	1
	141/101	2	4	3	1
	232/137	2	3	4	1
	144/370	4	2	3	1
	215/274	3	4	2	1
Group 3	108/490	3	2	4	1
	339/338	2	4	3	1
	212/220	4	3	2	1
	522/488	3	2	4	1
	213/515	4	3	2	1
	303/409	2	4	3	1

1= anterior, 4= posterior

Appendix B

Real-time PCR oligonucelotides designed in sheep

Primers for calpains I and II and calpastatin were designed by primer express (V1.5) in regions homologous to isoform. mRNA nucleotide sequences were created from sheep EST databases (NCBI) and aligned to known sequences of bovine mRNA. These sequences and position of primer and dual-labelled probe are shown.

Ovine Sequence for Calpain I

>Calpain I Ovine.
CGGTCCGGATTCCCGGGATCCCAGTTCAAAATCCGGCTGGAGGAGACGGATGACCCAGACCCCGACGATTACGGG
GGTCGCGAGTCAGGCTGCAGCTTCTTGCTCGCCCTCATGCAGAAGCACCGCCGTCGAGAGCGCCGATTCGGCCGT
GACATGGAGACCATAGGTTTCGCTGTCTACGAGGTCCCTCCGGAGCTGGTGGGCCAGCCGGCCGTGCATCTGAAG
CGAGACTTCTTCCTGGCCAATGCCTCCCGGGCCCGGTCTGAGCAGTTCATCAACCTGCGGGAGGTCAGCACCCGC
TTCCGCCTGCCGCCCGGGGAGTACGTGGTGGTGCCCTCTACCTTCGAGCCCAACAAGGAAGGCGACTTTGTGCTG
CGTTTCTTCTCAGAGAAGAGCGCAAGGACCCAAGAGCTGGATGACCAGGTCCAGGCCAATCTCCCTGATGAGCAA
GTGCTCTCAGAAGAGGAGATTGATGAGAACTTCAAGTCTCTCTTCAGACAACTGGCAGGGGAGGACATGGAGATC
AGCGTCAAGGAGCTGCGGACCATCCTCAACAGGATCATCAGCAAACACAAAGACCTGCGGACCACGGGCTTCAGC
GTGGAGTCCTGCCGCAGCATGGTCAACCTCATGGATCGCGACGGCAATGGCAAACTGGGCCTGGTGGAGTTCAAC
ATCCTATGGAACCGGATCCGGAATTACCTGTCCATCCTATGGAACCGGATCCGGAATTACCTGTCCATCTTCCGG
AAGTTTGACCTGGACAAGTCGGGCAGCATGAGTGCCTACGAGATGCGGATGGCCATTGAGTTTGCAGGCTTCCAG
CTCAACAAGAAGCTGTACGAGCTCATTATCACCCGCTACTCGGAGCCAGACCTGGCCGTGGACTTCGACACTTTG
TGTGCTGCCTGGTGCGGCTGGAGACCATGTTCCGGTTTTTCAAAACTCTGGACACCGATCTGGATGGAGTGGTGA
CCTTTGACTTGTTTAAGTGGCTACAGCTGACCATGTTTGCATGAGGCGGGGGCTCGGGCCCCTTGCTATGCTCCT
CTGCCCTCCTCGTCCGCCAAGCCACGCCTTCCTGCGACACCACGCCAGGCCACACCAGCTGCAAGTGCCTTCCTT
GGAGCAGGAGGCGGCCTCGACCTCCTGTCGCCTCTCCTTTCAGCCGCTGCGGTTCGTTTGCTATGGGCAGAGCCA
TGGGGCCCTCCCTGCCTCCAACAGGCGCACGTGGACTCCTCCAGCCCCTCCGGTCGCCAGACCAGGGAGGCAGCT
TTCGCTAGTTCCTGCCTCAGGATGGGGCTCCCCAGGGAGCTGGGGGCCCCTGGCCTCCCCCATCCTGACGTGTCC
TCTCTCCTGCCCCCGCAGGCCAACCATTCGCCCCCCTCCCCCACCTGCAAACTT

Primers:
Forward: TCCGGAAGTTTGACCTGGAC
Reverse: GCTCGTACAGCTTCTTGTTGAGC
[Reverse Antisense: *GCTCAACAAGAAGCTGTACGAGC]*
Probe: TGGCCATTGAGTTTGCAGGCTTCC
Product length: 102bp

Ovine Sequence for Calpain II

>Calpain II Ovine.
GCCTGCAGAAGCTGATCCGCATCCGGAATCCCTGGGGAGAGGTGGAGTGGACCGGGCAGTGGAATGACAACTGCC
CAAACTGGAACACAGTTGACCCCGAGGTGAGGGAAAGCCTGACCAGACGGCACGAGGATGGGGAATTCTGGATGT
CTTTCAGCGACTTCCTGAGACACTATTCCCGCCTGGAGATCTGCAACCTAACCCCTGATACACTCACCAGTGACA
CCTACAAGAAGTGGAAACTCACCAAGATGGATGGGAACTGGAGGCGAGGCTCGACCGCGGGCGGCTGCAGGAACT
ACCCAAACACGTTCTGGATGAACCCTCAGTACCTGATCAAGCTGGAGGAAGAGGACGAGGATCAGGAGGACGGGG
AGAGCGGCTGCACCTTCCTGGTGGGCCTCATCCAGAAGCACCGCCGGCGGCAGAGAAAGATGGGCGAGGACATGC
ACACCATTGGCTTCGGCATCTATGAGGTTCCAGAGGAGTTTACTGGGCAGACCAACATCCACCTCAGCAAAAAAT
TCTTCCTGACAACCAGAGCGCGGGAGCGGTCGGACACCTTCATCAACCTGCGGGAGGTGCTCAACCGCTTCAAGC
TGCCCCCCGGGGAGTACATCGTGGTGCCCTCCACCTTCGAGCCCAACAAGGACGGCGACTTCTGCATCCGGGTCT
TTTCTGAGAAGAAAGCCGACTACCAAGTTGTCGATGATGAAATCGAGGCCAACCTTGAGGAGATTGACATCAGCG
AGGACGACATCGATGATGGATTCAGGAGACTGTTTGCCCAGCTAGCTGGAGAGGATGCAGAGATTTCTGCCTTTG
AGTTGCAGACAATCCTGAGACGAGTGCTAGCAAAGCGCCAAGATATCAAGTCCGACGGCTTCAGTATTGAGACCT
GCAAGATCATGGTGGACATGCTCGACTCGGATGGGAGTGGCAAACTGGGTCTGAAGGAGTTCTACATTCTCTGGA
CCAAAAATCAAAAAT

Primers:
Forward: CCGGCGGCAGAGAAAGAT
Reverse: TGCTGAGGTGGATGTTGGTC
[Reverse Antisense: *GACCAACATCCACCTCAGCA*]
Probe: CACACCATTGGCTTCGGCATCTATGAG
Product length: 101bp

Ovine Sequence for Calpastatin

>Calpastatin Ovine. U66320,Ovis aries calpastatin mRNA, complete cds
AGGGAGCGACGCAGAGAGCCTCGTGTAGGTAGCAGCGGTACCGTAAACGTTCGACCACCATGTTCTGCGTACAGT
CATCTTCTTGGTCATCTAGCGGAAGGTTCAGTCCTCTTTTCTTTCCTAGTCTACTCTTTTTTTCGTTGTTCGGTT
GGGAGCAGTCAGCTCTCCAGAACTCAGGCTGGTGAAAAAGCCCCGGTCACCAAGGTAACTACTTCCTCTGCTTCA
GCCAGCAAGTCTTCCAGTATGAATCCCACAGAAGCCAAGGCTATTCCAGGCAGCAAACAGCTGGAAGGACCGCAT
TCTCCTAACAAGAAAAGACACAAAAAACAGGCTGTAAAAACAGAACCTGAGAAGAAGTCACAATCAACTAAGCCA
TCTGTGGTTCATGAGAAAAAAACCCAAGAAGTAAAGCCAAAGGAACACACAGAGCCAAAAAGCCAACCCAAGCAC
CCCTCAGATACAAGAAGCAAGCATGCTCCTAAGGAAAAAGCTGTTTCCAAGTCAAGCGAGCAGCCACCATCAGAG
AAATCAACAAAACCAAAGACCAAGTCACAGGACGAGATTTCCGGTGGTGGAAAGAGCGCTGTTCCTGCTGTTGCA
GCTGCAGCATCTGCCGAACCAGCTGACAAGAATAAAGAAAGTAAATTGTTAACATCGGCCGTGCCAGTTGAATCC
AAGCCAAGTAAACCTTCTGCAAAGTCAGACATGGATACTGCTCTGGATGACTTAATAGACACTTTAGGAGAACCT
GAAGAGACAAAAGAAGATACCACAACATATACTGGACCTGAAGTTTCGGATCCAATGAGTTCTACCTACATAGAG
GAACTGGGTAAAAGAGAAGTCACACTTCCTCCAAAATATAGGGAACTTTTGAATAAAGAAGAAGGGATCGCAGGG
CCTCCTCCAGACTCCTCGAAACCCCTGGGGCCCAATGACGCCATCGATGCCTTGTCATCAGACTTCACCTGCAGT
TCCCCTACAGCTGATGCAAAGAAAACTGAGAAAGAGAAATCTACAGAAGAGGCTTTAAAAGCTCAGTCAGCTGGG
GTGATCAGAAGTGCTGCTCCACCCAAAGAGAAAAGAAGAAAAGTGGAAGAGGATACCATGACTGAGCAAGCCCTG
GAGGCCCTGTCTGCCTCCCTGGGCACCCGGAAGCCAAGGCCAGAGCTCGACCCCAGCTCCATTAAGGAGGTCGAT
GAGGCAAAAGCCAAAGAAGAGAAAGTAAAGAAATGTGGTGAAGATGAGGAAAGAGTCCCATCGGAGTATAGATTA
AAGCCGGCCACAGATAAAGATGGAAAACCACTCTTGCCAGAGGCTGAAGAAAAACCCAAGCCCCTGAGTGAATCA
GAACTCATTGATGAACTCTCAGAAGATTTTGACCGGTCTAAGTGTAAAGAAAAACAATCTAAGCCAACTGAAAAA
AACAGAGGCATCCCCGGCCGCTGCCCCCGTGCCTGTGCGAGAGGATGTGCCTCGGACCTCCATGTGTTCTGTGCA
GTCAGCTCCACCCACAGCAGCTCCAGCGAAGGCATGGTGCCAGACGATGCTGTGGAAGCCCTTGCTGGAAGCCTG
GGCAAAAAGGAAGCAGATCCAGAAGACGGAAAGCCTGTGGAGGATAAAGTCAAGGAGAAAGCCAAAGAAGAGGAT
CGTGAGAAACTTGGTGAAAGAGAAGAAACGATTCCTCCTGATTACAGATTAGAAGAGGCCAAGGATAAAGACGGA
AAACCACTGCCGCCAAAAGAGGTCAAGGAACCGCTCCCACCCTTGAGTGAAGACTTCCTCCTCGATGCTCTGTCC
AAGGACTTCACTGTCCCCTCAGACACGTCATCGCCTCAATTTGAAGATGCTAAACTTTCAGTCGTCGTCTCTGAA
GTGGTTTCCCAAACCCCAGCTCCAACCACCCAGGCAGCCGGTCCACCCCGCGACTCTGCGCGTGACAACAAAGAA
CTTGACGATGCCCTGGATCAACTTTCTGACAGTCTCGGGCAAAGACAGCCTGATCCAGATGAGCATAAACCTGTA
GAGGATAAAGTCAAGGAAAAAGCCAAAGCTGAACACAGAGACAAGCTGGGAGAAAGAGATGACACCATCCCACCT
AAATACCAGCATCTTCTGGATGATAACAAGGAGGGCACACCCGGGAAGCCAAAGCGATCAGAAAGCCCGAGGCAT
CAGAGAAGCCCGAGGCATCAGAGAAACCTGCAGGTGCCCAGGACCCCATTGACGCCCTCTCAGGGGACTTGGACA
GCTGTCCCTCAACTACAGAAGCCTCAACAAACACAGCAAAGGACAAAGACAAGAAGCCTGCTTCCAAGTGACAAA
GCACCCAGGAATGGCGGGAAAGCAAAGGATTCCACAAAGGCAAAGGAGGAAACTTCCAAGCCAAAAGCTGATGGA
AAAAGTACAAGTTAAAATTCACAGTATTTGGTTCTGCATATAAAATCTGCAGCAGGTAGATGGTGACGTCTGAAG
AACAAAAGGCTTTGACAACAGAAACAATTCTGGGGTGGCTTCTAGACAGTGGTATTTGTTGAGTCTTTTGACATC
CTAAACATTGTCTGTTATTCTTTTTCCTGAAAAGAAACTGAATTTGTCTGGTTC

Primers:
Forward: GGTCCACCCCGCGACT
Reverse: TGCTCATCTGGATCAGGCTG
[Reverse Antisense: *CAGCCTGATCCAGATGAGCA]*
Probe: TGCGCGTGACAACAAAGAAC
Product length: 101bp

Ovine calbindin D-28K PCR product

A 263bp partial length cDNA for ovine calbindin D-28K was obtained from a 301bp amplicon generated by RT-PCR from ovine kidney cDNA (Miss C. Anike-Nkweze, University of Nottingham). This sequence has been BLAST (NCBI) searched against the human kidney calbindin D-28k cDNA and found to be 91% similar, thereby confirming the PCR amplicon as being calbindin D-28kDa from ovine kidney tissue. The primers used to generate this 300bp product are as follows:

Forward (CalBF): CTGCTCTTCCGATGCCAGC
Reverse (CalBR): CTTTCCCACACATTTTGATTCCC
[Reverse antisense: *GGGAATCAAAATGTGTGGGAAAG*]

>Calbindin reverse complement sequence RT-PCR product.
KTTCTGCTCTTCCGATGCCAGCAGCTAAAGTCCTGTGAGGAATTCATGAAGACATGGAGAAAATATGATACTGAT
CACAGTGGCTTCATAGAAACTGAGGAGCTTAAGAACTTTCTAAAGGACTTGCTGGAAAAAGCAAACAAGACTGTT
GATGATACAAAACTAGCTGAATATACAGACCTAATGCTGAAATTGTTTGATTCAAATAATGATGGAAAGCTGGAA
TTAACTGAGATGGCCCAGATTACTACCCGTGCTATGGA

Primers and probe were designed within this sequence for use in qRT-PCR

>Calbindin reverse complement sequence RT-PCR product.
KTTCTGCTCTTCCGATGCCAGCAGCTAAAGTCCTGTGAGGAATTCATGAAGACATGGAGAAAATATGATACTGAT
CACAGTGGCTTCATAGAAACTGAGGAGCTTAAGAACTTTCTAAAGGACTTGCTGGAAAAAGCAAACAAGACTGTT
GATGATACAAAACTAGCTGAATATACAGACCTAATGCTGAAATTGTTTGATTCAAATAATGATGGAAAGCTGGAA
TTAACTGAGATGGCCCAGATTACTACCCGTGCTATGGA

Primers:
Forward (Calb28F): ACAAGACTGTTGATGATACAAAACTAGCT
Reverse (Calb28R): GGCCATCTCAGTTAATTCCAGC
[Reverse antisense: *GCTGGAATTAACTGAGATGGCC*]
Probe: ACAGACCTAATGCTGAAATTGTTTGATTCAAATAATGA
Product length: 101bp

Ovine Sequence for β-actin

Primers and dual labeled probe for ovine β-Actin were designed to the mRNA sequence as shown below, by Zoë Daniel (University of Nottingham).

>gi|8809715|gb|AF129289.1|AF129289 Ovis aries strain Warhill beta-actin mRNA, partial cds
ACCTTCTACAACGAGCTGCGTGTGGCCCCCGAGGAGCACCCCGTGCTGCTGACGGAGGCTCCTCTGAACCCCAAG
GCCAACCGTGAGAAGATGACCCAGATCATGTTCGAGACTTTCAACACTCCTGCCATGTATGTGGCCATCCAGGCT
GTGCTGTCCCTGTACGCCTCTGGCCGCACCACTGGCATCGTCATGGACTCTGGGGACGGGGTCACCCACACGGTG
CCCATCTACGAGGGGTACGCCCTCCCCCACGCCATCCTGCGTCTGGACCTGGCTGGCCGGGACCTGACGGATTAC
CTCATGAAGATCCTCACGGAACGTGGTTACAGCTTTACCACCACAGCCGAGCGGGAAATTGTGCGTGACATCAAG
GAGAAGCTCTGCTACGTGGCCCTGGACTTCGAGCAGGAGATGGCCACTGCGGCGTCGTCCTCCTCCTTGGAGAAG
AGCTATGAGCTGCCCGATGGTCAGGTGATCACCATCGGCAACGAGCGGTTCCGGTGTCCCGAGGCGCTCTTCCAG
CCGTCCTTCCTGGGTATGGAGTCCTGCGGCATTCACGAGACCACCTTCAATTCCATCATGAAGTGTGACGTGGAC
ATCCGCAAGGACCTGTACGCCAACACGGTGCTGTCTGGTGGCACCACCATGTACCCTGGCATCGCCGACAGGATG
CAG

Forward: TGTGCGTGACATCAAGGAGAA
Reverse: CGCAGTGGCCATCTCCTG
[Reverse Antisense: CAGGAGATGGCCACTGCG]
Probe: CTGCTACGTGGCCCTGGACTTCGA
Product length: 67bp

Ovine MHC primers Developed by Krystal Hemmings

Primers and probes for detection of the myosin Heavy Chain genes by real-time RT-PCR were designed to three partial cDNA sequences which were generated by RT-PCR from ovine skeletal muscle total RNA by Krystal Hemmings (University of Nottingham).

MHC I/β

>gi|13488743|dbj|AB058898.1| Ovis aries OMyHCs mRNA for myosin heavy chain slow, partial cds

GAGCCCCTTTCCTCTCGAAGGCGCGTTTGAAAAGCCAAGCCGCAGCCATGGTGGAYGCGGAGATGGCCGCGTTTG
GGGAGGCCGCCCCCTACCTGCGCAAGTCAGAGAAGGAGCGGCTGGAAGCCCAGACCAGGCCTTTTGACCTCAAGA
AGGACGTCTTTGTGCCTGATGACAAAGAGGAGTTCGTCAAGGCCACAATTTTGTCTCGAGAGGGTGGCAAAGTCA
CCGCTGAGACAGAGCATGGCAAGACAGTGACCGTGAAGGAGGACCAGGTGTTGCAGCAGAACCCACCCAAGTTCG
ACAAGATCGAGGACATGGCCATGCTGACCTTCCTGCACGAGCCTGCCGTGCTC

MHC I/β Primers

Forward: TCG TCA AGG CCA CAA TTT TG
Reverse: CTG CTG CAA CAC CTG GTC CT
[Reverse Antisense: *AG GAC CAG GTG TTG CAG CAG]*
Probe: ACA GAG CAT GGC AAG ACA GTG ACC GTG
Product length: 101bp

MHC IIA

>gi|13488739|dbj|AB058896.1| Ovis aries OMyHC2a mRNA for myosin heavy chain 2a, partial cds

GCAGCCATGAGTTCAGACCAAGAAATGGCAGTCTTTGGGGAGGCTGCTCCTTACCTCCGAAAGTCCGAAAAGGAG
CGCATTGAAGCCCAGAATAAGCCTTTTGATGCCAAGACATCTGTCTTTGTGGCCGAGCCCAAGGAATCTTTTGTC
AAAGGGACTATCCAGAGCAGAGAAGGTGGGAAAGTGACGGTGAAGACCGAAGGAGGGGCGACTCTGACAGTGAAA
GAGGATCAAGTCTTCCCCATGAACCCTCCCAAATTCGACAAGATCGAGGACATGGCCATGATGACCCACCTGCAC
GAGCCCGCTGTGCTG

MHC IIA Primers

Forward: AAG CCT TTT GAT GCC AAG ACA T
Reverse: TTC ACC GTC ACT TTC CCA CC
[Reverse Antisense: *GG TGG GAA AGT GAC GGT GAA]*
Probe: TGT GGC CGA GCC CAA GGA AT
Product length: 101bp

MHC IIX/IIB

Sequence homogeneity between MHC IIX and MHC IIB was too high for real time primer and probes to be developed for these genes separately and as there is very little MHC IIB expressed in the ruminant species any expression of MHC IIB would be detected by primers and probes.

>gi|13488741|dbj|AB058897.1| Ovis aries OMyHC2x mRNA for myosin heavy chain 2x, partial cds
CACTTCAAGGTTACATCTCCAAGGCAGGGTCTTTGATTGGGCTGCCATCAATAACCTGCAGCCATGAGTTCAGAC
CAGGAAATGGCTATTTTTGGGGAGGCTGCTCCTTACCTCCGAAAGTCTGAAAAGGAGCGCATCGAGGCCCAGAAT
AAGCCTTTTGATGCCAAGACCTCAGTCTTCGTGGCGGACCCTAAGGAGTCCTTCGTGAAAGCGACCGTGCAGAGC
AGGGAAGGGGGGAAGGTGACAGCCAAGACCGAAGCTGGGGCGACAGTAACTGTGAAAGAAGACCAAGTCTTCCCC
ATGAACCCTCCCAAGTTTGACAAGATCGAGGACATGGCCATGATGACCCACCTGCACGAGCCCGGAGTACTG

MHC IIX/B Primers

Forward: CTT CGT GGC GGA CCC TAA G
Reverse: CAG TTA CTG TCG CCC CAG CT
[Reverse Antisense : *AG CTG GGG CGA CAG TAA CTG*]
Probe: AGC GAC CGT GCA GAG CAG GG
Product length: 102bp

Appendix C

Solutions used to create denaturing protein Gels

SDS- PAGE Acrylamide Solutions for two 20 x 10cm protein Gels

1 MAIN GEL	8%	10%	12%
acrylamide (ml)	13.3	16.7	20
2M Tris-HCL (pH 8.8) (ml)	9.4	9.4	9.4
10% SDS (ml)	0.5	0.5	0.5
distilled H_2O		to 50mls	
Mix Solutions before addition of 10% AMPS & TEMED			
10% (w/v) ammonium persulphate (AMPS) (ml)		0.4	
N,N,N'N'- tetramethyl-ethylenediamine (TEMED) (ml)		0.04	

2 STACK	25ml (for two gels)
acrylamide (ml)	3.4
1M Tris-HCL (pH 6.8) (ml)	2.52
10% SDS (µl)	200
distilled H_2O	to 20mls
Mix Solutions before addition of 10% AMPS & TEMED	
10% (w/v) ammonium persulphate (AMPS) (ml)	0.2
N,N,N'N'- tetramethyl-ethylenediamine (TEMED) (ml)	0.02

Appendix D

Protein Antibodies

For analysis of protein expression levels in sheep tissues the following antibodies were used using the conditions provided below. Antibodies were diluted in 5% (w/v) non fat dried milk- TBS-T solution at the concentration stated in the table below. Each protein required the separation of either supernatant or whole homogenate protein fraction by action of gels containing differing levels of acrylamide. The amount of protein loaded and the dilution of both primary and secondary antibodies were optimal for production of clear bands following western blotting.

Primary Antibody	Gel %	Protein (µg)	Tissue	Protein Fraction	Dilution	Source	secondary Antibody*	Source
Calpain I	10	100	L.D. Muscle	Supernatant	1:500	Parr, to Porcine	Anti-Rabbit	
Calpain II	10	100	L.D. Muscle	Supernatant	1:1000	Parr, to Porcine	Anti-Rabbit	
Calpastatin	8	100	L.D. Muscle	Whole Homogenate	1:1000	Monoclonal to Clone IF7E3D10	Anti-Mouse	
Calbindin	12	100	Kidney	Whole Homogenate	1:1000	Monoclonal to Clone DB-955	Anti-Mouse	Sigma
VDR	12	100	Kidney	Whole Homogenate	1:500	Monoclonal to chicken intestinal VDR, Abcam	Anti-Rat	
Desmin	8	100	L.D. Muscle	Whole Homogenate	1:500	Polyclonal, Abcam	Anti-Rabbit	
MHC fast	8	3	L.D. Muscle	Whole Homogenate	1:1000	Nova Castra	Anti-Mouse	
MHC slow	8	3	L.D. Muscle	Whole Homogenate	1:1000	Nova Castra	Anti-Mouse	

* All at Dilution 1:5000

Primary Antibody	Gel %	Protein (µg)	Source	Total Protein Ab	Phosporylated Protein Ab	secondary Antibody	Source	Luminescence
4EBP1	15	5	Cell Signalling	#9452*	#9459*	Anti Rabbit *	Sigma	ECL- Advance
MAPKinase ERK 1/2	10	5	Cell Signalling	#4695*	#4370*			

* All at Dilution 1:5000

Appendix E

Semi-quantitative oligonucleotides used in Rat

Rat MHC Primers

Primers to determine MHC expression profile of rat derived cells were used from two publications; primers for the neonatal and embryonic MHC were designed by Sanchez *et al.* (2006) while the primers for the four adult MHC isoforms were designed by Jaschinski *et al.* (1998). The regions of the sequences to which each primer set was designed to are shown below with highlighted forward primer in green, reverse antisense of the reverse primer highlighted in purple.

Rat Embryonic MHC Primers

Designed by Sanchez *et al.*, (2006) towards the 5' end of the sequence, product is 181bp.

Forward: AGC AGA GGA GGC TGA GGA ACA ATC
Reverse: GGC CTC CTC AAG ATG CGT TTA CTC
[Reverse antisense: *GAG TAA ACG CAT CTT GAG GAG GCC*]

```
> K02111 Rat embryonic myosin heavy chain gene, partial 5' region, mRNA
601 tacaagagac aagcagagga ggctgaggaa caatccaacg tcaacctggc caagttccgc
661 aagctgcagc acgagctgga ggaagccgag gagcgggcag acatcgccga gtcccaggtc
721 aacaagctgc gggtgaagag ccgcgaggtt cacaccaaaa tcagtgcaga gtaaacgcat
781 cttgaggagg ccgccaagtg gctgaaggaa aggcacagaa tgtgctgcct tgggtcgctt
841 gctgggtcgc ttgcctctcg tgtttacttt tctcccactg ctgactgaat aaaaccacaa
```

Rat Neonatal MHC Primers

Designed by Sanchez *et al.*, (2006) towards the 3' end of the sequence, product is 103bp.

Forward: TGA AAG TGA GGA GTG AGC ATG TCC
Reverse: GGG GTT ACG TGG AAA TTA AGC AGG
[Reverse antisense: *CCT GCT TAA TTT CCA CGT AAC CCC*]

```
>NM_012604 Rattus norvegicus myosin, heavy polypeptide 3, skeletal muscle,
embryonic (Myh3), mRNA
5761 aatgttcatc tcaccaagtt ccggaaagcc cagcatgagc tagaggaggc cgaggaacgt
5821 gcggatattg cagaatcgca agtcaataaa ctgcgggcta aaacccggga cttcacctct
5881 agccggatgg tggtccatga aagtgaggag tgagcatgtc ctcctggtga ggggcagaag
5941 atatgcagaa tgtatgtttt cgtggctcct gaccatcctg cttaatttcc acgtaacccc
6001 tttccacatg caataaaatt tgccttgttt caagt
```

RAT MHC I (Slow)

Designed by Jaschinski *et al.*, (1998) towards the 3' end of the sequence, product is 288bp.

Sense: ACA GAG GAA GAC AGG AAG AAC CTA C
Antisense: GGG CTT CAC AGG CAT CCT TAG
[Reverse antisense: *CTA AGG ATG CCT GTG AAG CCC*]

```
> NM_017240 Rattus norvegicus myosin, heavy polypeptide 7, cardiac
muscle, beta(Myh7), mRNA
5461 ccgggtccgg gagctggaga atgagctgga ggctgagcag aagcgcaatg cggagtcggt
5521 gaagggcatg aggaagagcg agcggcgcat caaggagctc acctaccaga cagaggaaga
5581 caggaagaac ctactgcgac tgcaggacct ggtggacaag ctgcagttaa aggtgaaggc
5641 ctacaagcgc caggctgagg aggcggagga acaggccaac accaacctgt ccaagttccg
5701 caaggtgcag cacgagctgg atgaggcaga ggagagggcg gacattgccg agtcccaggt
5761 caacaagctg cgggccaaga gccgtgacat tggcgccaag ggcctgaatg aagagtagat
5821 cttgtgctac ccaaccctaa ggatgcctgt gaagccctga gacctggagc ctttgaaaca
5881 gcaccttagg cagaaacaca ataaagcaat tttccttcaa gccaa
```

RAT MHC IIA

Designed by Jaschinski *et al.*, (1998) towards the 3' end of the sequence, product is 310bp.

Sense: TAT CCT CAG GCT TCA AGA TTT G
Antisense: TAA ATA GAA TCA CAT GGG GAC A
[Reverse antisense: *T GTC CCC ATG TGA TTC TAT TTA*]

```
>L1360 Rattus norvegicus skeletal muscle myosin heavy chain mRNA, 3' end
241 gttgaggctg tcaaagggct tcgaaaacac gagaggcgag tgaaggagct tacttaccag
301 acagaagaag accgaaaaaa tatcctcagg cttcaagatt tggtggataa actccaagca
361 aaagtaaaat cttacaagag acaagctgag gaggctgagg aacaatccaa cacaaatcta
421 tccaagttcc gcaagctgca gcatgagctg gaggaagccg aggagcgggc tgacatcgcc
481 gagtcccagg tcaacaagct gcgggtgaag agccgcgagg ttcacactaa agtcataagt
541 gaagagtaag gcagctctga tgctgtagaa tgaccgaaga gaggcacaaa atgtgaagcc
601 tttggtcatg tccccatgtg attctattta atcctattgt aa
```

Rat MHC IIX

Designed by Jaschinski *et al.*, (1998) towards the 3' end of the sequence, product is 120bp.

Sense: CGC GAG GTT CAC ACC AAA
Antisense: TCC CAA AGT CGT AAG TAC AAA ATG G
[Reverse antisense: *C CAT TTT GTA CTT ACG ACT TTG GGA*]

```
>XM_213345 Rattus norvegicus type 2X myosin heavy chain (Myh1),mRNA.
6001 gagctggagg aagccgagga gcgggctgac atcgccgagt cccaggtcaa caagctgcgg
6061 gtgaagagcc gcgaggttca caccaaaatc ataagcgaag agtgatcgat ccaaagcagg
6121 aaagtgacca aagagatgag caaaatgtga agatctttgt cactccattt tgtacttacg
6181 actttgggag ataaaaaatt tatctgcca
```

RAT MHC IIB

Designed by Jaschinski *et al.*, (1998) towards the 3' end of the sequence, product is 197bp.

Sense: CTG AGG AAC AAT CCA ACG TC

Antisense: TTG TGT GAT TTC TTC TGT CAC CT

[Reverse antisense: *AG GTG ACA GAA GAA ATC ACA CAA*]

```
> BC113948 Rattus norvegicus myosin, heavy polypeptide 4, skeletal
muscle,mRNA
5701 aggacctagt ggataaatta cagactaaag tgaaagccta caagagacaa gcagaggagg
5761 ctgaggaaca atccaacgtc aacctggcca agttccgcaa gatccagcac gagctggagg
5821 aagccgagga gcgggccgac atcgccgagt cccaggtcaa caagctgcgg gtgaagagcc
5881 gagaggttca caccaaagtc ataagcgaag aatagctcaa ttccttctgt tgaaaggtga
5941 cagaagaaat cacacaatgt gacgttcttt gtcactgtcc tgtatatcaa ggaaataaaa
6001 gctgcagata attttgcaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa
```

Appendix F

Oligonucleotides used in Mouse

Mouse MHC primers

Primers and probes designed for real time PCR to mouse MHC genes were designed by da Costa *et al.* (2007), to isotype the C2C12 cell line the primers were used in semi-quantitative RT-PCR reactions and products electrophoresed in a 1.5% agarose gel. The regions of the sequences to which each primer set was deigned to are shown below with highlighted forward primer in green, reverse antisense of the reverse primer highlighted in purple, dual labelled fluorescent probe is highlighted blue.

Mouse MHC Embryonic

Sense: TCC GAC AAC GCC TAC CAG TT
Antisense: CCC GGA TTC TCC GGT GAT
[Reverse antisense: *ATC ACC GGA GAA TCC GGG*]
Probe: ATG CTG ACT GAT CGT GAG AAC CAG TCT ATC CT

```
> XM_908146 Mus musculus myosin, heavy polypeptide 3, skeletal muscle,
embryonic (Myh3), mRNA.
421 tcaaccccta caaatggctg ccagtgtaca accccgaggt ggtggacggc taccgaggca
481 aaaaacgcca ggaggctccg ccccacatct tctccatctc cgacaacgcc taccagttca
541 tgctgactga tcgtgagaac cagtctatcc tgatcaccgg agaatccggg gccgggaaga
601 cggtgaacac caagcgggtc atccagtact ttgcaacaat tgcagccact ggggaccttg
```

MOUSE MHC Neonatal

Sense: GGA GGC CAG GGT ACG TGA A
Antisense: GAG CAC ATT CTT GCG GTC TTC
[Reverse antisense: *GAA GAC CGC AAG AAT GTG CTC*]
Probe: AGG AAC TTA CCT ACC AGA CTG

```
> NM_177369 Mus musculus myosin, heavy polypeptide 8, skeletal muscle,
perinatal (Myh8), mRNA.
5581 tctggacgag gctgagcagc tggcgctgaa gggcggcaag aagcagatcc agaaactgga
5641 ggccagggta cgtgaacttg aaggagaggt cgaaaatgaa cagaaacgca atgctgaggc
5701 tgttaaaggg ttaaggaagc atgaaagaag agtaaaggaa cttacctacc agactgagga
5761 agaccgcaag aatgtgctcc ggctgcagga cctggtggac aaattacagg cgaaggtgaa
5821 atcctacaag agacaggctg aggaggctga ggaacaatcc aatgccaacc tggccaagtt
```

MOUSE MHC I (Slow)

Sense: GCC TGG GCT TAC CTC TCT ATC AC
Antisense: CTT CTC AGA CTT CCG CAG GAA
[Reverse antisense: *TTCCTGCGGAAGTCTGAGAAG*]
Probe: CGT TTG AGA ATC CAA GGC TCA

```
> AY056464 Mus musculus beta myosin heavy chain mRNA, complete
cds.

1   tgagcattct cctgctgttt ccttacttgc taccctcagg tggctccgag aaaggaagcc
61  tcagcagagg agtacagctc tcctacaggc ctgggcttac ctctctatca ctagacacgt
121 ttgagaatcc aaggctcagc catggcggat gcagagatgg ctgcatttgg ggctgcagcc
181 ccctttcctgc ggaagtctga gaaggagagg ctggaggcac agaccaggcc ctttgacctc
```

MOUSE MHC IIA

Sense: CAG CTG CAC CTT CTC GTT TG
Antisense: CCC GAA AAC GGC CAT CT
[Reverse antisense: *AG ATG GCC GTT TTC GGG*]
Probe: TGA GTT CAG CAG TCA TGA G

```
> NM_001039545 Mus musculus myosin, heavy polypeptide 2, skeletal
muscle, adult.
1   tagctagcca tataaaagag tcccgaacga ggctgactcg tcctgcttta aaaagctcca
61  aggaccctct tatttcccag ctgcaccttc tcgtttgcca gtaagggtct gtgagttcag
121 cagtcatgag ctccgacgcc gagatggccg ttttcgggga ggctgccccc tacctccgga
```

MOUSE MHC IIX

Sense: GGA CCC ACG GTC GAA GTT G
Antisense: GGC TGC GGG CTA TTG GTT
[Reverse antisense: *AACCAATAGCCCGCAGCC*]
Probe: CTA AAG GCA GGC TCT CTC ACT GGG CTG

```
> NM_030679 Mus musculus myosin, heavy polypeptide 1, skeletal
muscle, adult
1   aaagcttcaa gtttggaccc acggtcgaag ttgcatccct aaaggcaggc tctctcactg
61  ggctgcaacc aatagcccgc agccatgagt tccgacgccg agatggccgt tttcggggag
121 gctgctcctt acctccggaa gtctgaaaag gagcgaatcg aggctcagaa caagcctttt
```

MOUSE MHC IIB

Sense: CAA TCA GGA ACC TTC GGA ACA C
Antisense: GTC CTG GCC TCT GAG AGC AT
[Reverse antisense : *ATGCTCTCAGAGGCCAGGAC*]
Probe: TGC TGA AGG ACA CAC AGC TGC ACC T

```
> NM_010855 Mus musculus myosin, heavy polypeptide 4, skeletal muscle
(Myh4)
4921 agcaggaatg atgctctgag gattaagaag aagatggagg gagacctcaa tgagatggag
4981 atccagctga accatgccaa ccgccaggct gcggaggcaa tcaggaacct tcggaacacc
5041 cagggaatgc tgaaggacac acagctgcac ctggacgatg ctctcagagg ccaggacgac
5101 ctgaaagagc agctggccat ggttgagcgc agagccaacc tgatgcaggc tgagatcgag
```

Mouse real-time RT-PCR Targets

In addition to the real-time RT-PCR primers and probes designed to the MHC genes, primers and probes were required for signaling factors and markers of differentiation designed to mouse sequences. Primers and probes to the myogenic regulatory factor Myf5 and the internal control β-actin were designed by da Costa *et al*. (2007). Primers and probes to measure desmin as a marker for the presence of differentiated muscle cells were designed using Primer Express V. 1.5. A fragment of sequence 30 base pairs of nucleotides either side the Desmin product was BLAST searched to detect any homogeneity with other sequences. The search returned only mouse desmin fragments and it was concluded that the primers and probes were specific to the sequence and were used in real time qRT-PCR analysis.

Mouse cell culture targets

Target Protein	Sequence	Primers designed by	Product Size
myf5	X56182	da Costa *et al*., 2007	117
β-Actin	NM_007393	da Costa et al., 2007	72
Desmin	BC031760	Primer Express V. 1.5	101

Mouse Myf5

Sense: CAG CCC CAC CTC CAA CTG
Antisense: GCA GCA CAT GCA TTT GAT ACA TC
[Reverse antisense: *GATGTATCAAATGCATGTGCTGC*]
Probe: TGT CTG GTC CCG AAA GAA CAG CAG CTT

```
> X56182 M.musculus myf-5 mRNA.
421 caggtggaga actattacag cctgccggga cagagctgct ctgagcccac cagccccacc
481 tccaactgct ctgacggcat gcctgaatgt aacagccctg tctggtcccg aaagaacagc
541 agctttgaca gcatctactg tcctgatgta tcaaatgcat gtgctgcaga taaaagctcc
601 gtgtccagct tggattgctt gtccagcatt gtggatcgga tcacgtctac agagccatct
```

Mouse β-actin

Sense: CGT GAA AAG ATG ACC CAG ATC A
Antisense: CAC AGC CTG GAT GGC TAC GT
[Reverse antisense: *ACG TAG CCA TCC AGG CTG TG*]
Probe: TTG AGA CCT TCA ACA CCC CAG CCA TG

```
> NM_007393 Mus musculus actin, beta (Actb), mRNA.
361 gcgtgtggcc cctgaggagc accctgtgct gctcaccgag gccccctga accctaaggc
421 caaccgtgaa aagatgaccc agatcatgtt tgagaccttc aacaccccag ccatgtacgt
481 agccatccag gctgtgctgt ccctgtatgc ctctggtcgt accacaggca ttgtgatgga
541 ctccggagac ggggtcaccc acactgtgcc catctacgag ggctatgctc tccctcacgc
```

Mouse Desmin

Sense: GCG CAG AAT CGA ATC CCT C
Antisense: GGA CCT GCT GTT CCT GAA GC
[Reverse antisense: *GC TTC AGG AAC AGC AGG TCC*]
Probe: CGC GTT CCT TAA GAA AGT GCA TGA AGA GG

```
> BC031760 Mus musculus desmin, mRNA
661 aaatccaact aagagaagaa gcagagaaca acttggctgc cttccgagcg gatgtggatg
721 cagccactct agctcgtatt gacctggagc gcagaatcga atccctcaac gaggagatcg
781 cgttccttaa gaaagtgcat gaagaggaga tccgtgagct tcaggcccag cttcaggaac
841 agcaggtcca ggtggagatg gacatgtcca agccggacct cacagctgcc ctcagggaca
```

This product was nucleotide BLAST searched against the Mouse Genomic + Transcript database and returned one mRNA transcript sequence, NM_010043.1 with 100% similarity (E value: $8e\text{-}10^{6}$), this was a mouse desmin mRNA sequence. No other mRNA sequences were recovered with this product and so it was deemed specific to desmin in mouse.

Appendix G

"Assay on Demand" Oligonucleotides (ABI)

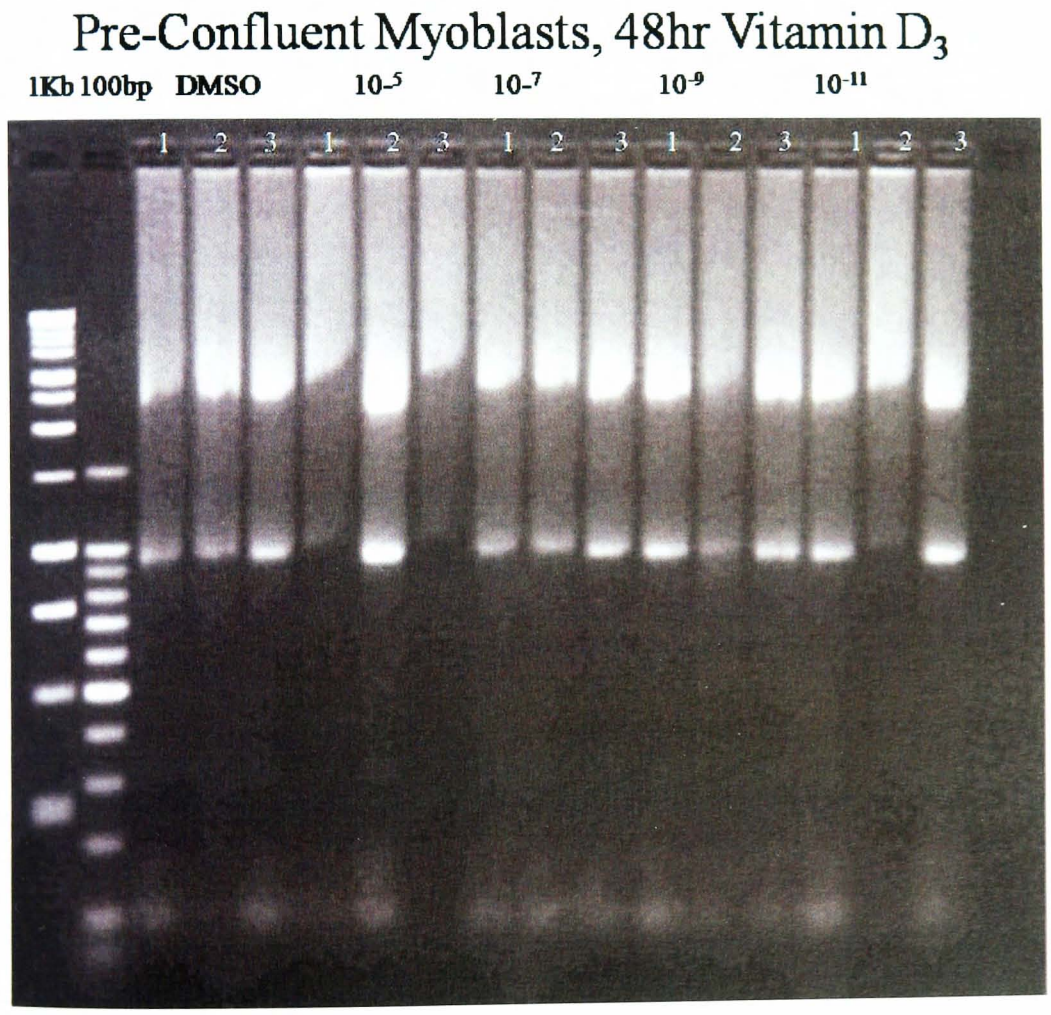
Assays on demand used on C2C12 real time PCR analysis. All assays were purchased from Applied Biosystems and were mouse specific.

Target Gene	Gene Name	Assay ID	Category	Gene Group
Cyclophilin A	peptidylprolyl isomerase A	Mm02342430_g1	Isomerase	Other isomerase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Mm03302249_g1	Oxidoreductase	Dehydrogenase
Myogenin	myogenin	Mm00446195_g1	Transcription factor	Basic helix-loop-helix transcription factor
C/EBPβ	CCAAT/enhancer binding protein (C/EBP), beta	Mm00843434_s1	Transcription factor	Other transcription factor
c-Myc	myelocytomatosis oncogene	Mm00487804_m1	Transcription factor	Basic helix-loop-helix transcription factor
Rbp4	Retinol Binding Protein 4	Mm00803266_m1	Transfer/carrier protein	Other- transfer/carrier protein
Metallothioenin 2A	metallothionein 2	Mm00809556_s1	Miscellaneous function	Other miscellaneous function protein
Parvalbumin	parvalbumin	Mm00443100_m1	Select calcium binding protein	Calmodulin related protein

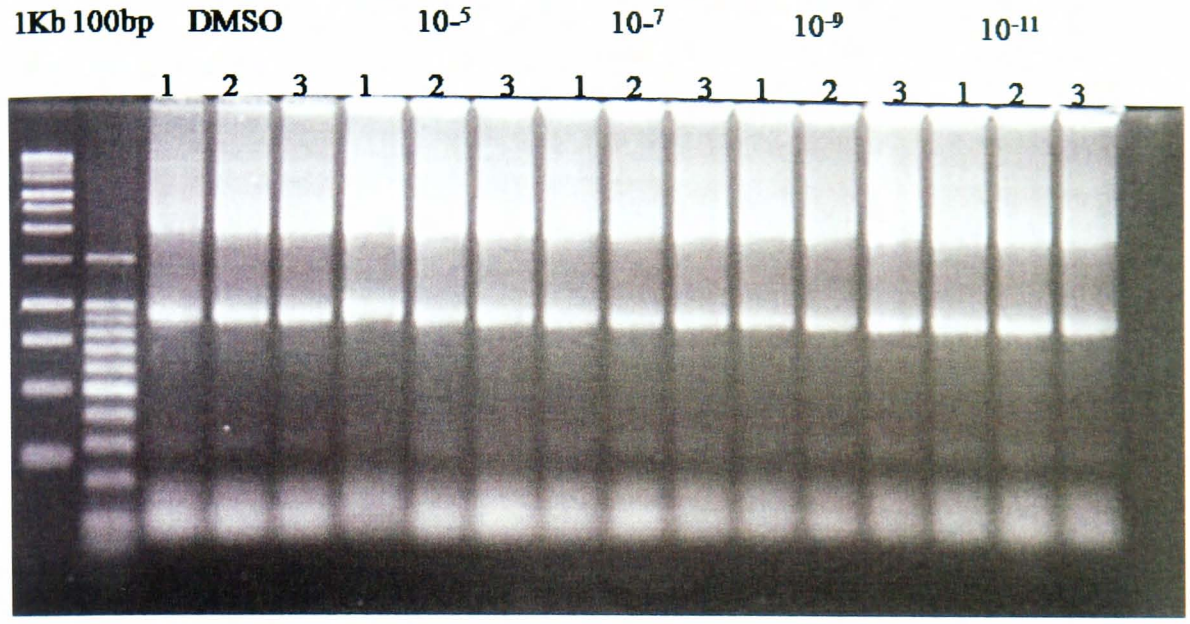
Appendix H

C2C12 RNA agarose gel electrophoresis

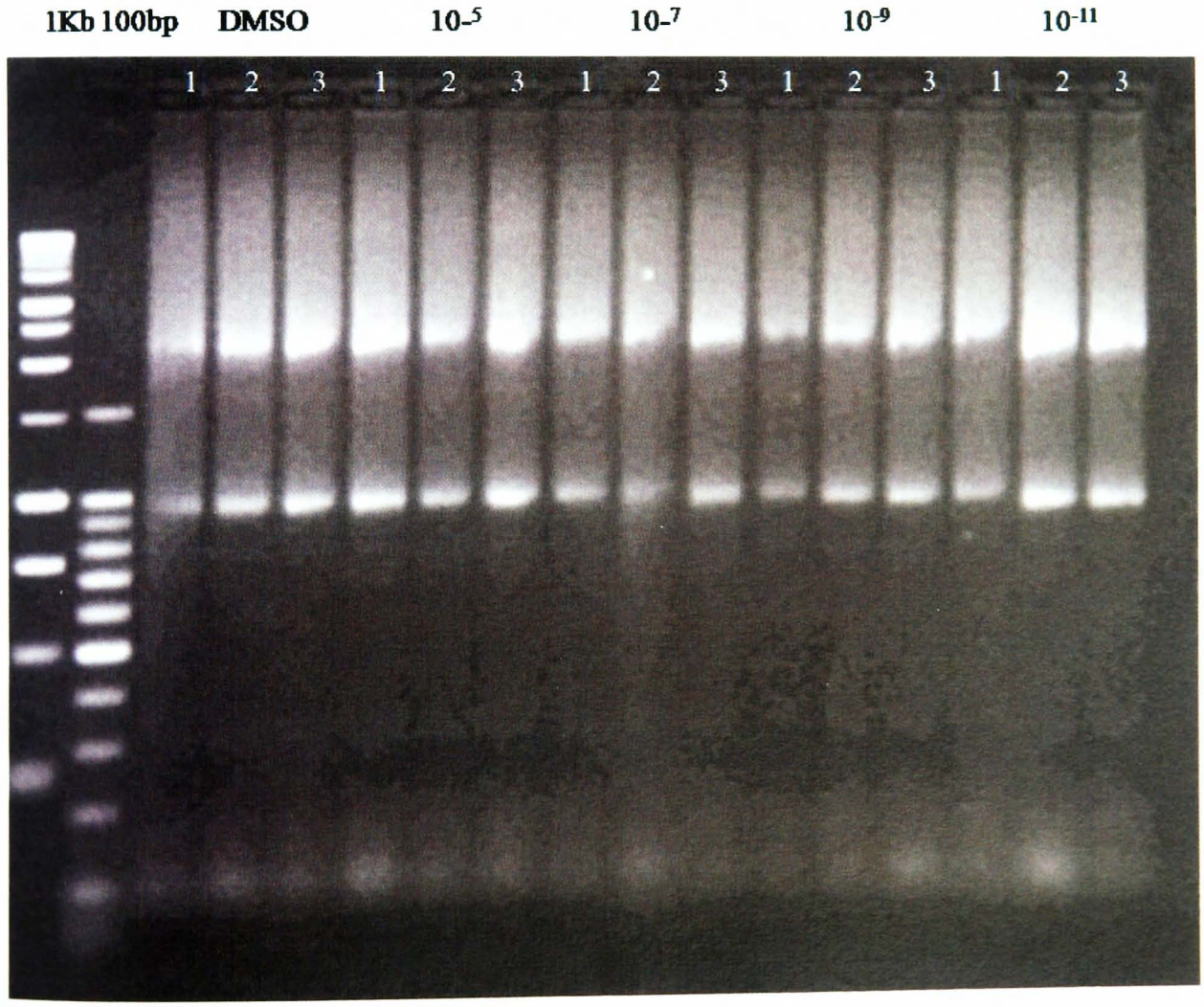
The preparation of C2C12 RNA was evaluated by both Nanodrop spectrometer and by visual assessment after agarose gel electrophoresis. The mRNA was diluted to a constant concentration of 0.1µg/µl before 0.5µg was loaded onto a 1.5% agarose gel and separated by electrophoresis and imaged using ethidium bromide stain and GelDoc software and equipment (Biorad, Hemel Hempstead, UK). These images of the mRNA provide evidence to show that all mRNA was intact for both 28s and 18s rRNA and for tRNA and would therefore provide a high quality template for cDNA synthesis without the need to normalise to a housekeeping gene at real-time RT-PCR level.



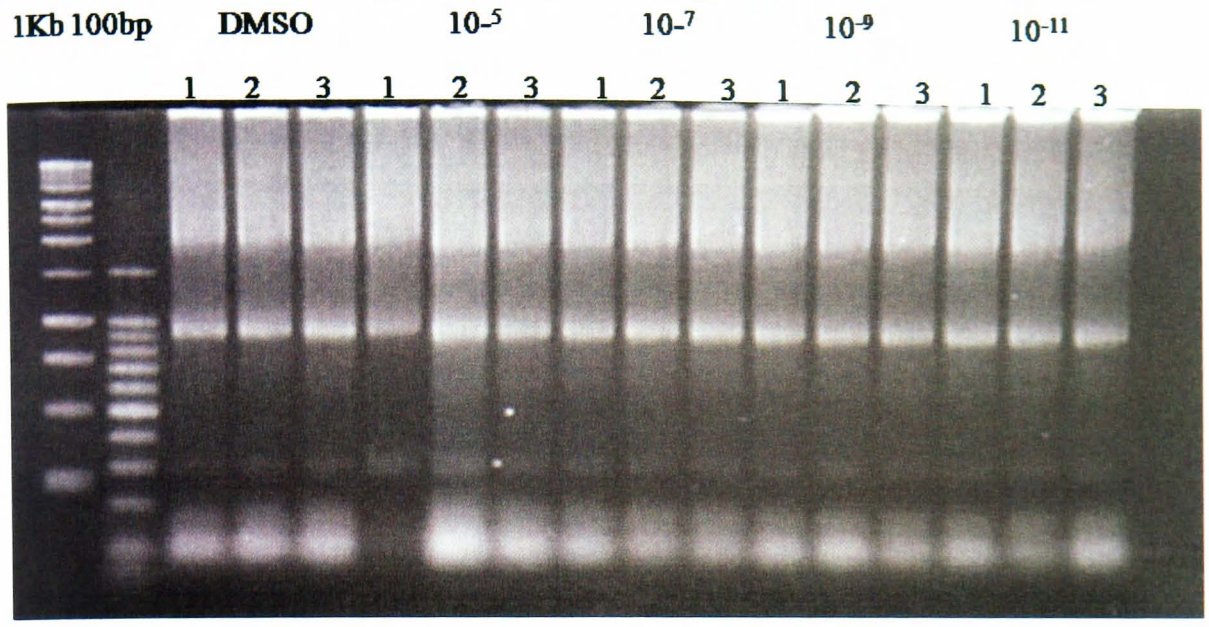
Day 0 C2C12 Myoblasts



Day 3 C2C12 Differentiating Myoblasts



Day 7 C2C12 Myotubes



Appendix I

Trial 2 Feed Refusal Data

Feed refusal data for vitamin D plus calcium fed animals in trial 2 and the effects of feed refusals on plasma vitamin D_3, serum calcium, pH, weight at slaughter (liveweight) and carcass weight. Total feed refused is in g, over the course of the week 9800g was presented to all animals.

Vitamin D animals	SLG	Refusal vit d only diet (g)	Refusal Ca diet (g)	Total Refused g
511	1	0	0	**0**
308	1	0	0	**0**
158	1	0	0	**0**
281	1	0	0	**0**
455	1	541	1384.7	1925.7
145	1	0	211.8	211.8
296	2	0	706.2	706.2
228	2	0	147.3	147.3
141	2	0	88.9	88.9
232	2	0	0	0
144	2	0	0	0
215	2	1149.8	1115.3	2265.1
108	3	0	1798.4	1798.4
339	3	0	0	0
212	3	764.2	1554	2318.2
522	3	0	321.9	321.9‡
213	3	1998.9	985.5	2984.4*
309	3	0	0	0

Vitamin D + Calcium Animals	Plasma D_3 (ng/ml)	Serum Calcium (mg/L)	pH 45 mins	pH 24Hrs	Liveweight	Carcass Wt
Ate all feed (n=8)	**1202**	**145.3**	**6.493**	**5.543**	**49.21**	**25.12**
Range (min-max)	1045.7 to 1430.8	132.95 to 164.4			45.3 to 51.0	
Refused feed (n=10)	**1252**	**140.6**	**6.436**	**5.483**	**47.44**	**25.36**
Range (min-max)	728.4‡ to 1428.8	126.2 to 159.7			45.2 to 49.9	
S.E.D.	113.7	5.596	0.122	0.0315	0.784	0.44
P	0.664	0.411	0.646	**0.091**	**0.038**	0.603

Animal with lowest plasma vitamin D_3 (728.4ng/ml) did not have the largest feed refusal (321.9g), denoted with ‡. Animal with highest feed refusal, denoted with *, had plasma vitamin D_3 level of 1152.2ng/ml and serum calcium concentration of 148.25 mg/L.

Appendix J

Real-time PCR data of C2C12 myoblasts

mRNA extracted from C2C12 cells was quantified using Nanodrop spectrometer prior to dilution to constant concentration before samples were visually checked for integrity using agarose gel electrophoresis. mRNA expression data obtained for all genes measured by qRT-PCR in C2C12 cells across differentiation analysed by two-way ANOVA, results presented in section 2.6:

Days post differentiation	2				5				9				S.E.D.			ANOVA p value		
Gene \ Treatment	DMSO	10-11	10-9	10-7	DMSO	10-11	10-9	10-7	DMSO	10-11	10-9	10-7	Age	Treatment	A*T	Age	Treatment	A*T
Cyclophilin A (LD)	0.0687	0.0943	0.0555	0.0828	0.0813	0.1006	0.0953	0.0784	0.0508	0.0568	0.0232	0.0422	0.01165	0.01345	0.02329	0.002*	0.302	0.808
Cyclophilin A (Day 10)	0.0413	0.0591	0.0329	0.0511	0.0498	0.0628	0.0592	0.0479	0.0298	0.0341	0.0128	0.0248	0.00763	0.00881	0.01527	0.002*	0.294	0.81
GAPDH (LD)	0.01286	0.01484	0.0111	0.01193	0.01343	0.01498	0.01263	0.01236	0.01178	0.01051	0.00786	0.00927	0.001351	0.00156	0.002702	0.038*	0.245	0.975
GAPDH (Day 10)	0.043	0.0541	0.0347	0.0389	0.046	0.0537	0.0419	0.0408	0.0382	0.0325	0.0213	0.0284	0.00615	0.0071	0.0123	0.044*	0.218	0.966
Beta Actin (LD)	0.2038	0.2094	0.18	0.2047	0.1847	0.198	0.1734	0.1487	0.1279	0.1404	0.1128	0.1187	0.00613	0.00708	0.01226	<.001*	0.002*	0.158
Beta Actin (Day 10)	0.1528	0.1564	0.1371	0.1533	0.1404	0.1491	0.133	0.1166	0.1024	0.1112	0.092	0.0959	0.00404	0.00466	0.00807	<.001*	0.002*	0.156

Days post differentiation	2				5				9				S.E.D.			ANOVA p value		
Gene	DMSO	10-11	10-9	10-7	DMSO	10-11	10-9	10-7	DMSO	10-11	10-9	10-7	Age	Treatment	A*T	Age	Treatment	A*T
Desmin	0.4988	0.4738	0.3922	0.4704	0.4692	0.4652	0.45	0.456	0.477	0.4806	0.3982	0.4912	0.01877	0.02167	0.03753	0.988	0.016*	0.607
Myogenin	1.724	2.188	1.342	1.553	1.46	1.469	1.592	1.547	0.863	0.928	0.479	0.757	0.1838	0.2122	0.3676	<.001*	0.345	0.654
Myf 5	0.0289	0.0339	0.0258	0.0316	0.0467	0.0626	0.052	0.049	0.0381	0.0542	0.0343	0.0535	0.00542	0.00626	0.01084	0.001*	0.154	0.819
Metallothionein 2A	0.0811	0.0323	0.0176	0.0213	0.0196	0.0149	0.0309	0.0183	0.0098	0.0171	0.0047	0.0152	0.00642	0.00742	0.01284	0.001*	0.054	0.005*
Parvalbumin	0.01	0.021	0.0066	0.0114	0.0156	0.023	0.0215	0.0188	0.0165	0.0194	0.0102	0.034	0.00347	0.00401	0.00694	0.058	0.076	0.138
C/EBPβ	0.08	0.331	0.218	0.274	0.051	0.398	0.289	0.374	0.035	0.295	0.082	0.17	0.0557	0.0643	0.1113	0.077	0.001*	0.889
c-Myc	0.0709	0.0781	0.0624	0.0642	0.1077	0.1311	0.0736	0.081	0.0455	0.0405	0.0399	0.0337	0.00919	0.01061	0.01838	<.001*	0.08	0.45

Days post differentiation	2				5				9				S.E.D.			ANOVA p value		
Gene \ Treatment	DMSO	10-11	10-9	10-7	DMSO	10-11	10-9	10-7	DMSO	10-11	10-9	10-7	Age	Treatment	A*T	Age	Treatment	A*T
Embryonic MHC	0.1993	0.1896	0.165	0.1962	0.1744	0.1577	0.1622	0.1827	0.128	0.1153	0.1029	0.1297	0.0057	0.00658	0.0114	<.001*	0.002*	0.7
MHC I (slow)	0.402	0.3388	0.2919	0.3828	0.2356	0.2202	0.2428	0.3499	0.2	0.1498	0.2067	0.2232	0.02434	0.0281	0.04867	<.001*	0.032*	0.347
MHC IIA	0.00427	0.00453	0.0025	0.00339	0.00458	0.00498	0.00608	0.00737	0.02588	0.0221	0.02371	0.02788	0.00111	0.001282	0.00222	<.001*	0.275	0.39
MHC IIX	0.0481	0.0511	0.0444	0.0564	0.0568	0.0577	0.0564	0.0664	0.096	0.0958	0.0818	0.1079	0.00629	0.00726	0.01257	<.001*	0.204	0.974
MHX IIB	0.00192	0.00181	0.0015	0.00216	0.00298	0.0025	0.00286	0.00216	0.00579	0.00547	0.00447	0.00625	0.00036	0.000419	0.00073	<.001*	0.431	0.432

Appendix K

Average Housekeeping gene values for real-time PCR data of C2C12 myoblasts across differentiation

Analysis of Average Housekeeping values in timecourse experiment																		
Days Post Differentiation	**2**				**5**				**9**				**S.E.D.**			**ANOVA P Value**		
	DMSO	**10-11**	**10-9**	**10-7**	**DMSO**	**10-11**	**10-9**	**10-7**	**DMSO**	**10-11**	**10-9**	**10-7**	**Age**	**Treatment**	**A* T**	**Age**	**Treatment**	**A* T**
Average of all three Housekeeping genes																		
LD Standard Curve	0.095	0.106	0.082	0.1	0.093	0.1045	0.094	0.08	0.064	0.0693	0.048	0.057	0.00583	0.00673	0.01166	**<.001**	**0.058**	0.652
Day 10 Standard Curve	0.079	0.09	0.068	0.081	0.079	0.0885	0.078	0.068	0.057	0.0593	0.042	0.05	0.00552	0.00637	0.01103	**<.001**	**0.083**	0.843
Average GAPDH and Cyclophilin A																		
LD Standard Curve	0.041	0.055	0.033	0.047	0.047	0.0578	0.054	0.045	0.031	0.0337	0.016	0.026	0.00646	0.00746	0.01293	**0.003**	0.303	0.843
Day 10 Standard Curve	0.042	0.057	0.034	0.045	0.048	0.0582	0.051	0.044	0.034	0.0333	0.017	0.027	0.0068	0.00786	0.01361	**0.008**	0.274	0.925

Appendix L

Protein data of C2C12 myoblasts across differentiation

Level of phosphorylation of two protein targets in C2C12 cells treated with 1α(OH)D at three time points post differentiation, data presented in section 3.6.11.

Days Post Differentiation	Day 0				Day 3				Day 7				S.E.D.			P		
Protein / 1α(OH)D Dose	DMSO	10-11	10-9	10-7	DMSO	10-11	10-9	10-7	DMSO	10-11	10-9	10-7	Age	Treatment	A*T	Age	Treatment	A*T
ERK 1/2Phosphorylation	0.787	0.617	0.498	0.723	1.533	0.670	0.577	1.063	1.178	0.683	0.679	0.578	0.0469	0.0541	0.0937	**0.016**	**<.001**	**0.007**
4EBP1 Phosphorylation	0.827	0.902	0.847	0.802	0.841	0.757	0.710	0.722	0.730	0.761	0.815	0.693	0.0317	0.0366	0.0635	**0.019**	0.852	0.428

References

Alzuherri H. & Chang K.C. 2003. Calcineurin activates NF-κB in skeletal muscle C2C12 cells. Cell. Sign. 15: 471-478.

Aniansson A., Hedberg M., Henning G.B. & Grimby G. 1986. Muscle morphology, enzymatic activity, and muscle strength in elderly men: a follow up study. Mus. & Nerve. 9: 585-591.

Antrobus S.D., Siaw E.K.O. & Walters M.R. 1995. Protacted 1,25-dihydroxyvitamin D treatment stimulates multiple calmodulin binding proteins in rat kidney. Endocrinology 136: 1516-1522.

Apostovla M.D., Ivanova I.A. & Cherian M.G. 1999. Metallothionein and apoptosis during differentiation of myoblasts to myotubes: Protection against free radical toxicity. Toxicol. Appl. Pharmacol. 159: 174-184.

Arany Z., Lebrasseur N., Morris C., Smith E., Yang W., Ma Y., Chin S. & Spiegelman B.M. 2007. The transcriptional coactivator PGC-1β drives the formation of oxidative type IIx fibres in skeletal muscle. Cell. Metab. 5: 35-46.

Ariano M.A., Edgerton V.R. & Armstrong R.B. 1973. Hindlimb muscle fibre populations of five animals. J. Histochem. Cytochem. 21: 51-55.

Armstrong R.B., Saubert IV C.W., Seeherman H.J. & Taylor C.R. 1982. Distribution of fiber types in locomotory muscles of dogs. J. Am. Anat. 163: 87-98.

Armstrong R.B. & R.O. Phelps. 1984. Muscle fiber type composition of the rat hindlimb. Am. J. Anat. 171: 259-272.

Arthur J.S.C., Elce J.S., Hegadorn C., Williams K. & Greer P.A. 2000. Disruption of the murine calpain small subunit gene, *capn4*: calpain is essential for embryonic development but not for cell growth and division. Moll. Cell Biol. 20: 4478-4481.

Au Y. 2004. The muscle ultrastructure: a structural perspective of the sarcomere. Cell. Moll. Life Sci. 61: 3016-3033.

Azam M., Andrabi S.S., Sahr K.E., Kamath L., Kuliopulos A. & Chishti A.H. 2001. Disruption of the Mouse μ-Calpain Gene Reveals an Essential Role in Platelet Function. Moll. Cell. Biol. 21: 2213-2220.

Bandman E. & Zdanis D. 1988. An immunological method to assess protein degradation in post-mortem muscle. Meat Sci. 22: 1-19.

Bardsley R.G., Allcock S.M.J., Dawson J.M., Dumelow J.M., Higgins T.A., Lasslet Y.V., Lockley A.K., Parr T. & Buttery P.J. 1992. Effect of β-agonists on expression of calpain and calpastatin activity in skeletal muscle. Biochimie 74: 267-273.

Beerman D.H. 2002. Beta-adrenergic receptor agonist modulation of skeletal muscle growth. J. Anim. Sci. 80: E18-E23.

Benezra R.,Davis R.L., Lockshon D., Turner D.L. & Weintraub H. 1990.The protein Id: A negative regulator of helix-loop-helix DNA binding proteins. Cell 61: 49-59.

Berkes C. A. & Tapscott S. J. 2005. MyoD and the transcriptional control of myogenesis. Sem. Cell. Dev. Biol. 16: 585-595.

Berry D.M. & Meckling-Gill K.A. 1999. Vitamin D analogs, 20-Epi-22-Oxa-24a,26a,27a,-Trihomo-1α,25$(OH)_2$-Vitamin D_3, 1,24$(OH)_2$-22-Ene-24-Cyclopropyl-Vitamin D_3 and 1α,25$(OH)_2$-Lumisterol$_3$ prime NB4 Leukemia cells for monocytic differentiation via non-genomic signalling pathways, involving calcium and calpain. Endocrinology 140: 4779-4788.

Bindon B.M. & Jones N.M. 2001. Cattle supply, production systems and markets for Australian beef. Aus. J. of Exp. Agri. 41: 861 – 877.

Birchmeier C. & Brohmann H. 2000. Genes that control the development of migrating muscle precursor cells 12: 725-730.

Birge S.J. & Haddad J.G. 1975. 25-Hydroxycholecalciferol stimulation of muscle metabolism. J. Clin. Invest. 56: 1100-1107.

Bischoff H.A., Stähelin H.B., Dick W., Akos W., Knecht M., Salis C., Nebiker M., Theiler R., Pfeifer M., Begerow B., Lew R.A. & Conzelmann M. 2003. Effects of vitamin D and calcium supplementation on falls: a randomized control trial J. Bone Miner. Res. 18: 343-351.

Bischoff-Ferrari H.A., Orav J.E. & Dawson-Hughes B. 2006. Effect of cholecalciferol plus calcium on falling in ambulatory older men and women. Arch. Intern. Med. 166: 424-430.

Boleman C.T., McKenna D.R., Ramsey W.S., Peel R.K. & Savell J.W. 2004. Influence of feeding vitamin D_3 and aging on the tenderness of four lamb muscles. Meat Sci. 67: 185-190.

Boleman S.J., Miller R.K., Taylor J.F., Cross H.R., Wheeler T.L., Koohmaraie M., Shackelford S.D., Miller M.F., West R.L., Johnson D.D. & Savell J.W. 1997. Consumer evaluation of beef of known categories of tenderness. J. Anim. Sci. 75: 1521 – 1524.

Bonjour J.P., Preston C. & Fleisch H. 1977. Effect of 1,25-Dihydroxyvitamin D_3 on the renal handling of Pi in thyroparathyroidectomized rats. J. Clin. Invest. 60:1419-1428.

Braun T., Rudnicki M.A., Arnold H.H. & Jaenisch R. 1992. Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. Cell 71: 369-382.

Brown A.J., Dusso A. & Slatopolsky E. 1999. Vitamin D. Am. J Phsyiol. 277: (Renal Physiol 46:) F157-175.

Buckingham M. 1992. Making muscle in mammals. Trends Genet. 8: 144-149.

Buckingham M. 2001. Skeletal muscle formation in vertebrates. Curr. Opin. Genet. Dev. 11: 440-448.

Buckingham M., Bajard L., Chang T., Daubas P., Hadchouel J., Meilhac S., Montarras D., Rocancourt D. & Relaix F. 2003. The formation of skeletal muscle: from somite to limb.J. Anat. 202: 59–68.

Buitrago C.G. & Boland R.L. 2008. Calcitriol induces PKC/PTPα/c-Src activation upstream to ERK 1/2 and p38 MAPK phosphorylation in skeletal muscles. J. Bone 43: S133.

Buitrago C.G., Ronda A.C., De Boland A.R. & Boland R. 2006. MAP kinases p38 and JNK are activated by the steroid hormone 1α,25$(OH)_2$-vitamin D_3 in the C2C12 cell line. J. Cell. Biochem. 97: 698-708.

Buitrago C., Vazquez G., De Boland A.R. & Boland R. 2001. The vitamin D receptor mediates rapid phosphorylation changes in muscle protein tyrosine phosphorylation induced by 1,25$(OH)_2D_3$. Biochem. Biophys. Res. Comms. 289: 1150-1156.

Cabane C., Englaro W., Yeow K., Ragno M. & Dérijard B. 2003. Regulation of C2C12 myogenic terminal differentiation by MKK3/P38 pathway. Am. J. Cell Physiol. 284: C658-666.

Capiati D., Benassati S. & De Boland R. 2002. 1,25(OH)2-vitamin D_3 induces translocation of the vitamin D receptor (VDR) to the plasma membrane in skeletal muscle cells. J. Cell. Biochem. 86: 128-135.

Carlberg C., Bendike I., Wyss A., Meier E., Sturzenbecker L.J., Grippo J.F. & Hunziker W. 1993. Two signalling pathways for vitamin D. Nat. 361: 657-660.

Chang K.C. 2007. Key signalling factors and pathways in the molecular determination of skeletal muscle phenotype. Animal. 1: 681-698.

Chin E.R., Olson E.N., Richardson J.A., Tang Q., Humphries C., Shelton J.M., Wu H., Zhu W., Bassel-

Duby R. & Williams R.S. 1998. A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. Genes Dev.12: 2499-2509.

Chin E.R. 2005. Role of Ca^{2+}/calmodulin-dependant kinases in skeletal muscle plasticity. J. Appl. Physiol. 99: 414-423.

Chikuni E., Muroya A. & Nakajima I. 2004. Absence of the functional myosin heavy chain 2b isoforms in equine skeletal muscles. Zool. Sci. 21: 589-596.

Cho Y.M., Choi H., Hwang I.H., Kim Y.K. & Myung K.H. 2006. Effects of 25-hydroxyvitamin D_3 and manipulated dietary cation-anion difference on the tenderness of beef from cull native Korean cows. J. Anim. Sci. 84: 1481-1488.

Christakos S., Barletta F., Heuning M., Dhawan P., Lui Y., Porta A. & Peng X. 2003. Vitamin D target proteins: Function and regulation. J. Cell. Biochem. 88: 238-244.

Clare T.L., Jackson S.P., Miller M.F., Elliot C.T. & Ramsey C.B. 1997. Improving tenderness of normal and callipyge lambs with calcium chloride.J. Anim. Sci. 75: 377-385.

Clément K., Viguerie N., Diehn M., Alizadeh A., Barbe P., Thalamas C., Story J.D., Brown P.O., Barsh G.S. & Languin D. 2002. In vivo regulation of human skeletal muscle gene expression by thyroid hormone. Genome Res. 12: 281-291.

Coolican S.A., Samuel D.S., Ewton D.Z., McWade F.J. & Florini J.R. 2997. The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signalling pathways. J. Biol. Chem. 272: 6653-6662.

Cooper G.M. & Hausman R.E. 2007. The Cell: A molecular Approach. 4th Edition ASM Press Washington, D.C. U.S.A.

Cossu G., Tajbakhsh S. & Buckingham M. 1996. How is myogenesis inititated in the embryo? Trends Genet. 12: 218-223.

Curry O.B., Basten J.F., Francis M.J.O. & Smith R. 1974. Calcium uptake by sarcoplasmic reticulum of muscle from vitamin D-deficient rabbits. Nature. 249: 83-84.

Da Costa N., Edgar J., Ooi P.T., Su Y., Meissner J.D. & Chang K.C. 2007. Calcineurin differentially regulates fast myosin heavy chain genes in oxidative muscle fibre type conversion. Cell Tissue Res. 329: 515-527.

Dayton W.R., Goll D.E., Zeece M.G., Robson R.M. & Reveille W.J. 1976. A Ca^{2+} activated protease possibly involved in myofibrillar protein turnover. Purification from porcine muscle. Biochem. 15: 2150-2158.

De Arcangelis V., Coletti D., Canato M., Molinaro M., Adamo S., Reggianni C. & Naro F. 2005. Hypertrophy and transcriptional regulation induced in myogenic cell line L6-C5 by an increase of extracellular calcium. J. Cell. Phys. 202: 787-795.

De Haes P., Garmyn M., carmeliet G., Degreef H., Vantieghem K., Bouillon R. & Segaert S. 2004. Molecular pathways involved in the anti-apoptotic effect of 1,25-dihydroxyvitamin D_3 in primary human keratinocytes. J. Cell. Biochem. 93: 951-967.

De Macedo R.M.G., Pai-Silva M.D., Pai V.D., De Macedo F.A.F. & Martins E.N. 2000. Morphology and histochemistry of muscle fibre types of skeletal muscle tissue of lambs during growth. Acta. Sci. 22: 581-585.

De Viragh P.A., Haglid K.G. & Celio M.R. 1989. Parvalbumin increases in the caudate putamen of rats with vitamin D hypervitaminosis. Proc. Natl. Acad. Sci. 86: 3887-3890.

Deeb K.K., Trump D.L. & Johnson C.S. 2007. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nat. Rev. 7:684-700.

Dedieu S., Mazères G., Cottin P. & Brustis J.J. 2002. Involvement of myogenic regulator factors during fusion in cell line C2C12. Int. J. Dev. Biol. 46: 235-241.

Delling U., Tureckova J., Lim H.W., De Windt L.J., Rotwein P. & Molkentin J.D. 2000. A calcineurin-NFATc3-dependant pathway regulates skeletal muscle differentiation and slow myosin heavy chain expression. 20: 6600-6611.

Dransfield E. 1994. Optimisation of tenderization, aging and tenderness. Meat Sci. 36:105-121.

Drittanti L., De Boland A.R. & Boland R. 1989. Modulation of DNA synthesis in cultured muscle cells by 1,25-dihydroxyvitamin D_3. Biochim. Biophys. Act. 1014: 112-119.

Duckett S.K., Klein T.A., Dodson M.V. & Snowder G.D. 1998. Tenderness of normal and callipyge lamb aged fresh or after freezing. Meat Sci. 49: 19-26.

Duckett S.K., Snowder G.D. & Cockett N.E. 2000. Effect of the callipyge gene on muscle growth, calpastatin activity, and tenderness of three muscles across the growth curve. J. Anim. Sci. 78: 2836-2841.

Dunnet C.W. 1964. New tables for multiple comparisons with a control. Biometrics. 20: 482-491.

Eastwood J.B., Stamp T.C.B., DeWardner H.E., Bordier P.J. & Arnaud C.P. 1977. The effect of 25-hydroxyvitamin D_3 in the osteomalacia of chronic renal failure. Clin. Sci. Mol. Med. 52: 499–508.

Edmondson D.G. & Olson E.N. 1993. Helix-loop-helix proteins as regulators of muscle-specific transcription. J. Biol. Chem. 268: 755-758.

Endo I., Inoue D., Mitsui T., Umaki Y., Akaike M., Yoshizawa T., Kato S & Matsumoto T. 2003. Deletion of vitamin D receptor gene in mice results in abnormal skeletal muscle development with deregulated expression of myoregulatory transcription factors. Endocrinology 144: 5138-5144.

Finck B.N., Bernal-Mizrachi C., Han D.H., Coleman T., Sambandam N., LaRiviere L.L., Holloszy J.O., Semenkovick C.F. & Kelly D.P. 2005. A potential link between muscle peroxisome proliferator-activated receptor-α signaling and obesity-related diabetes. Cell Metab. 1:133-144.

Foote M.R., Horst R.L., Huff-Lonergan E.J., Trenkle A.H., Parrish F.C. & Beitz D.C. 2004. The use of vitamin D_3 and its metabolites to improve meat tenderness. J. Anim. Sci. 82: 242-249.

Geesink G.H., Kuchay S., Chishti A. H. & Koohmaraie M. 2006. μ-Calpain is essential for postmortem proteolysis of muscle proteins. J. Anim. Sci. 84: 2834-2840.

Gerrard D.E., Okamura C.S., Ranalletta M.A.M. & Grant A.L. 1998. Developmental expression and location of IGF-I and IGF-II mRNA and protein in skeletal muscle. J.Anim. Sci. 76: 1004-1011.

Gniadecki R. 1998. Nongenomic signalling by vitamin D. Biochem. Pharmacol. 56: 1273-1277.

Goll D.E., Thompson V.F., Taylor R.G & Ouali A. 1998. The calpain system and skeletal muscle growth. Can. J. Anim. Sci. 78: 503–512.

Goll D.E., Thompson V.F., Li H., Wei W. & Cong J. 2003. The Calpain System. Physiol. Rev. 83:731-801.

Gredinger E., Gerber A.N., Tamir Y., Tapscott S.J. & Bengal E. 1998. Mitogen-activated protein kinase pathway is involved in the differentiation of muscle cells. J. Biol. Chem. 273: 10436-10444.

Grounds M.D. 2002. Reasons for the degeneration of aging skeletal muscle: a central role for IGF-I signalling. Biogerentology 3: 19-24.

Handel S.E. & Stickland N.C. 1987. The growth and differentiation of porcine skeletal muscle fibre types and the influence of birth weight. J. Anat. 152: 107-119.

Harrison M.A. & Rae I.F. 1997. General techniques of cell culture. Cambridge University Press, UK.

Hasty P., Bradley A., Morris J.H., Edmondson D.G., Venuti J.M., Olson E.N. & Klein W.H. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature 364: 501-506.

Hidiroglou M. & Karpinski K. 1989. Providing vitamin D to confined sheep by oral supplementation Vs ultraviolet irradiation. J. Anim. Sci. 67: 794-802.

Hidiroglou M., Proulx J.G. & Roubos D. 1979. 25-Hydroxyvitamin D in plasma of cattle. J. Dairy Sci. 62: 1076-1080.

Holick M.F. 1999. Vitamin D: Physiology, molecular biology and clinical applications. Humana Press Inc. New Jersey, USA.

Hoover L. C., Cook K. D., Miller M. F., Huffman K. L., Wu C. K., Lansdell J. L. & Ramsey C.B. 1995. Restaurant consumer acceptance of beef loin strip steaks tenderized with calcium chloride. J. Anim. Sci. 73: 3633-3638.

Huff-Lonergan E., Mitsuhashi T., Beekman D.D., Parrish F.C. Jr., Olsen D.G. & Robson R.M. 1996. Proteolysis of specific muscle structural proteins by μ-calpain at low pH and temperature is similar to degradation in post mortem bovine muscle. J. Anim. Sci. 74: 993-1008.

Hwan S.F. & Bandman E. 1989. Studies of desmin and α-actinin degradation in bovine semitendinosus muscle. J. Food Sci. 54: 1426-1430.

Izumiya Y., Hopkins T., Morris C., Sato K., Zeng L., Viereck J., Hamilton J.A., Ouchi N., LeBrasseur N.K. & Walsh K. 2008. Fast/Glycolytic muscle fibre growth reduces fat mass and improves metabolic parameters in obese mice. Cell Metab. 7: 159-172.

Jaschinski F., Schuler M., Peuker H. & Pette D. 1998. Changes in myosin heavy chain mRNA and protein isoforms of rat muscle during forced contractile activity. Am. J. Physiol. Cell Physiol. 271: 365-370.

Jeacocke R.E. 1993. The concentrations of free magnesium and free calcium both increase in skeletal muscle fibres entering *rigor mortis*. Meat Sci. 35: 27-45.

Jen Y., Weintraub H. & Benezra R. 1992. Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins. Genes Dev. 6: 1466-1479.

Karamanlidis G., Karamitri A., Docherty K., Hazelrigg D.G & Lomax M.A. 2007. C/EBPβ reprograms white 3T3-L1 preadipocytes to a brown adipocyte pattern of gene expression. J. Biol. Chem. 282: 24660-24669.

Karges K., Brooks J.C., Gill D.R., BreazileJ.E., Owens F.N. & Morgan J.B. 2001. Effects of supplemental vitamin D_3 on feed intake, carcass characteristics, tenderness, and muscle properties of beef steers. J. Anim. Sci. 79: 2844-2850.

Kent M. P., Spencer M. J. & Koohmaraie M. 2004. Postmortem proteolysis is reduced in transgenic mice overexpressing calpastatin. J Anim Sci 82: 794-801.

Kerth C.R., Montgomery J.L., Lansdell J.L., Ramsey C.B. & Miller M.F. 2002. Shear gradient in longissimus steaks. J. Anim. Sci. 80: 2390-2395.

Klont R.E., Brocks L. & Eikelenboom G. 1998. Muscle fibre type and meat quality. Meat Sci. 49:S219-229.

Koohmaraie M. 1992. Ovine skeletal muscle multicatalytic proteinase complex (proteasome): purification, characterization and comparison of its effects on myofibrils with μ-calpains.j. Anim. Sci. 70: 3697-3708.

Koohmaraie M., Babiker A.S., Schroeder A.L., Merkel R.A & Dutson T.R. 1988. Acceleration of postmortem tenderization in ovine carcasses through activation of Ca2+ -dependent proteases. J Food Sci. 53: 1638-1641.

Koohmaraie M., Crouse J. D. & Mersmann H. J. 1989 Acceleration of postmortem tenderization in ovine carcasses through infusion of calcium chloride: Effect of concentration and ionic strength. J. Anim. Sci. 67: 934-942.

Koohmaraie M., Shackelford S.D., Wheeler T.L., Lonergan S.M. & Doumit M.E. 1995. A muscle hypertrophy condition in lamb (callipyge): characterization of effects on muscle growth and meat quality traits. J. Anim. Sci. 73: 3595-3607.

Koohmaraie M., Geesink G.H. 2006. Contribution of post-mortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. Meat Sci. 74: 34-46.

Koohmaraie M. & Shackelford S.D. 1991. Effect of calcium chloride infusion on the tenderness of lambs fed a beta-adrenergic agonist. J. Anim. Sci. 69: 2463-2471.

Krappel H.P & Goll D.E. 1989 Effect of Ca2+ on binding of the calpains to calpastatin. J. Biol. Chem. 264: 17888-17896.

Kurokawa R., Yu V.C., Näär A., Kyakumoto S., Han Z., Silverman S., Rosenfield M.G. & Glass C.K. 1993. Differential orientations of the DNA-binding domain and carboxy-terminal dimerization interface regulate binding site selection by nuclear receptor heterodimers. Genes Dev. 7: 1423-1435.

Lametsch R., Roepstorff P., Møller M.S. & Bendixen E. 2004. Identification of myofribrillar substrates for μ-calpain. Meat Sci. 68: 515-521.

Larsson L., Grimby G. & Karlsson J. 1979. Muscle strength and speed of movement in relation to age and muscle morphology. J. Appl. Physiol. 46: 451-456.

Larsson L., Müller U., Li X., Schiaffino S. 1995. Thyroid hormone regulation of myosin heavy chain isoform composition in young and old rats, with special reference to IIX myosin. Acta. Physiol. Scan. 153: 109-116.

Lecker S.H., Jagoe R.T., Gilbert A., Gomes M., Baracos V., Bailey J., Price S.R., Mitch W.E. & Goldberg A.L. 2004. Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. FASEB J. 18: 39-51.

Latres E., Amini A.R., Amini A.A., Griffiths J., Martin F.J., Wei Y., Lin H.C., Yancopoulos G.D. & Glass D.J. 2005. Insulin-like growth factor (IGF-1) inversely regulates atrophy-induced genes via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway. J/ Biol. Chem. 280: 2737-2744.

Lee G.S., Lee K.Y., Choi K.C., Ryu Y.H., Paik S.G., Oh G.T. Jeung E.B. Phenotype of a calbindin D-9k gene knockout is compensated for by the induction of other calcium transporter genes in a mouse model. J. Bone Miner. Res. 22: 1968-1978.

Leeuw T. & Pette D. 1993. Coordinate changes in the expression of troponin subunit and myosin heavy chain isoforms during fast-to-slow transition of low-frequency stimulated rabbit muscle. Eur. J. Biochem. 213: 1039-1046.

Lewin B. 2008. Genes IX. Ninth edition, Jones & Bartlett publishers, Massechussets USA.

Li H. & Capetenaki Y. 1994. An E box in the desmin promoter cooperates with the E box and MEF-2 sites of a distal enhancer to direct muscle specific transcription. EMBO J. 13: 3580-3589.

Li X., Hughes S.M., Salviati G., Teresi A., & Larsson L. 1996. Thyroid hormone effects on contractility and myosin composition of soleus muscle and single fibres from young and old rats. J. Physiol. 494: 555-567.

Lin J., Wu H., Tarr P.T., Zhang C.Y., Wu Z., Boss O., Micheal L.F., Puigserver P., Isotani E., Olson E.N., Lowell B.B., Bassel-Duby R. & Spiegelman B.M. 2002. Transcriptional co-activator PGC-1α drives the formation of slow-twitch muscle fibres. Nature 418: 797-801.

Littledike E.T. & Goff J. 1987. Interactions of calcium, phosphorous, magnesium and vitamin D that influence their status in domestic meat animals. J. An. Sci. 65: 1727-1743.

Locker J. 2001. Transcription factors. Oxford : BIOS Scientific publishers Ltd., Oxford, UK.

Lösel R. & Wehling M. 2003. Nongenomic actions of steroid hormones. Nat. Rev. Mol. Cell. Biol. **4:** 46–56.

Lowry O.H., Rosebrough N.J., Farr L.A. & Randall R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Ludolph D.C. & Koneiczny S.F. 1995. Transcription factor families: muscling in on the myogenic program. FASEB J. 9: 1595-1604.

Maccatrozzo L., Patruno M., Toniolo L., Reggiani C. & Mascerello F. 2004. Myosin heavy chain 2B isoforms is expressed in specialized eye muscles but not in trunk and limb muscles of cattle. Euro. J. Histochem. 48: 357-366.

Maltin C., Balcerzak D., Tilley R. & Delday M. 2003. Determinants of meat quality: tenderness. Proc. Nutr. Soc. 62: 337-347.

Maltin C. & Plastow G. 2004. Functional genomics and proteomics in relation to muscle tissue. In: M.F.W. te Pas, M.E. Everts and H.P. Haagsman, Editors, *Muscle development in livestock*, CAB International, Wallingford, UK, pp. 267–296.

Martini F.H & Welch K. 2001. Fundamentals of anatomy and physiology. 5th Edition. Prentic-Hall International (UK) Ltd, London UK.

Matsukara V.A., Okitani T., Nishimuro T. & Kato H. 1981. Mode of degradation of myofibrillar proteins by an endogenous protease, cathepsin L . Biochim. Biophys. Acta 662: 41.

McDonald P. et al 2002. Animal Nutrition. 6th Edition Harlow : Prentice Hall

Melloni E., Michetti M., Salamino F., Minafra R. & Pontremoli S. 1996. Modulation of the calpain autoproteolysis by calpastatin and phospholipids. Biochem. Biophys. Res. Comm. 229: 193-197.

Melloni E., Averna M., Stifanese R., De Tullio R., Defrandi E., Salamino F. & Pontremoli S. 2006. Association of calpastatin with inactive calpain: A novel mechanism to control the activation of the protease? J. Biol. Chem. 281: 24945-24954.

Meissner J.D., Umeda P.K., Chang K.C., Gros G. & Scheibe R.J. 2006. Activation of the β myosin heavy chain promoter by MEF-2D, MyoD, p300, and the calcineurin/NFATc1 pathway. J. Cell. Phys. 211: 138-148.

Meissner J.D., Chang K.C., Kubis H.P., Nebrada A.R., Gros G. & Scheibe R.J. 2007. The p38α/β mitogen-activated protein kinases mediate recruitment of CREB-binding protein to preserve fast myosin heavy chain IId/x gene activity in myotubes. J. Biol. Chem. 282: 7265-7275.

Michel R.N., Dunn S.E. & Chin E.R. 2004. Calcineruin and skeletal muscle growth. Proc. Nut. Soc. 63: 341-349.

Millward C.A., Heaney J.D., Sinasac D.S., Chu E.C., Bederman I.R., Gilge D.A., Orevis S.F. & Cringer C.M. 2007. Mice with a deletion in the gene for CCAAT/enhancer-binding protein β are protected against diet induced obesity. Diabetes 56: 161-167.

Minghetti P.P. & Norman A.W. 1988. 1,25$(OH)_2$-vitamin D_3 receptors: gene regulation and genetic circuitry. FASEB J.2: 3043-3053.

Molina M.E., Johnson D.D., West R.L. & Gwartney B.L. 2005. Enhancing palatability traits in beef chuck muscles. Meat Sci. 71: 52-61.

Montgomery J.L., Parrish F.C. Jr., Beitz D.C., Horst R.L., Huff-Lonergan E.J. & Trenkle A.H. 2000. The use of vitamin D_3 to improve beef tenderness. J. Anim. Sci. 78: 2615-2621.

Montgomery J.L., Carr M.A., Kerth C.R., Hilton G.G., Price B.P., Galyean M.L., Horst R.L. & Miller M.F. 2002. Effect of vitamin D_3 supplementation level on the post-mortem tenderization of beef from steers. J. Anim. Sci. 80: 971-981.

Montgomery J.L., King M.B., Gentry J.G., Barham A.R., Barham B.L., Hilton G.G., Blanton J.R. Jr., Horst R.L., Galayean M.L., Morrow K.J. Jr., Wester D.B. & Miller M.F. 2004. Supplemental Vitamin D_3 concentration and biological type of steers II. Tenderness, quality, and residues in beef. J. Anim. Sci. 82: 2092-2104.

Morelli S., de Boland A.R. & Boland R. L. 1993. Generation of inositol phosphates, diacylglycerol and calcium influxes in myoblasts with $1,25(OH)_2D_3$. Biochem. J. 289: 675-679.

Morelli S., Buitrago C., Boland R. & de Boland A.R. 2001. The stimulation of MAP kinase by $1,25(OH)_2$-vitamin D_3 in skeletal muscle cells is mediated by protein kinase C and calcium. Mol. Cell. Endocrinol. 173: 41–52.

Nabeshima Y., Hanaoka K., Hayasaka M., Esuml E., Li S., Nonaka I. & Nabeshima Y.I. 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature 364: 532-535.

Naumann K. & Pette D. 1994. Effects of chronic stimulation with different impulse patterns on the expression of myosin isoforms in rat myotube cultures. Differentiation 55: 203-211.

Nemere I., Schwartz Z., Pedrozo H., Sylvia V.L., Dean D.D. & Boyan B.D. 1998. Identification of a membrane receptor for $1,25(OH)_2D_3$ which mediates rapid activation of protein kinase C. J. Bone Miner. Res. 13: 1353-1359.

Norman A.W. & Collins E.D. 2001. Nutrient-gene interactions in health and disease. Edited by Moustaïd- Moussa M. & berdanier C.D. CRC Press, USA.

Norman A.W., Okamura W.H., Bishop J.E. & Henry H.L. 2002. Update on biological actions of $1\alpha,25(OH)_2$-vitamin D_3 (rapid effects) and $24R,25(OH)_2$-vitamin D_3. Moll. Cell. Endocrin. 197: 1-13.

Nwoye L. & Mammaerts W.F.H.M. 1981. The effects of thyroid status on some properties of rat fast-twitch muscle. J. Mus. Res. Cell. Motil. 2: 307-320.

Olson E.N. 1990. MyoD family: A paradigm for development? Genes Dev. 4: 1454-1461.

Okitani A., Matsukura U., Kato H, & Fujimaki M. 1980. Purification and Some Properties of a Myofibrillar Protein-Degrading Protease, Cathepsin L, from Rabbit Skeletal Muscle J. Biochem. 87: 1133-1143.

Parr T., Sensky P.L., Bardsley R.G. & Buttery P.J. 2001. Calpastatin expression in porcine cardiac and skeletal muscle and partial gene structure. Arch. Biochem. Biophys. 395: 1-13.

Penner G., Gang G., Sun X., Wray C. & Hasselgren P.O. 2002. C/EBP DNA-binding activity is upregulated by a glucocorticoids-dependant mechanism in septic muscle. Am. J. Regulatory Integrative Comp. Physiol. 282: R439-444.

Pérez-Palacios T., Ruiz J., Martín D., Mureil E. & Antequera T. 2008. Comparison of different methods for total lipid quantification in meat and meat products. Food Chem. 110: 1025-1029.

Peter J.B., Barnard R.J., Edgerton V.R., Gillespie C.A. & Stempel K.E. 1972. Metabolic profiles of three fibre types of skeletal muscles in guinea pig ands rabbit. Biochem. 11: 2627-2633.

Peterson G.V., Madié P. & Blackmore D.K. 1991. Veterinery aspects of meat quality. Veterinary continuing education, The New Zealand Veterinary Association.

Peterson J.S., Henckel P., Maribo H., Oksbjerg N. & Sørenson M.T. 1997. Muscle metabolic traits, post mortem-pH-decline and meat quality in pigs subjected to regular physical training and spontaneous activity. Meat Sci. 46: 259-275.

Pette D. & Staron R.S. 2000. Myosin isoforms, muscle fibre types, and transitions. Micro. Res. Tech. 50: 500-509.

Pfeiffer M., Begerow B., Minnie H.W. 2002. Vitamin D & muscle function. Osteosporos. Int. 13: 187-194.

Polidori P., Trabazal Marinucci M., Fantuz F., Renieri C. & Polidori F. 2000. Tenderization of wether lambs meat through pre-rigor infusion of calcium ions. Meat Sci. 55: 197-200.

Pösö A.R. & Puolanne E. 2005. Carbohydrate metabolism in meat animals. Meat Sci. 70: 423-434.

Randall D., Burggren W. & French K. 2002. Eckert Animal Physiology: mechanisms and adaptations. 5th Edition. W.H. Freeman and Company, New York, USA.

Ravid A., Koren R., Rotem C., Garach-Jehoshua O., Glaser T. & Liberman U.A. 1994. 1,25-Dihydroxyvitamin D_3 increases the cellular content of the calcium-activated neutral protease μ-calpain in renal cell carcinoma. Endocrinology 135: 2822-2825.

Rehfeldt C., Fiedler I., & Stickland N.C.. 2004. Number and size of muscle fibers in relation to meat production. In Muscle Development of Livestock Animals: Physiology, Genetics, and Meat Quality. M. F. W. te Pas, M. E. Haagsman, and H. P. Everts, ed. CAB Int., Wallingford, UK. pp1.

Reiling B.A. & Johnson D.D. 2003. Effects of implant regimens (trenbolone acetate-estradiol administered alone or in combination with zeranol) and vitamin D_3 on fresh colour and beef quality. J Anim. Sci. 81: 135- 142.

Ringe JD, Faber H, Fahramand P, Schacht E. 2005. Alfacalcidol versus plain vitamin D in the treatment of glucocorticoid/inflammation-induced osteoporosis. J Rheumatol. Suppl.76: 33-40.

Rider-Sell N., Mikel W.B., Xiong Y.L. & Brehends J.M. 2004. Vitamin D_3 supplementation on cull cows: effects on Longissimus and ST muscle tenderness. J. Anim. Sci. 82: 225-230.

Reggiani C. & Mascarello F. 2004. Fibre type identification and functional characteristics in adult livestock animals. In: M.F.W. te Pas, M.E. Everts and H.P. Haagsman, Editors, Muscle development in livestock, CAB International, Wallingford, UK, pp. 39-68.

Rodman J.S. & Baker T. 1978. Changes in kinetics of muscle contraction in vitamin D depleted rats. Kid. Int. 13: 189-193.

Ronda A.C., Buitrago C., Colicheo A., De Boland A.R., Roldán E. & Boland R. 2007. Activation of MAPKs by 1,25$(OH)_2$-Vitamin D_3 and 17β-Estradiol in skeletal muscle cells leads to phosphorylation of Elk-1 and CREB transcription factors. J. Ster. Biochem. Mol. Biol. 103: 462-466.

Roth J., Brown D., Norman A.W., Orci L. 1989. Localization of the vitamin D-dependent calcium-binding protein in mammalian kidney. A,. J. Phsyiol. 243: F423-F252.

Rudnicki M. A., Braun T., Hinuma S. & Jaenisch R. 1992. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development Cell 71: 383-390.

Russel J.A. 1994. Osteomalacic myopathy. Musc. & Nerve 17:578-580.

Sanchez H., Chapot R., banzet S., Koulmann N., Birot O., Bigard A.X. & Peinnequin A. 2006. Quantification by real-time PCR of developmental and adult myosin mRNA in rat muscles. Biochem. Biophys. Res. Comms. 340: 165-174.

Sato Y., Iwamoto J., Kanoko T., & Satoh K. 2005. Low-Dose Vitamin D Prevents Muscular Atrophy and Reduces Falls and Hip Fractures in Women after Stroke: A Randomized Controlled Trial. Cerebrovasc. Dis. 20: 187-192.

Sazili A.Q. 2003. Calpastatin and meat tenderness in sheep and cattle. University of Nottingham Theses.

Scanga J.A., Belk K.E., Tatum J.D. & Smith G.C. 2001. Supranutritional oral supplementation with vitamin D_3 and calcium and the effects on beef tenderness. J. Anim. Sci. 79- 912-918.

Schiaffino S. & Reggianni C. 1996. Molecular diversity of myofibrillar proteins: gene regulation and functional significance. Phsyiol. Rev. 76: 371-423.

Schott G.D. & Willis M.R. 1976. Muscle weakness in osteomalacia. Lancet. 307: 626-629.

Schuler M., Ali F., Chambon C., Diteil D., Bornert J.M., Tardivel A., Desvergne B., Wahli W., Chambon P. & Metzger D. 2006. PGC1α expression is controlled in skeletal muscles by PPARβ, whose ablation results in fibre-type switching, obesity and type 2 diabetes. Cell Met. 4: 407-414.

Sensky P.L., Jewell K.K., Ryan K.J.P., Parr T., Bardsley R.G. & Buttery P.J.B. 2006. Effect of anabolic agents on calpatstain promoters in porcine skeletal muscle and their responsiveness to cyclic adenosine monophosphate- and calcium- related stimuli. J. Anim. Sci. 84: 2973-2982.

Sentrandau M.A., Coulis G. & Ouali A. 2002. Role of muscle endopeptidases and their inhibitors in meat tenderness. Trends in Food Sci. & Tech. 13: 400-421.

Sears R., Nuckells F. Haura E., Taya Y., Tamai K. & Nevins J.R. 2000. Multiple Ras-dependant phosphorylation pathways regulate Myc protein stability. Genes Dev. 14: 2501-2514.

Shi H., Scheffler J.M., Pleitner J.M., Zeng C., Park S., Hannon K.M., Grant A.L. & Gerrard D.E. 2008. Modulation of skeletal muscle fibre type by mitogen-activated protein kinase signalling. FASEB J. 22: 2990-3000.

Shirakata M., Friedman F.K., Wei Q. & Paterson B.M. 1993, Dimerization specificity of myogenic helix-loop-helix DNA-binding factors directed by nonconserved hydrophilic residues. Genes Dev. 7: 2456-2470.

Short K.R., Vittone J.L., Bigelow M.L., Proctor D.N., Coenenschimke M J.M., Rys P. & Nair K.S. 2005. Changes in myosin heavy chain mRNA and protein expression in human skeletal muscle with age and endurance exercise training. J. Appl. Physiol. 99: 95-102.

Simpson R.U., Thomas G.A. & Arnold A.J. 1985. Identification of 1,25-dihydroxyvitamin D_3 receptors and activities in muscle. J. Biol. Chem. 25: 8882-8891.

Simpson R.U., Hsu T., Begley D.A, Mitchell B.S. & Alizadeh B.N. 1987. Transcriptional regulation of the c-myc protooncogene by 1,25-dihydroxyvitamin D_3 in HL-60 promyelocytic leukemia cells. J. Biol. Chem. 262: 4104-4108.

Simpson R.U., Hsu T., Wendt M.D. & Taylor J.M. 1989. 1,25-dihydroxyvitamin D_3 regulation of c-myc protooncogene transcription. J. Biol. Chem. 264: 19710-19715.

Snijder M.B., van Schoor N.M., Pluijm S.M.F., van Dam R.M., Visser M. & Lips P. 2006. Vitamin D Status in Relation to One-Year Risk of Recurrent Falling in Older Men and Women J. Clin. Endocrinol. Metab. 91: 2980-2985.

Sorensen O.H., Lund B., Saltin B., Lund B., Andersen R.B., Hjorth L., Melsen F. & Mosekilde L. 1979. Myopathy in bone loss of ageing: improvement by treatment with 1 alpha-hydroxycholecalciferol and calcium. Clin. Sci. 56: 157–161.

Suzuki A. & Tamate H. 1988. Distribution of myofiber types in the hip and thigh musculature of sheep. Anat. Rec. 221: 494-502.

Swanek S.S. Morgan J.B., Owens F.N., Gill D.R., Strasia C.A., Dolezal H.G. & Ray F.K. 1999. Vitamin D_3 supplementation of beed steers increases Longissimus tenderness. J. Anim. Sci. 77: 874-881.

Tajbakhsh S., Rocancourt D., Cossu G. & Buckingham M. 1997. Redefining the genetic hierarchies controlling skeletal myogenesis: Pax3 and Myf5 act upstream of MyoD. Cell. 89: 127-138.

Taylor R.G., Geesink G.H., Thompson V.F., Koohmaraie M. & Goll D.E. 1995. Is Z-disk degradation responsible for postmortem tenderization? J. Anim. Sci. 73: 1351-1367.

Vazquez G., Boland R. & De Boland A.R. 1995. Modulation by $1,25(OH)_2$-Vitamin D_3 of the adenylyl cyclase/cAMP pathway in rat and chick myoblasts. Biochim. Biophys. Act. 1269: 91-97.

Vestergaard M., Henckel P., Oksbjerg N. & Sejrsen K. 1994. The effect of cimaterol on muscle fibre characteristics, capillary supply and metabolic potentials of longissimus and semitendinosus muscles from young Friesian bulls. J. Anim. Sci. 72: 2298-2306.

Voet D.J, Voet J.G & Pratt C.W. 2008. Principles of biochemistry. 3rd edition. John Wiley & Sons Inc. NJ USA.

Walters M.R. 1992. Newly identified actions of the vitamin D endocrine system. Endocr. Rev. 13:719-764.

Wang Y.X., Zhang C.L., Yu R.T., Cho H.K., Nelson M.C., Bayuga-Ocampo C.R., Ham J., Kang H. & Evans R.M. 2004. Regulation of Muscle Fiber Type and Running Endurance by PPARδ. PLoS Biology 10: 1532-1539.

Warris P.D. 2000. Meat Science: An introductory text. CABI publishing, Oxon, UK.

Wassner S.J., Li J.B., Sperduto A. & Norman M. E. 1983. Vitamin D deficiency, hypocalcaemia, and increased muscle degradation in rats. J. Clin. Invest. 72: 102-112.

Weiss A., McDonough D., Wertman B., Acakpo-Satchivi L., Montgomery K., Kucherlapati R., Leinwand L. & Krauter K. 1999. Organization of human and mouse skeletal myosin heavy chain gene clusters is highly conserved. Proc. Natl. Aca. Sci. 96: 2958-2963.

Wegner J., Albrecht E., Fiedler I., Teuscher F., Papstein H.J. & Ender K. 2000. Growth- and breed-related changes of muscle fibre characteristics in cattle. J. Anim. Sci. 78: 1485-1496.

Wendt A., Thompson V.F. & Goll D.E. 2004. Interaction of calpastatin with calpain: a review. Biol. Chem. 385: 465-472.

Wheeler, T.L. & Koohmaraie M., 1992. Effects of the β-adrenergic agonist $L_{644,969}$ on muscle protein turnover, endogenous proteinase activities, and meat tenderness in steers. J. Anim. Sci. 70: 3035–3043.

Wheeler T.L. & Koohmaraie M. 1994. Prerigor and postrigor changes in tenderness of ovine longissimus muscle.J. Anim. Sci. 72: 1232-1238.

Wheeler T.L., Shackelford S.D., Johnson L.P., Miller M.F., Miller R.K. &Koohmaraie M. 1997. A comparison of Warner-Bratzler shear force assessments within and among institutions. J. Anim. Sci. 75: 2423-2432.

Whipple G. & Koohmaraie M. 1993. Calcium chloride marination effects on beef steak tenderness and calpain proteolytic activity. Meat Sci. 33: 265-275.

Wiegand B.R., Sparks J.C., Beitz D.C., Parrish F.C. Jr., Horst J.L., Trenkle A.H. & Ewan R.C. 2002. Short-term feeding of vitamin D_3 improves colour but does not change tenderness of pork-loin chops. J. Anim. Sci. 80: 2116-2121.

Wiegand B.R., Parrish F.C., Morrical D.G. Jr. & Huff-Lonergan E. Feeding high levels of vitamin D_3 does not improve tenderness of callipyge lamb loin chops. J.Anim. Sci.79: 2086-2091.

Wilborn B.S., Kerth C.R., Owsley W.F., Jones W.R. & Frobish L.T. 2004. Improving pork quality by feeding supranutritional concentrations of vitamin D_3. J. Anim. Sci. 82: 218-224.

Wu Z., Woodring P.J., Bhakta K.S., Tamura K., Wen F., Feramisco J.R. Karin M., Wang J.Y.J. & Puri P.L. 2000. P38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps. Moll. Cell. Biol. 20: 3951-3964.

Yamamoto M., Kawanobe Y., Takahashi H., Shimazawa E., Kimura S. & Ogata E. 1984. Vitamin D deficiency and renal calcium transport in the rat. J. Clin. Invest. 74:507-513.

Yang Y.T. & McElligot M.A. 1989. Multiple actions of β-adrenergic agonists on skeletal muscle and adipose tissue. Biochem. J. 261: 1-10.

Yang H., Mammen J., Wei W., Menconi M., Evenson A., Fareed M., Petkova V. & Hasselgren P.O. 2005. Expression and activity of C/EBP beta and delta are upregulated by dexamethasone in skeletal muscle.J. Cell. Physiol. 204: 219–226.

Yun K. & Wold B. 1996. Skeletal muscle determination and differentiation: story of a core regulatory network and its context. Curr. Opin. Cell Biol. 8: 877-889.

Zhang W., Behringer R.R. & Olson E.N. 1995. Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. Genes Dev. 9: 1388-1399.

Zhiamaras K. & Dagogo-Jack S. 1997. Reversible muscle weakness in patients with vitamin D deficiency. West. J. Med. 167: 435-439.