

**DNA Methylation Analysis of Sox2 Regulatory Regions SRR1 and  
SRR2 in Undifferentiated and Differentiated Mouse Embryonic  
Stem Cells**

**Sajida Batool**

**Thesis submitted to the University of Nottingham for the degree of Doctor  
of Philosophy (PhD)**

**July 2013**

## **Abstract**

One hallmark of embryonic stem cells (ES) is their ability to renew themselves indefinitely and still retain the potential to develop into any specialized cell type once triggered by specific exogenous signals. This very versatile nature has made them an attractive model to study developmental events occurring during embryogenesis and also to employ them for regenerative medicine. The idea to exploit their developmental potential for intended therapeutic applications requires a detailed knowledge of the molecular regulation of differentiation. Thus pluripotent embryonic stem cells can be employed to investigate the molecular framework regulating pluripotency. The major aim of this research endeavour is to explore the role of DNA methylation in regulation of endogenous Sox2 transcription factor in context of mouse ES cells following their transition from the pluripotent to a differentiated state. An insight into molecular regulatory mechanisms will shed light on developmental programming and also aid in refining of methodologies for differentiation and nuclear reprogramming increasing their chances of success and efficiency.

Mouse embryonic stem cells were differentiated towards osteogenic and neural cell types through the formation of embryoid bodies (EBs) – cellular aggregates partially recapitulating the early embryonic development. These EBs were then disaggregated and single cells plated in medium containing supplements to promote osteogenic or neural differentiation while control cells were grown in medium lacking those factors. Cells were harvested undifferentiated and at different time points during differentiation. Molecular characterization was carried out by expression profiling of lineage specific genes and proteins using RT-PCR and immunofluorescence respectively. DNA methylation analysis of two regulatory regions of Sox2 i.e. SRR1 and SRR2 was carried out by MS-PCR and bisulphite sequencing.

Embryonic stem cells were observed to be differentiating as evidenced by changes in cellular morphologies and lineage-specific markers expression. Two regulatory regions of Sox2, namely SRR1 and SRR2, were found to be methylated by methylation sensitive PCR at all time-points chosen for analysis in differentiating cells. Three individual CpGs in SRR2 region were then analysed further by bisulphite sequencing which appeared unmethylated in both undifferentiated and differentiated embryonic stem cells. This hints towards the possible role of DNA methylation in regulating the expression of Sox2 in differentiating embryonic stem cells and need further investigation.

## **Acknowledgements**

I would like to thank my principal supervisor Dr Cristina Tufarelli for her support and advice. She has helped me with the design of experiments and reviewing all the writing process. I am also thankful to my second supervisors; first Dr Virginie Sottile and then Dr Raheela Khan for their help.

I would like to convey my special thanks to Dr Lee Buttery and Professor Lorraine Young for their kind help and support to complete my experimental work.

I am grateful to my laboratory colleagues Mr. Hassan Rashidi, Mr. Omar Quatachi, Ms. Nafeesa Abdul Rahman, Ms. Stephanie Stroubecker, Mr. Jayson Bispham, Ms Elena Masta and Ms Maria M. Barbadillo for their technical help, suggestions and guidance.

I would like to express my special gratitude to Higher Education Commission (HEC), Government of Pakistan and University of Karachi for providing funding to carry out this research project.

Finally, I am thankful to my family especially my parents Fazal Hussain and Ghulam Sughra, and friends for their love, support and encouragement throughout.

## **Table of Contents**

1. Introduction.....	8
1.1.Stem cells – classification and sources.....	9
1.2.Mouse embryonic stem cell – derivation and maintenance.....	11
1.3.Pluripotency or stemness of mouse ES cells at molecular level.....	13
1.3.1. Transcription factors.....	15
1.3.2. Chromatin modification.....	17
1.3.3. DNA methylation and CpG islands.....	24
1.3.4. Regulatory RNAs.....	27
1.4.Development and cellular differentiation.....	31
1.5.Sox2.....	35
1.5.1. Sox2 – targets.....	37
1.5.2. Sox2 – functions.....	38
1.5.3. Sox2 – regulation.....	38
1.6.Aims of the research.....	40
2. Methods and Materials.....	42
2.1.Chemicals, reagents and medium.....	43
2.2.Cell culture.....	43
2.2.1. Mouse ES cell culture.....	43
2.2.2. Embryo body formation.....	44
2.2.3. Osteogenic differentiation.....	45
2.2.4. Neural differentiation.....	46
2.2.5. Mouse MSC culture and osteogenic differentiation.....	46
2.3.Molecular Analysis.....	47
2.3.1. Reverse transcription PCR.....	47

2.3.2. Methylation sensitive PCR.....	49
2.3.3. Bisulphite conversion of genomic DNA and PCR.....	51
2.4.Biochemical Analysis.....	52
2.4.1. Alizarin red staining and quantification.....	52
2.4.2. DNA quantification.....	53
2.4.3. Immunofluorescence .....	53
3. DNA methylation analysis of Sox2 regulatory regions in mouse embryonic stem cells (E14Tg2a) cultured in osteoblast differentiation medium.....	55
3.1.Introduction.....	56
3.1.1. Bone formation/osteogenesis.....	56
3.1.2. ES cells as a model to study osteogenesis.....	59
3.1.3. Sox2 in osteogenesis.....	60
3.2.A schematic representation of experimental work plan.....	62
3.3.Results.....	63
3.3.1. Cell culture.....	63
3.3.2. Alizarin red dye extraction and quantification.....	68
3.3.3. Immunocytochemistry.....	70
3.3.4. Molecular Analysis.....	91
3.4.Discussion.....	102
4. DNA methylation analysis of Sox2 regulatory regions during <i>in vitro</i> differentiation of mouse embryonic stem cells in neurogenic medium.....	109
4.1.Introduction.....	110
4.1.1. Neurogenesis.....	110
4.1.2. ES cells as a model to study neurogenesis.....	111
4.1.3. Sox2 in neurogenesis.....	114

4.2.Experimental work plan.....	116
4.3.Results.....	117
4.3.1. <i>In vitro</i> differentiation of mES cells towards neural lineage.....	117
4.3.2. Characterization of neural differentiation by immunofluorescence....	117
4.3.3. Molecular characterization of differentiated cells.....	123
4.4.Discussion.....	128
5. DNA methylation analysis of Sox2 regulatory regions in mouse mesenchymal stem cells (MSCs) cultured in osteoblast differentiation medium.....	141
5.1.Introduction.....	142
5.1.1. Mesenchymal stem cells as a model to study osteogeneiss.....	142
5.1.2. Sox2 in MSCs.....	143
5.2.Experimental work plan.....	145
5.3.Results.....	146
5.3.1. Differentiation of mMSCs (D1 cell line) into osteoblasts.....	146
5.3.2. Molecular Analysis.....	163
5.4.Discussion.....	169
6. Discussion.....	173
7. Appendices.....	180
7.1.Chemicals, medium and reagents.....	181
7.2.Medium and buffer compositions.....	184
7.3.Primer sequences and their annealing temperatures.....	187
7.4.Primer optimization.....	189
7.5.Sequencing chromatograms.....	193
8. References.....	194

## **Chapter 1 - Introduction**

## **1. Introduction**

Embryonic stem cells are master cells having the potential to develop into any of about 200 different cells types a multicellular creature is composed of and, due to this very versatile nature, are an attractive candidate in regenerative medicine (Prelle *et al.*, 2002). The first successful attempt of *in vitro* culture of embryonic stem cells with their subsequent maintenance and propagation outside a living system in undifferentiated state has opened a new arena for researchers to explore developmental pathways at one end, and a new hope for millions of those suffering from debilitating disorders at another (Evans and Kaufman, 1981; Martin, 1981).

The reprogramming of somatic genomes to a pluripotent state at will using transcription factors now termed as “core regulatory circuitry” i.e. Oct3/4, Sox2, Myc-4 and Klf-4 indicates the central role of transcription factors in the maintenance of differentiation potential. These transcription factors together with DNA methylation, chromatin modifications and regulatory RNAs result in interactive and extended epigenetic network of regulatory mechanisms (Jaenisch and Young, 2008). Thus pluripotent stem cells can be employed to understand epigenetic changes that accompany cell differentiation and how they contribute to the regulation of differentiation into multiple lineages.

### **1.1. Stem Cells – Classification and Sources**

Stem cells can be broadly classified in to two categories: embryonic or adult (somatic) depending upon their origin of extraction. In mammals, the term “ES cell” refers to stem cells derived from mammalian embryo in general and was specifically coined to distinguish these

cells from “EC cells” – teratocarcinoma derived pluripotent embryonal carcinoma cells which are similar to them in morphology, growth behaviour and marker expression (Martin, 1981). ES cells are also able to produce teratocarcinoma but they tend to consist of well differentiated mesodermal, ectodermal and endodermal tissues and cell types in contrast to those derived from EC cells that are dominated by undifferentiated cells (Evans and Kaufman, 1983).

Embryonic stem cells are derived from the inner cell mass (ICM) of the blastocyst stage of embryo. The surrounding tissues of the ICM form the trophoblast which ultimately forms into placenta and other supportive tissues required for fetal development (Wobus and Boheler, 2005). Pluripotent stem cells give rise to somatic stem cells of limited differentiation potential and hence referred to as multipotent stem cells which can develop into specified cell types of a particular lineage such as haematopoietic stem cells, neural stem cells etc. as shown in Figure 1:

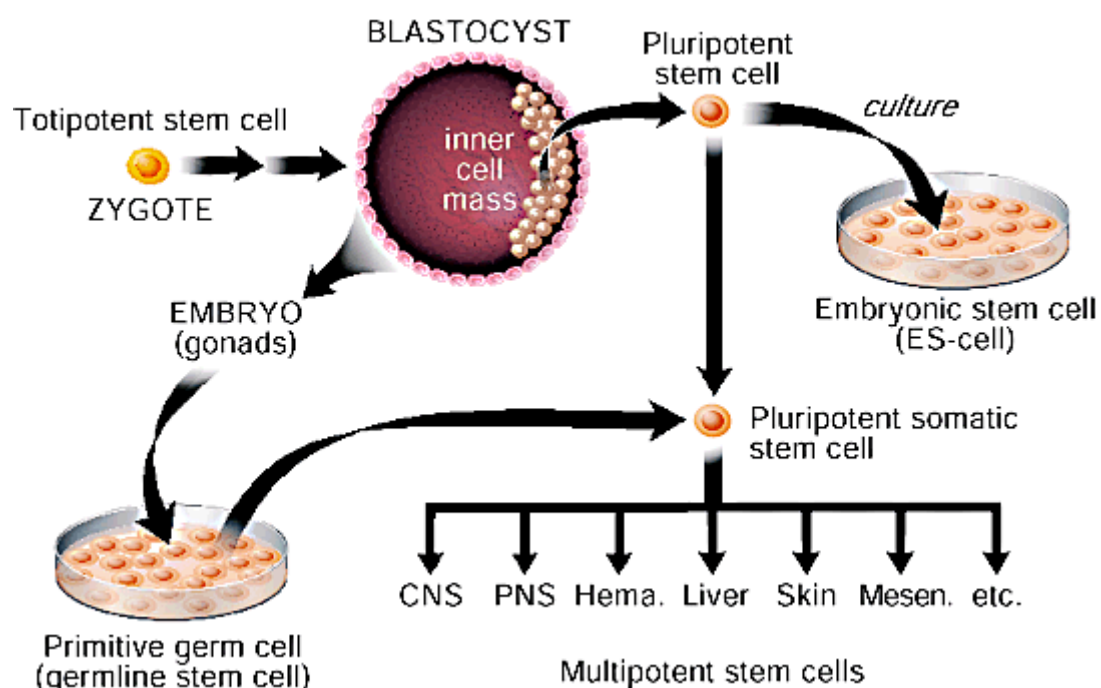


Figure 1: Classification of stem cells based on their differentiation potential (Anderson *et al.*, 2001)

Multipotent stem cells are localized to tissues or organs and are generally regarded as adult or somatic stem cells to differentiate them from their embryonic counterparts. These cell populations mainly act as an active or passive reservoir to renew depleting/dying/damaged cells based on their localization and therefore serve as a continuous source of cell renewal inside a living system (Anderson *et al.*, 2001). It was believed that these cells could have limited differentiation potential restricted to one lineage but recent evidence indicates that under appropriate conditions they can differentiate across different lineages, a phenomenon known as transdifferentiation (Blau *et al.*, 2001).

### **1.2. Mouse Embryonic Stem Cells - Derivation and Maintenance**

Mouse is an established animal model for experimental studies in mammals because of its genetic and physiological similarity with human beings (Fields and Johnston, 2005). Mouse embryonic stem cells are derived from inner cell mass of blastocyst stage embryo. Tissue culture medium enriched with 10-20% fetal calf serum and 2-mercaptoethanol is routinely used for their propagation either with or without feeders (Smith, 2001; Robertson, 1987). Mouse ES cells propagation and maintenance in an undifferentiated and pluripotent state is also dependent on the presence of LIF, Leukaemia inhibitory factor, so called because it was originally isolated as a haemopoietic regulator (Williams *et al.*, 1988; Smith *et al.*, 1988). LIF is a cytokine of the IL-6 family and acts through the gp130 receptor resulting in the activation of a series of signalling pathways involving STAT3, SHP-2, Hck, ERK1 and ERK2 (Burdon *et al.*, 1999a, b). The exact role of every single factor and signalling pathway in keeping the self-renewal ability is not known yet (Smith, 2001). A pictorial representation of different stages of mouse embryogenesis and stem cells at different time points is shown in Figure 2:

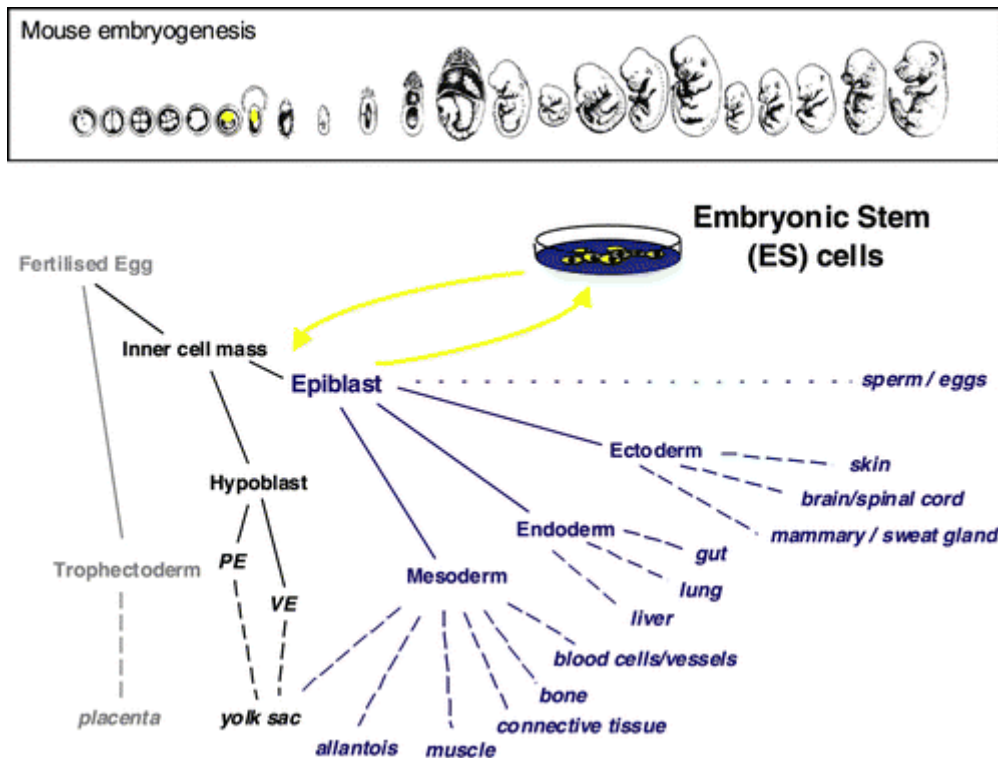


Figure 2: Developmental pathway in mouse (upper panel) and ES cells at different stages (lower panel) in this context (Smith, 2001)

One of the rationales for growing embryonic stem cells *in vitro* is to create an environment similar to that found inside the embryo to understand complex developmental events. But development is largely a context-dependent phenomenon involving cell-cell interactions at tissue and organ level besides various environmental stimuli. So functional assessment criteria are needed to interpret and validate the information obtained from *in vitro* studies to complement that with real physiological systems. The lowest level of this functional assessment regarding the validation of pluripotent state of embryonic stem cells is firstly achieved through *in vitro* differentiation of cultured cells. This is followed by teratoma formation after injection into host and then contribution to germ line by injected cells and chimera formation. The extreme level of control is exercised by injection of laboratory grown cells into 4n host blastocyst to get an animal composed only of injected donor cells (Jaenisch and Young, 2008; Eggan *et al.*, 2001; Nagy *et al.*, 1990). The ability to produce chimeras or

to validate germ line contribution for humans is not possible due to ethical and technical reasons hence primate ES cell lines other than human are used as an accurate *in vitro* model to understand differentiation (Thomson *et al.*, 1995; Thomson *et al.*, 1998). Also the process of gastrulation and germ layer formation are difficult to characterize *in vivo* due to the small size of the embryo and rapid start of development after implantation in the uterus. Embryonic stem (ES) cells are derived from the inner cell mass of early blastocysts or from the epiblast of late blastocysts (human ES cells and mouse EpiES), and have been shown to be able to self-renew or differentiate in most tissues of an adult organism under specific culture conditions *in vitro*, therefore providing an attractive model to understand pluripotency and its regulation at a molecular level (Smith, 1992; Pesce *et al.*, 1999).

### **1.3. “Pluripotency” or “stemness” of mouse ES cells at molecular level**

Pluripotency is a cellular state of embryonic stem cells characterized by their potential to differentiate into cell types originating from all three germ layers of a developing embryo i.e. ectoderm, mesoderm and endoderm (Niwa, 2001). A complex and dynamic interplay mediated by a diverse range of networks has been shown to contribute towards pluripotency rather than a single universal mechanism. It comprises of transcription factors, chromatin modifiers, regulatory RNAs and signalling pathways which are generally described together as epigenetic regulators of pluripotency (Jaenisch and Young, 2008).

Conard Waddington (1905-1975) is credited for introducing the term epigenetic and he defined it as, “the branch of biology which studies the casual interactions between genes and their products, which bring the phenotype into being” (Waddington, 1942; Jablonka and Lamb, 2002). An updated and currently prevailing definition of epigenetic is, “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by

changes in DNA sequence” (Bird, 2007). Hence epigenetic in its true essence is a sort of bridge between genotype and phenotype to explain any observed disparity in terms of phenotypic outcome at a particular locus or chromosome but without associated DNA sequence changes (Goldberg *et al.*, 2007).

Epigenetic influences on cell fate decisions are inherited in a non-Mendelian way in contrast to genetic changes and are usually triggered by a signal event (Rassoulzadegan *et al.*, 2006). There are many allowed combinations of epigenetic marks to be taken by a cell during differentiation and Figure 3 shows a visual representation of epigenetic landscape based on Waddington’s idea.

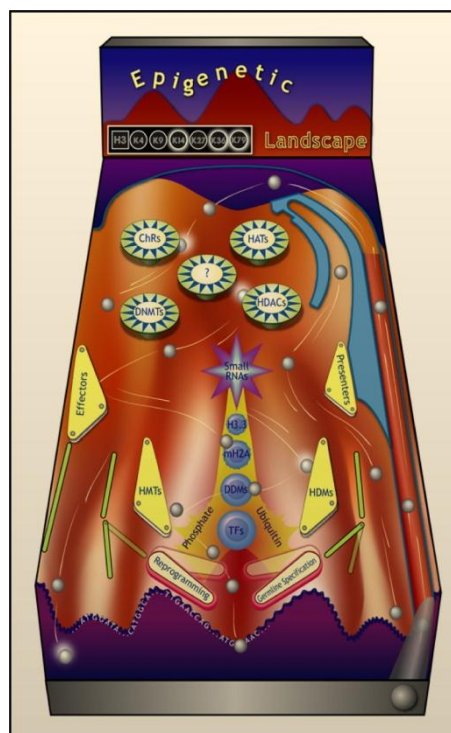


Figure 3: A current view of the epigenetic molecular machinery. Known factors that regulate epigenetic phenomena are shown directing the complex movement of pinballs (cells) across the elegant landscape proposed by Waddington. No specific order of molecular events is implied; as such a sequence remains unknown. Effector proteins recognize specific histone modifications, while presenters are proposed to impart substrate specificity for histone-modifying enzymes (Ruthenburg *et al.*, 2007). H3.3 and macroH2A are shown only as representative histone variants involved in transcriptional activation or repression, respectively. For simplicity, other histone (and non-histone) proteins are not shown. (Key: ChR, chromatin remodelers; DNMTs, DNA methyltransferases; HATs, histone acetyltransferases; HDACs, histone deacetylases; HMTs, histone methyltransferases; HDMs, histone demethylases; DDMs, DNA demethylases [unidentified in mammals to date]; and TFs, transcription factors [reflecting the genetic component of the epigenetic process]). Text and figure are from (Goldberg *et al.*, 2007)

After the cell has decided to take on a specific differentiation pathway in response to an external stimulus, self-perpetuating epigenetic memory allow cells to maintain their identity through the involvement of polycomb proteins even in the absence of inducing signal (Ringrose and Paro, 2004). This is also true for those environments that are rich in signals favouring induction of other cell types, hence important in maintaining the identity of stem cells over time (Martin and Zhang, 2007). This epigenetic memory is a specific cell state comprised of selective expression of some transcription factors acting in conjunction with chromatin modifications – covalent modifications of histone tails, DNA methylation at CpG dinucleotides, and localization of chromatin to specialized nuclear domains (Li, 2002).

### **1.3.1 Transcription Factors**

The process by which genetic information encoded in double helix of DNA is expressed through the mediumtion of mRNA is called transcription and is facilitated by transcription factors to a large extent (Watson *et al.*, 2008). Transcription factors can be grouped into two categories based on their expression: basal transcription factors or general transcription factors which are expressed ubiquitously and, regulatory transcription factors expressed in tissue-specific manner (Lee and Young, 2000). Those associated with expression of tissue-specific genes have been found to act via different interacting positive and negative feedback loops on their target to maintain irreversible cell differentiation. They act on multiple differentiation pathways in temporal order to control and co-ordinate their output through self-maintained nuclear networks (Schulaz and Hoffmann, 2007).

The transcription factor network involved in the maintenance of pluripotent state in embryonic stem cells has been studied in detail (Boyer *et al.*, 2005; Loh *et al.*, 2006). This network is suggested to be ranked at higher position in a hierarchy of pluripotency controlling

elements (Silva and Smith, 2008) as only a trinity of factors, Oct4, Sox2 and Nanog referred to as “core regulatory circuitry”, are required to maintain that state (Chambers and Smith, 2004; Niwa, 2007).

Oct4 is a member of the POU-family of transcription factors, it is encoded by Pou5f1 and is shown to be expressed in totipotent and pluripotent cells in the mouse life cycle (Pesce *et al.*, 1998a). Targeted gene deletion experiments in mouse embryos have resulted in failure of fetal development indicating its importance in pluripotency as cells differentiated to trophectoderm lineage (Nichols *et al.*, 1998). Oct4 can heterodimerize with Sox2 – an HMG box transcription factor which in turn regulates the Oct4 expression level (Masui *et al.*, 2007). Nanog has been shown to stabilize the pluripotent state rather than being essential for its maintenance (Chambers *et al.*, 2007).

These three factors bind together at their own promoters and form an interconnected auto-regulatory loop besides co-occupying their target genes. They collectively target two sets of genes, one that is actively expressed and another that is silent in embryonic cells but remain poised for expression during differentiation (Boyer *et al.*, 2005; Jaenisch and Young, 2008). The majority of genes occupied by these master regulators of pluripotency are those of transcription factors, signal transduction components and chromatin modifying enzymes that promote ES cell self-renewal. But they also co-occupy and silence genes whose products are required in lineage commitment and cellular differentiation hence maintaining stemness of ES cells (Boyer *et al.*, 2006; Loh *et al.*, 2006).

Most of the developmentally silent promoters are also occupied by Polycomb group (PcG) proteins that maintain cell state through gene silencing and hence are epigenetic regulators

(Bernstein *et al.*, 2006; Lee *et al.*, 2006). These proteins form multiple polycomb repressive complexes (PRCs) which mediate gene silencing at the transcription level (Schuettengruber *et al.*, 2007). The presence of stalled RNA polymerase at promoters of genes targeted by PcG and core transcription regulators involved in developmental regulations suggests that they are prone to transcription once differentiation is triggered (Mikkelsen *et al.*, 2007; Bernstein *et al.*, 2006). The mechanisms triggering selective activation of genes encoding specific developmental regulators have not been revealed yet, but most likely involve signaling pathways leading to establishment of a specific signature event such as nucleosome modifications e.g. demethylation of core histone 3 at lysine 27 (Lan *et al.*, 2007).

### **1.3.2 Chromatin Modifications**

The majority of DNA in mammalian cells is found in a condensed state called chromatin which is composed of a basic repeating unit called the nucleosome. In all eukaryotic cells each individual nucleosome consists of an octamer of four core histone proteins (H2A, H2B, H3 and H4) around which 147 base pairs of DNA are wrapped. Nucleosomes are linked together by linker DNA whose length is species dependent but typically fall in the range of 20-60 bps and can be bound by another histone protein H1 usually referred to as linker histone (Kornberg and Lorch, 1999).

Histones are positively charged proteins rich in basic amino acids i.e. lysine and arginine, and heterodimerization of specific histone pairs is mediated by a conserved histone fold domain. Histone proteins pairs get an ordered structure only after binding to DNA in a sequential manner; first a H3.H4 tetramer binds DNA which is successively joined in by two dimers of H2A.H2B to form nucleosome. These core histones have amino-terminal extensions called “histone tails” which are not required for the formation of nucleosome (Luger *et al.*, 1997),

but are indispensable for nucleosome remodelling and accessibility of DNA to replication and transcription machinery. These tails are targets for different type of dynamic covalent modifications which give the nucleosome an individual function and are mostly modified by phosphorylation, acetylation and methylation on serine, lysine and arginine residues (Strahl and Allis, 2000). These modifications appear in different combinations on a variety of target amino acids in histone tails depending on the set of conditions and they confer cells an enormous functional diversity to keep up with the changing cellular contexts (Kouzarides, 2007).

The observation that some combinatorial patterns of histone tail modifications have been shown to display identical phenotype (e.g. acetylation at certain specific amino acids is correlated with transcriptional upregulation in most instances) has led to the emergence of the “Histone code” concept. This code symbolizes the extension of genetic code at post genetic level and the enzymes carrying out these reactions are highly specific for their target amino acids with respect to changes and positions thus generating interdependent series of modifications (Strahl and Allis, 2000). This concept has been further extended to “nucleosome code” whereby concentration and combination of differentially modified individual nucleosomes in higher order chromatin structure dictates multiple readouts of genetic information e.g. gene activation v/s silencing or cell proliferation v/s differentiation (Jenuwin and Allis, 2001). But discrepancies have been reported between combination of signal events and generated output limiting the use of the term code in a universal sense (Liu *et al.*, 2005). A brief account of common histone modifications is detailed below.

**1.3.2.1 Acetylation** at lysine (represented by K) residues has been the most studied modifications of histone tails so far. One possible reason might be the

concomitant discovery of enzymes maintaining a balance between acetylated and non-acetylated state of target residues namely histone acetyl transferases (HATs) and histone deacetylases (HDACs) and also the observation that it is almost invariably associated with transcription activation (Gurnstein, 1997; Struhl, 1998). HAT is an enzyme that catalyses the acetylation of specific lysine residues in core histones resulting in changes in chromatin structure compactness and hence, effecting gene expression (Lee and Workman, 2007). Acetylation of lysine residues neutralizes the positive charge of histone tails leading to weak histone-DNA, nucleosome-nucleosome, histone-regulatory protein interactions making chromatin environment more permissive for transcription (Masumi, 2011; Roth *et al.*, 2001). Most common sites observed being acetylated in histones are K9, K14, K18 and K23 in H3 and K5, K8, K12 and K16 in H4 in most species (Thorne *et al.*, 1990; Strahl and Allis, 2000).

p300/CBP (CBP is phosphorylated form of cAMP-response-element-binding protein) is example of the two most studied HATs which were identified as separate protein factors initially but later grouped together due to structure and functional relatedness (Roth *et al.*, 2001). Several distinct domains are present in p300/CBP including a bromodomain (a frequent feature of mammalian HATs), three histidine-cysteine rich domains (mediates protein-protein interactions) and two more domains that interact with different transcription factors e.g. c-Jun, nuclear hormone receptors, TFIID (a general transcription factor). p300/CBP are recruited to their target by interactions with DNA-bound transcription factors instead of directly binding to the DNA and regulate many different transcription and signalling pathways (Chan and Thangue, 2001; Roth *et al.*, 2001).

**1.3.2.2 Phosphorylation** occurs at serine (represented by S) and sometimes on threonine (marked as T) residues and specifically this modification on H1 and H3 histone tails is associated with chromosome condensation during mitosis (Bradbury, 1992; Koshlan and Strunikov, 1996). H3S10 phosphorylation is specifically associated with upregulation of “immediate early” genes such as *c-fos*, *c-jun* and *c-myc* (Thomson *et al.*, 1999; Chadee *et al.*, 1999). This modification has been reported to have most significant influence on signalling pathways. The phosphorylation of cytoplasmic proteins initiates a cascade of signalling networks which in turn affects gene expression e.g. mitogen activated protein (MAP) kinase pathway which is linked with H3 phosphorylation (Thomson *et al.*, 1999).

**1.3.2.3 Methylation** has been shown to occur on arginine and lysine residues in histone N-terminal chains and output effects can be either activating or repressive on transcription based on location of residues methylated and groups of methyl added to target residue i.e. mono, di or tri (Bannister and Kouzarides, 2005). This methylation is catalyzed by three distinct families of proteins: protein arginine methyl transferase (PRMT1) family, SET domain containing proteins and DOT1/DOT1L histone methyl transferase family (Martin and Zhang, 2005). Methylation of H3K4, H3K36 and H3K79 is associated with active genes while H3K9, H3K27 and H4K20 are found to be related with transcription repression (Strahl *et al.*, 1999). An interesting feature is differential marking of methylation pattern i.e. H3K4me3 associated with transcription start sites, H3K36me3 with gene sequences, H3K9me3 abundant on silent genes and H3K36me3 on exons but at lower levels on alternatively spliced exons (Kolasinska-Zwierz *et al.*,

2009). The consequences of arginine methylations are not well understood so far and PRMTs – protein arginine methyl transferases have been found to be recruited to promoter by transcription factors (Lee *et al.*, 2005a, b) but no protein has been identified to reverse arginine methylation (Kouzarides, 2007).

**1.3.2.4 Ubiquitylation** has been shown to occur on H2AK119 and H2BK20. This modification is mediated by the Bmi/Ring1A protein found in polycomb complexes and is associated with transcriptional repression (Wang *et al.*, 2006). SUMO (small ubiquitin-related modifier) is a small protein having three dimensional structure similar to that of ubiquitin but distinct cellular functions (Hay, 2001). Sumoylation have been shown to modulate protein-protein interactions, nuclear localization of subcellular components, chromatin organization, protein-DNA interactions, enzyme activity, and as antagonist of ubiquitylation which marks proteins for degradation (Melchior, 2000; Verger *et al.*, 2003; Johnson, 2004). It has also been shown that histone H4 modification by sumoylation lead to transcription repression through recruitment of histone deacetylases (HDACs) and heterochromatin protein 1 (HP1) both *in vivo* and *in vitro* (Shiio and Eisenman, 2003). HDACs catalyse the acetyl group removal from the histone tail residues, acetylation is a modification almost invariably associated with transcription activation as described above in section 1.3.2.1. HP1 is a known structural protein of heterochromatin that binds to methylated lysine (K) of H3 and leads to transcriptional repression (Nielsen *et al.*, 2001). Histone sumoylation is also reported to act as negative regulator of transcription in *Saccharomyces cerevisiae* (Nathan *et al.*, 2006).

There are two proposed mechanisms by which histone modifications function: by disruption of nucleosomes-DNA contact in chromatin and/or by recruiting non histone proteins having enzymatic activities which depending upon the type of modifications that recruited them will facilitate the unfolding or the compaction of nucleosomes thus affecting downstream cellular processes (Kouzarides, 2007). The transcriptionally active chromatin is referred to as euchromatin and is characterized by high level of acetylation and trimetylation mainly at H3K4, H3K36 and H3K79. By contrast, the term heterochromatin is used to describe the chromatin with little or no transcription and associated with low levels of acetylation, increased methylation at residues associated with silent loci and phosphorylation (Strahl and Allis, 2000). These two regions are demarcated by boundary elements in mammals because each is associated with distinct modifications which are maintained to preserve identities of these domains.

Boundary elements are *cis*-acting sequences in genomes of higher eukaryotes that define the distinct functional domains of genome by acting as a barrier to prevent spread of inactive heterochromatin into euchromatin and also restrict the action of regulatory elements to the appropriate target region (Mishra and Karch, 1999). Similar kind of elements are also proposed to be present in CpG islands that protect them from *de novo* DNA methylation and induce local demethylation during development. They are thought to contain binding sites for other proteins e.g. Sp1 and, together they form a physical barrier preventing the binding of other regulatory elements and methylating enzymes to the target sequence (Brandeis *et al.*, 1994; Macleod *et al.*, 1994). Sp1 is a ubiquitous transcription factor that regulates a diverse set of genes involved in cell differentiation, signal transduction and apoptosis along with housekeeping genes. Binding sites for Sp1 and related transcription factors (GC/GT box is the consensus binding site) are frequent in regulatory sequences such as promoters, enhancers

and CpG islands of genes (Bouwman and Philipsen, 2002). The boundary elements also recruit enzymes to modify the associated chromatin e.g. PC2 is recruited by H3K27me to maintain inactive X chromosome (Plath *et al.*, 2003; Umlauf *et al.*, 2004). Furthermore, histone modifications are not static *cis* acting phenomena rather dynamic and co-operative process where many cross talks between histone tail residues facilitate binding of a target protein but can at same time disrupt the binding of another repressor /activator on adjacent residue due to steric hindrances (Kouzarides, 2007).

The pluripotent state of embryonic stem cells is maintained mainly by polycomb proteins and trithorax proteins which are involved in mitotic inheritance of lineage specific genes and, have been shown to catalyse H3K27 and H3K4 methylation respectively (Ringrose and Paro, 2004). H3K4 trimethylation has been reported as a signature event resulting in recruiting histone acetylases signalling up regulation of transcription while H3K27 has been found to down regulate transcription by compacting chromatin (Bernstein *et al.*, 2005). These two contrasting signatures have been shown to coexist in highly conserved non coding elements (HCNEs) in the mammalian genome and so named “bivalent domains”. These regions encode transcription factors required for embryonic development and their existence as bivalent domains keeps expression of these TFs at low level yet prone to transcription if ES cells differentiate. Once ES cells have taken any differentiation pathway, only one of the mark is perpetuated either repressive H3K27 or activating H3K4 (Bernstein *et al.*, 2006). This finding has opened up a possibility for manipulating the pluripotent state of embryonic stem cells by altering the regulatory balance of these modifications. However, the overall structure of chromatin in ES cells and how it is linked to pluripotency maintenance have not been detailed yet (Szutorisz and Dollin, 2005).

### **1.3.3 DNA Methylation and CpG Islands**

DNA methylation is an epigenetic modification associated with repression of tissue specific genes, genomic imprinting, X chromosome inactivation and transposon silencing, and in mammals occurs almost invariably at position 5 of the Cytosine ring found in CG dinucleotides (Bester and Bourc'his, 2004; Bortvin *et al.*, 2003; Li, 2002). CpG islands refers to those GC rich regions ( 65%) of about 1kb length that are methylation free and found to be associated with promoters of 50% of all mammalian genes (Antequera and Burd., 1993), making them a reliable feature for promoter prediction in mammalian genome (Hannenhalli and Levy, 2001; Ioshikhes and Zhang, 2000). CpG islands are enriched in transcription factor binding sites, have an open chromatin conformation and are different from the rest of the genome which is GC poor (40% on average) and heavily methylated at CpG dinucleotide (Bird, 1986; Delgado *et al.*, 1998).

These islands were originally referred to as “HTF islands” – “*HpaII* tiny fragment fraction” owing to their sensitivity to the *HpaII* (CCpGG) restriction enzyme. *HpaII* cuts at methylated CpG and sensitivity to this enzyme was found to be associated with promoters of housekeeping genes and some of tissue specific genes in digested fragments (Bird *et al.*, 1985). The distribution of CG dinucleotide in terms of frequency ratio is 1 in CpG islands in contrast to 0.2 observed in the rest of the genomic DNA. This disparity points towards a direct consequence of methylation in CpG islands. Unmodified cytosine is prone to deamination which converts it to uracil and is removed by uracil-glycosylation by the DNA repair machinery (Lindahl, 1982). By contrast, deamination of methylated cytosines converts them to thymines and results in C to T transition mutations that remain fixed as TpG (or CpA on the complimentary strand) by the DNA repair system thus accounting for the high

frequency of mutation rates observed at CpGs (Bird, 1980). That explains why CpG islands have displayed a more stable existence over the course of evolution as the U:G mismatch is removed far more efficiently and generation of new CpG sites by point mutations counter balance to maintain 20% of the expected frequency (Sved and Bird, 1990).

Methylation of CpG islands is found to be associated with transcription repression highlighting the importance of methylation and its maintenance at those sites (Keshet *et al.*, 1985). Enzymes mediating methylation of CpG islands are generally classified in two categories: *de novo* methyltransferase (DNMT3a and DNMT3b) which adds a methyl moiety at a cytosine residue and maintenance methyltransferase (DNMT1) which copies the existing methylation pattern onto newly replicated DNA molecule hence keeping the methylated status over successive cell divisions (Klose and Bird, 2006).

*De novo* methylation activity has been shown to occur mainly in ES cells, embryonic carcinoma cells, early post implantation embryo and developing germ cells and is absent in differentiated somatic cells (Chen *et al.*, 2003). How *de novo* methyltransferases are recruited to their target during germ line development to establish methylation patterns has not been characterized in detail due to unavailability of biological material at that stage (Reik *et al.*, 2001). Cell culture systems therefore have been widely exploited to understand this mechanism and three possible explanations have been put forward: the DNMT3 enzymes might recognize targets via specific domains in their structure, or they may be recruited to their targets by protein-protein interactions, or through RNA interference mediated mechanisms (Klose and Bird, 2006). Mouse embryonic stem cells lacking DNA methylation can survive and proliferate in an undifferentiated state but undergo apoptotic cell death upon differentiation. Therefore it remains to be determined whether this methylation is involved in

gene expression or maintenance of pluripotent state highlighting the importance of this event particularly if ES cells have to be employed in regenerative medicine (Fouse *et al.*, 2008).

Two mechanisms have been proposed for gene repression by methylation: either the modification of target sequence abolishes the target site of DNA binding factors (Watt and Molloy, 1988); or methylated sites recruit protein complexes that have transcription inhibitory activity (Handrich and Bird, 1998; Boyes and Bird, 1991). Methyl-CpG-binding proteins (MBPs) are associated with transcriptional repressor molecules that repress transcription with concomitant modification of surrounding chromatin linking DNA methylation and chromatin modification (Klose and Bird, 2006). Histone methylation at H3K4 has been implicated in promoter protection from de novo methylation in somatic cells (Weber *et al.*, 2007) and maternal imprinting control regions (ICRs) are rich in H3K4me2 which become methylated during oogenesis (Delaval *et al.*, 2007). Figure 4 illustrates the possible gene repression mechanism by DNA methylation and how DNA methylation pattern is faithfully copied during replication.

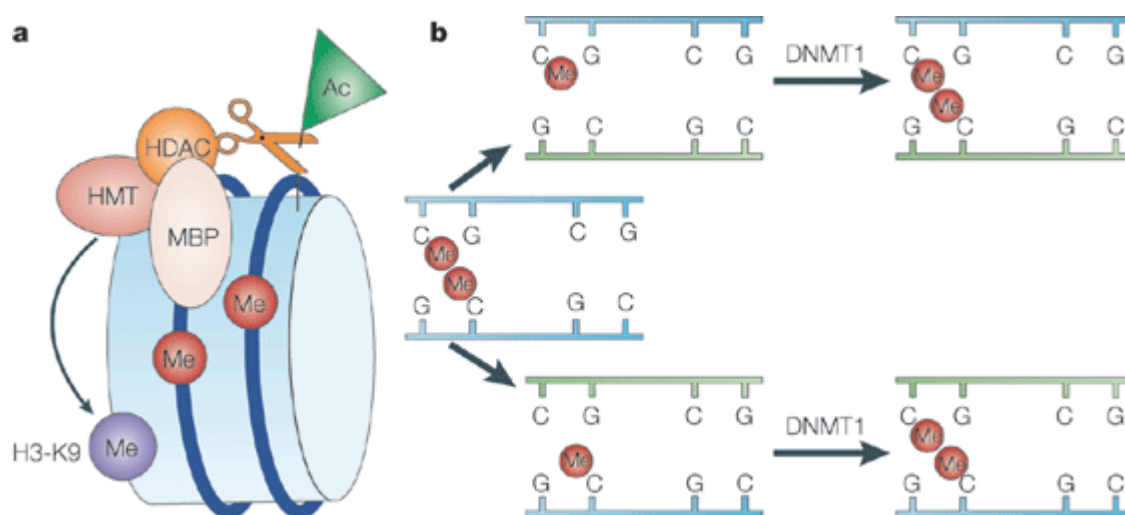


Figure 4: a) DNA methylation of promoters inhibits transcription by several molecular strategies. More commonly, methylated CpGs are recognized by methyl-binding domain proteins (MBP) that can recruit histone deacetylase (HDAC) and histone methyl transferase (HMT) and in this way bring about histone deacetylation and H3K9 methylation that alters the local chromatin environment. b) During replication, the newly made DNA strand (green) is generated using unmodified cytosine. DNA methyltransferase (cytosine 5) 1 (DNMT1) then progressively modifies only those sites that already have a methyl moiety on the parental DNA strand. In this way, the pattern of methylation is faithfully copied to the daughter cells, where it then contributes to reconstructing the chromatin structure and factor composition at the promoter (Text and Figure from Land-Diner and Cedar, 2005). [Ac, acetylation; Me, methylation.]

### 1.3.4 Regulatory RNAs

RNA interference is a vast and newly discovered regulatory network of gene expression in eukaryotes sharing functional similarities with prokaryotes (Cerutti and Casas-Mollano, 2006). This is a method by which small non-protein coding RNA molecules of about 21-22 nucleotides interact with mRNA and modulate the expression repertoire of eukaryotic cells (He and Hannon, 2004; Chapman and Carrington, 2007). These non-coding RNAs have been shown to modulate various epigenetic regulatory mechanisms such as chromatin modifications, RNAi-directed DNA methylation leading to silencing of genes, imprinting mainly dosage compensation, cellular decision making and differentiation (Bernstain and Allis, 2005). RNA interference can be classified into three branches based on type of regulatory RNA pathway involved (Matranga and Zamore, 2007):

- a) Small interfering RNA (siRNA) pathway providing defense against viruses and transposable elements.
- b) Micro RNA (miRNA) pathway which regulates gene expression.
- c) Piwi-interacting RNA (piRNA) involved in retrotransposon and germ line gene silencing.

miRNA and siRNA mediated pathways have some underlying differences. For instance miRNAs are endogenous non-protein coding RNA encoded by genes present in genome while siRNAs are degradation product of exogenous (e.g. virus) double stranded RNAs or transcribed from transposable elements integrated in genome or from inverted repeats or from overlapping and oppositely transcribed gene pairs. siRNAs show absolute complementarity to their target sequence in contrast to miRNAs where complementarity to target is partial particularly in animals (Shabalina and Koonin, 2008). And among these complementary sequence 2-8 residues of miRNA are most conserved among homologous miRNAs (Lim *et al* 2003).

The mechanism by which transcription and/or post transcriptional regulation of gene expression can be mediated by miRNA or siRNA depends on the level of complementarity: miRNA or siRNA with high level of complementarity can recruit RNA-induced silencing complex (RISC) and thus direct mRNA degradation; partial complementarity will lead instead to translational repression (Hutvagner and Zamore, 2002; Zeng *et al.*, 2002, 2003; Doench *et al.*, 2003). The choice of the post transcriptional mechanism is therefore independent of the identity of the RNA; rather the sequence similarity to target is the major determinant (Bartel, 2004). After cleavage of mRNA, the miRNA or siRNA remains intact and can further undergo similar cycles of target recognition and destruction (Tang *et al.*, 2003). Figure 5 represents the mode of action of different type of regulatory microRNAs either at

transcription or post transcription level. Endogenous miR-145 has been shown to directly target 3'-untranslated regions of SOX2, OCT4 and KLF4 in embryonic stem cells and switch the cell fate from undifferentiated state to a differentiated state i.e. increase expression of miR-145 represses the expression of these core pluripotency associated genes and induce differentiation (Xu *et al.*, 2009). Another example whereby gene expression is controlled by regulatory RNAs in stem cell is miR-124 which directly target the Sox9 gene, whose down regulation is the key for neural differentiation (Cheng *et al.*, 2009).

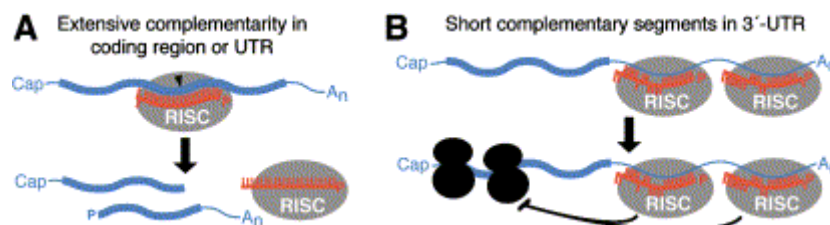


Figure 5: The Actions of Small Silencing RNAs(A) Messenger RNA cleavage specified by a miRNA or siRNA. Black arrowhead indicates site of cleavage.(B) Translational repression specified by miRNAs or siRNAs (Bartel, 2004).

Eukaryotes also express another class of non-protein coding RNAs greater than 200 nucleotides in size known as large non-coding RNAs (lncRNAs) (Kapranov *et al.*, 2007). These lncRNAs can be cytosolic, nuclear, spliced, polyadenylated and originate from either strand within a protein coding locus (Ponting *et al.*, 2009). These lncRNAs have been reported to be playing diverse functional and regulatory roles (summarized in Figure 6) such as chromatin remodeling, telomere biology, subcellular structural organization, transcriptional and post-transcriptional gene regulation (Mercer *et al.*, 2009). Given their diverse roles and cell and tissue specific expression, they are considered to be having crucial roles in development and cellular differentiation and their aberrant expression can also lead to different diseases including cancer (Caley *et al.*, 2010). The most notable example of gene regulation by lncRNA is X chromosome inactivation (XCI) in female cells by *Xist* lncRNA

that coats one of the X chromosome making it transcriptionally inactive in a phenomenon called dosage compensation (Lee, 2010). *Evx1as* and *Hoxb5/6as* ncRNAs have been shown to have a role in epigenetic regulation of homoeotic loci during differentiation of ES cells where both found to be associated with trimethylated H3K4 histones and histone methyltransferase MLL1 (Dinger *et al.*, 2008).

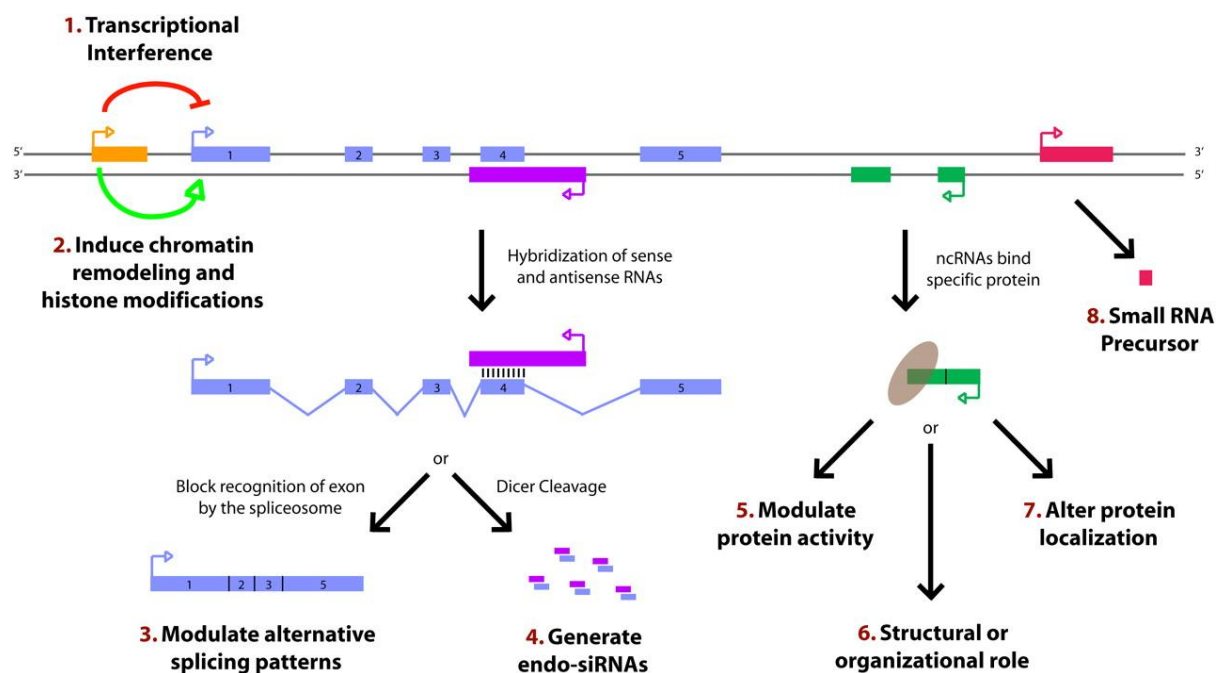


Figure 6: Paradigms for how long ncRNAs function. Recent studies have identified a variety of regulatory paradigms for how long ncRNAs function, many of which are highlighted here. Transcription from an upstream noncoding promoter (orange) can negatively (1) or positively (2) affect expression of the downstream gene (blue) by inhibiting RNA polymerase II recruitment or inducing chromatin remodeling, respectively. (3) An antisense transcript (purple) is able to hybridize to the overlapping sense transcript (blue) and block recognition of the splice sites by the spliceosome, thus resulting in an alternatively spliced transcript. (4) Alternatively, hybridization of the sense and antisense transcripts can allow Dicer to generate endogenous siRNAs. By binding to specific protein partners, a noncoding transcript (green) can modulate the activity of the protein (5), serve as a structural component that allows a larger RNA-protein complex to form (6), or alter where the protein localizes in the cell (7). (8) Long ncRNAs (pink) can be processed to yield small RNAs, such as miRNAs, piRNAs, and other less well-characterized classes of small transcripts. (Text and figure from Wilusz *et al.*, 2009)

## 1.4 Development and Cellular Differentiation

Development is a sequential process resulting in cellular specification through the process of cellular differentiation i.e. cells become specific in their function and localized in the developing animal body (Davidson *et al.*, 2002). The key of this process is differential control of gene expression mediated mainly by transcription factors and their interactions with *cis*-regulatory DNA sequence elements e.g. enhancers, silencers, insulators and locus control regions that can be found either upstream, downstream or in introns of a gene (Istrail and Davidson, 2004; Maston *et al.*, 2006). *Cis*-acting elements are DNA sequences that lie in physical vicinity of the target gene being regulated mostly found in promoters, upstream or downstream regions while *trans*-acting elements can diffuse to their target and may be a product of a gene locating several base pairs away from the target site (Albert *et al.*, 2002). It is the combinatorial action of these *cis*-regulatory DNA elements with transcription factors that produces a specific gene expression pattern and/or multiple patterns in spatial and temporal manner corresponding to specific cell type giving its identity (Levine and Davidson, 2005). These complex interactions can be regarded as gene regulatory networks (GRNs) consisting of hierarchy of interactions among transcription factors, *cis* acting regulatory sequences and signalling molecules (Davidson *et al.*, 2003). A regulatory gene with its multiple control elements is the central hub of these networks that receive inputs and generate output signals in the form of transcription factors and signalling molecules received from and, directed to these interacting regulatory networks as shown below in Figure 7 (Leon and Davidson, 2007).

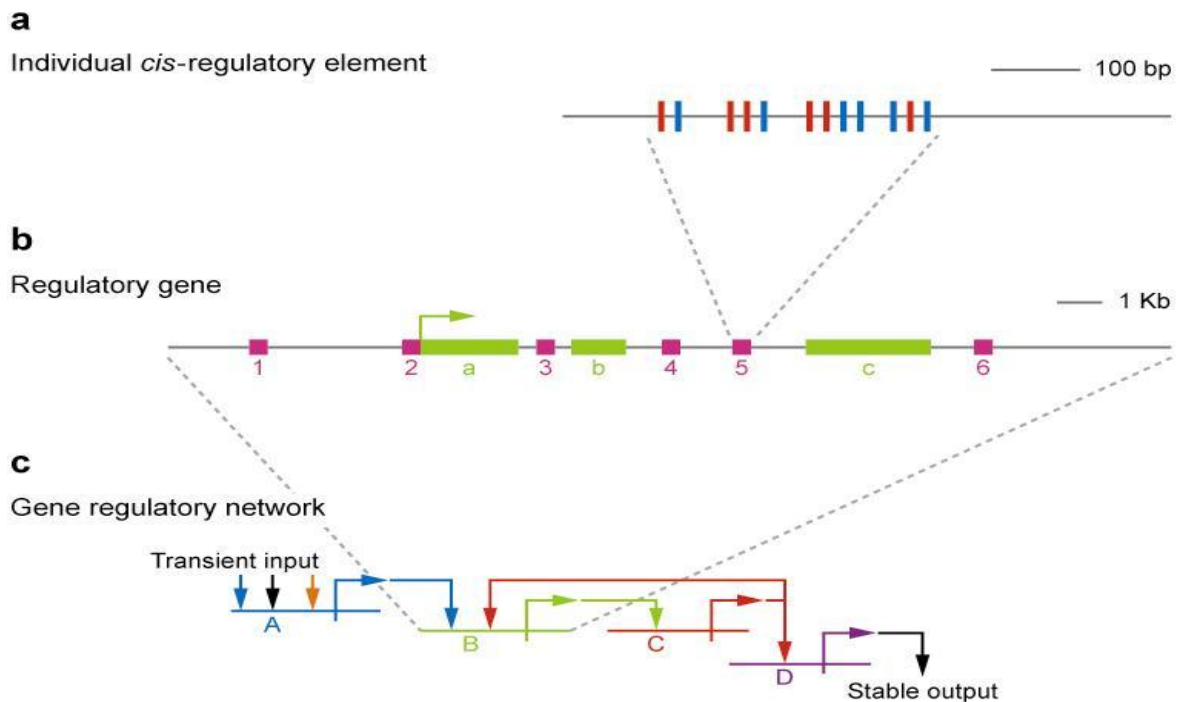


Figure 7: The gene regulatory hierarchy: (a) An individual *cis*-regulatory module contains a cluster of several transcription factor binding sites, indicated in red and blue boxes. (b) A gene contains a number of *cis*-regulatory modules (pink boxes) that control its expression at different times and lineages in the developing embryo. The exons are indicated in light green boxes. (c) The inter-regulating transcription factors and signalling molecules form a network that is essentially the genomic program for specification. In this diagram the colour codes of the three levels match, so panel a presents the *cis*-regulatory module of gene B (see panels b and c), which has binding sites of transcription factors A (blue) and C (red) (Text and figure from Leon and Davidson, 2007).

Transcription regulation is mostly done by enhancers, DNA sequences having multiple binding sites for variety of transcription factors and can activate transcription independent of their location, distance and orientation to that of their target genes' promoters and even for genes located in other chromosomes (Ong and Corces, 2011). A typical gene regulatory region is shown schematically in Figure 8. Silencers are sequences that negatively affect the rate of transcription and share most of the structural features with enhancers (Ogbourne and Antalis, 1998). Insulators or boundary elements limit the transcriptional activity of adjacent region of DNA so as to create pockets of expression domain within the genome (Maston *et al.*, 2006). Locus control regions (LCRs) regulate the transcriptional activity of whole gene cluster in tissue specific and position independent manner and are composed of multiple *cis*

acting elements including enhancers, silencers and insulators (Q *et al.*, 2002; Maston *et al.*, 2006).

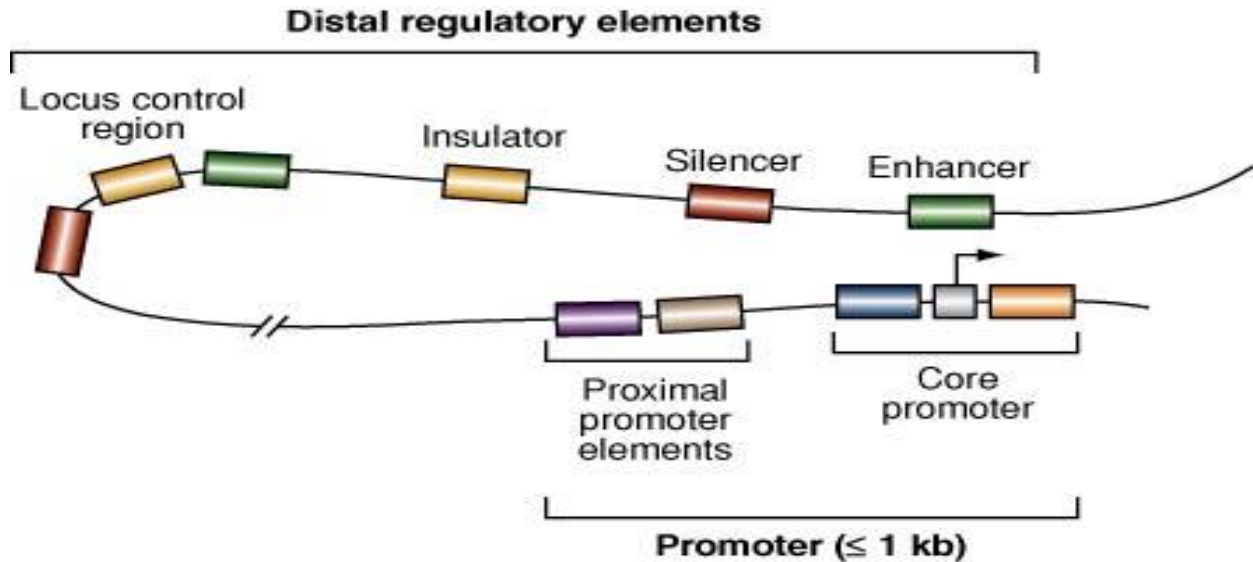


Figure 8: Schematic of a typical gene regulatory region. The promoter, which is composed of a core promoter and proximal promoter elements, typically spans less than 1 kb pairs. Distal (upstream) regulatory elements, which can include enhancers, silencers, insulators, and locus control regions, can be located up to 1 Mb pairs from the promoter. These distal elements may contact the core promoter or proximal promoter through a mechanism that involves looping out the intervening DNA (Text and Figure form Maston *et al.*, 2006).

Enhancers have been shown to carry epigenetic signatures e.g. specific histone marks that are usually established early in development and keep changing as cells differentiate hence modulating gene expression with and without non-coding RNAs (Ong and Corces, 2011). As already detailed in section 1.3.2 that distinct histone modification act sequentially or in combination to give specific downstream signals according to histone code hypothesis. For example specific histone marks that have been shown to be associated with increased gene activity are H3K4 and H3K36 trimethylation, H3 and H4 acetylation while H3K9 and H3K27 trimethylation, H4K20 di and trimethylation signals the transcriptionally repressed chromatin (Bernstein *et al.*, 2006; Van Leeuwen and Steensel., 2005; Meshorer and Misteli, 2006). These all are the modifications occurring in histone tails, similarly modification in core

histone sequences have also been shown to effect nucleosome structure e.g. phosphorylation of H3T45 particularly in apoptotic cells (Hurd *et al.*, 2009). Further these histone modification mainly methylation and acetylation have been shown to cross talk with DNA methylation (Fuks *et al.*, 2005). Hence a complex and co-ordinated interplay of these epigenetic and genetic control elements coordinates the cellular differentiation during vertebrate development making it irreversible and error free (Meissner, 2010).

It has been now well established that Sox2, an endogenous transcription factor in combination with Oct3/4 and Nanog, maintains the differentiation potential of embryonic stem cells, and forced expression of these factors can even reprogram somatic genomes to undifferentiated state (Takahashi *et al.*, 2006; 2007; Park *et al.*, 2008). As described above, development and differentiation are achieved by a precise and tight control of gene expression, it is therefore very important to understand the regulatory mechanisms being used by cells to control expression of these transcription factors and how they become altered during the course of differentiation. The aim of this study was to gain the deeper understanding of the role of different epigenetic regulatory mechanisms during differentiation of mouse embryonic stem cells. The work presented in this thesis has mainly focused on DNA methylation of regulatory regions of Sox2 in mouse embryonic stem cells and a transformed mesenchymal stem cell line to determine whether DNA methylation patterns at these regions could play any role in maintaining differentiation potential or in regulating their differentiation.

## 1.5 Sox2

Sox proteins have been implicated as crucial players of embryonic development mainly due to their regulatory roles in cellular differentiation pathways hence influencing cell specification by functioning as both transcriptional factors and architectural component of chromatin (Pevny and Lovell-Badge, 1997). They initially came under investigation by identification of their DNA binding domain highly homologous to the Sry box – a sex determining factor located on Y-chromosome in mouse and human (Sinclair *et al.*, 1990; Gubbay *et al.*, 1990). This Sry box encodes a 70-80 amino acid motif highly similar to DNA-binding domain of HMG (high mobility group proteins – so called because of their high electrophoretic mobility due to their small molecular mass <30KDa) box superfamily and hence named Sox (Laudet *et al.*, 1993). HMG box proteins are an extended family of proteins and classified in to two subgroups based on their DNA-binding sequence specificity and number of HMG domains present within a single protein (Laudet *et al.*, 1993). The Sox family of proteins is categorized as having a HMG-box sharing at least 50% sequence similarity with mouse Sry and at present consists of 20 different proteins which are grouped together as subfamilies based on homology within and outside the HMG-box of same family members (Bowles *et al.*, 2000; Schepers *et al.*, 2002).

The HMG domain of Sox factors mediates their binding with their target DNA in a sequence specific manner which is unique to Sox proteins and the consensus sequence for them has been identified as the heptameric sequence 5'-(A/T)(A/T)CAA(A/T)G-3' (Harley *et al.*, 1994). The structure of Sry-related HMG domain has been reported both with and without DNA binding and consists of three alpha helices arranged in twisted L-shape whereby helix I and helix II forming a long arm in an antiparallel arrangement and helix III

forms the short arm of L-shape in association with N-terminal extension (van Houte *et al.*, 1995; Werner *et al.*, 1995). The overall structure is maintained by a hydrophobic core whose constituent amino acids are highly conserved among Sox proteins as base specific DNA contacts. HMG domain conformation remains unaffected after binding with target DNA, however a large conformational change is induced into the target DNA structure forcing minor groove towards the concave binding domain of HMG and introducing an overall 70-85° bend thus helically unwinding it (Wegner, 1999; Ferrari *et al.*, 1992). The binding in to the minor groove of DNA by Sox proteins is sterically more feasible in its close proximity and is distinct from other transcription factors which bind in the major groove of DNA (van de Watering *et al.*, 1993). This feature of Sox proteins in combination with their ability to bend DNA has led to the speculation that they also serve as architectural component by organizing chromatin structure and assembling other DNA bound transcription factors in to biologically active and sterically defined multiprotein complexes (Wegner, 1999; Werner and Burley, 1997; Wolffe, 1994).

It has been shown by Sox binding sites analysis in the regulatory regions of target genes identified for Sox2 and Sox9 that a partner protein is required to bind with a nearby sequence to enable Sox proteins to act (Kamachi *et al.*, 1995, 2000; Pevny and Lovell-Badge, 1997). Figure 8 illustrates the general structure of a Sox protein and how their binding to their target DNA is stabilized when the target DNA is bound by an additional protein factor able to interact with Sox protein as well.

Sox2 is a transcription factor belonging to the Sox family of proteins described above. Sox2 expression is subjected to both temporal and spatial regulation in mouse: it is first expressed throughout the inner cell mass and later on its expression is restricted to primitive ectoderm,

developing CNS, the lens and primordial gut (Collington *et al.*, 1996; Que *et al.*, 2007; Wiebe *et al.*, 2000). Murine Sox2 maps to 3 A2-B locus and is an intronless gene of 2.4 Kb encoding a 319 amino acids protein which shares an overall 98% similarity with human SOX2 (Collington *et al.*, 1996). It belongs to Group B1 of Sox proteins – a subgroup of Group B which is broadly considered as activating members of Sox gene family (Uchikawa *et al.*, 1999).

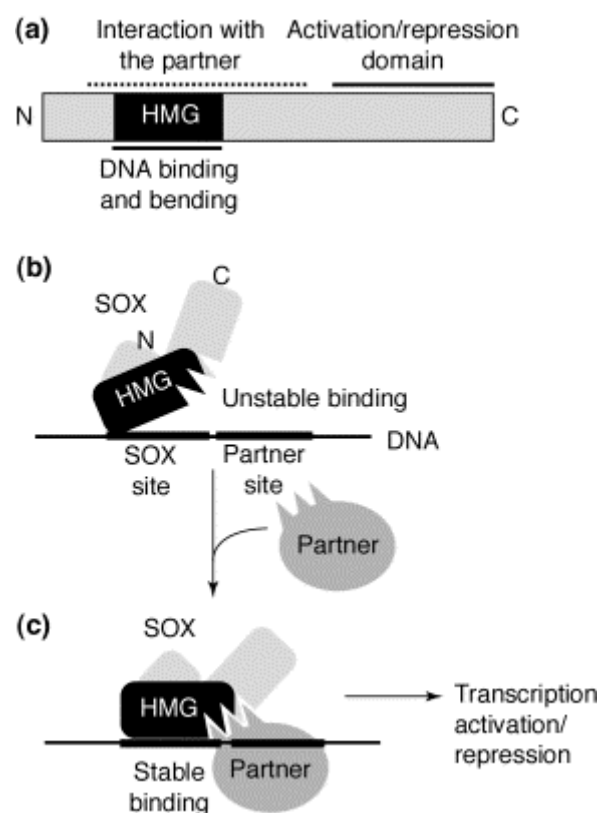


Figure 8: Sox proteins bind with their target stably only in presence of co-factor (Kamachi *et al.*, 2000)

### 1.5.1 Sox2 – targets

A lens specific gene  $\delta$ -crystallin was the first described target for Sox2 in chicken which gets activated upon binding with Sox2 in its minimal enhancer element DC5 (Kamachi *et al.*, 1995; 1998). Two important genes for maintaining the pluripotent state of embryonic stem

cells i.e. fibroblast growth factor 4(FGF-4) and undifferentiated embryonic cell transcription factor 1 (UTF1), were later shown to be activated by Sox2/Oct-3/4 complex (Yuan *et al.*, 1995; Ambrossetti *et al.*, 1997; Nishimoto *et al.*, 1999). Now many gene networks targeted by Sox2 alone or in complex with other core transcriptional regulatory circuitry members have been reported (Avillio *et al.*, 2003; Boyer *et al.*, 2005; 2007). Sox2 has also been reported to act as transcriptional repressor, for example as observed at the osteopontin gene (Botquin *et al.*, 1998). It is therefore possible that activating or repressive activity of Sox2 at different regulatory regions is dependent on its surrounding sequences which might recruit different partners. Also depending on which protein it is interacting in specific differentiation pathway, it can act as either activator or repressor.

### **1.5.2 Sox2 – functions**

Different roles have been ascribed to Sox2 so far by different research studies, including maintenance of pluripotent state of embryonic stem cells, specification and maintenance of neural stem cell identity, induction of lens fibre cell differentiation, regulation of various anterior pituitary hormones and taste bud development by endodermal progenitor cell differentiation (Lefebvre *et al.*, 2007).

### **1.5.3 Sox2 – regulation**

Identification of regulatory regions in the genome is quite a complex endeavour given the redundancy of consensus sequence of TFs, compactness of chromatin, concentration of TFs in cell at the given moment, competitive or synergistic binding of TFs to the same or neighbouring region and hence leading to transcription up regulation or down regulation (Pan

*et al.*, 2010). A common method for the study of gene regulation is to first use computational approaches to identify TF binding sites and then to validate them experimentally (Kolchanov *et al.*, 2007). Phylogenetic foot printing is an extensively used approach for the prediction of regulatory sites. Evolutionary conserved non-coding sequence elements of single gene found through cross-species alignment of homologues sequences are thought to be involved in regulation (Pavesi *et al.*, 2007). Affinity data, conformational and physico-chemical properties of TF in complex with its regulatory sequences aid in refining the target prediction (Pan *et al.*, 2010). ECR browser (Ovcharenko *et al.*, 2004) is an extensively used tool to compare genomes (available at <http://ecrbrowser.dcode.org/>) and Figure 9 is a screen shot showing Sox2 DNA sequence across different vertebrates in coding regions (blue and brown), upstream and downstream non-coding elements (red).



Figure 9: A screenshot from ECR browser. ECR is widely used to compare genomes and here in this screen shot genome sequences coding for Sox2 gene are compared across different vertebrates. Blue and yellow regions indicate the coding sequences of the gene and red regions are non-coding elements found upstream and downstream of the coding region. SRR1 and SRR2 regulatory regions of Sox2 gene are present about 4Kb upstream and downstream of the transcription start site and have been shown to be evolutionary conserved among different species as shown here by genome comparison.

The Sox2 gene has been reported to contain several regulatory regions i.e. core promoter (Wiebe *et al.*, 2000), an ambiguous 5' flanking region (Zappone *et al.*, 2000), and the evolutionary conserved upstream and downstream (~4kb from transcription start sites) enhancers which are designated Sox Regulatory Region 1 and 2 (SRR1 and SRR2) in mouse and humans (Tomioka *et al.*, 2002; Sikorska *et al.*, 2008). SRR1 contains binding site for POU transcription factor and direct neural-specific Sox2 expression while SRR2 binds Oct4-Sox2 and is essential for Sox2 expression both in ES cells and neural stem cells. These enhancers have been shown to exhibit differential DNA methylation and histone H3 acetylation during human neural progenitor's differentiation in astrocytes and neurons making Sox2 permanently or transiently silenced (Sikorska *et al.*, 2008). It is therefore feasible to propose that regulatory mechanisms might have been conserved in mouse and humans, making mouse embryonic stem cells a good model system to study regulation of Sox2 during *in vitro* differentiation.

### **1.6. Aims of the research**

This study was aimed to understand the molecular regulation of endogenous Sox2 in mouse embryonic stem cells. As described above, Sox2 is a key transcription factor required for maintenance of stem cell like state i.e. their ability to self-renew whilst retaining their differentiation potential. The major focus was on the analysis of DNA methylation of two regulatory regions of Sox2 namely SRR1 and SRR2, in undifferentiated and differentiated mouse embryonic stem cells and mesenchymal stem cells (a transformed cell line). These regulatory regions of Sox2 were analysed at different time points following transition of embryonic stem cells from an undifferentiated state to differentiated state to identify potential onset of DNA methylation changes and how this is correlated with cellular decision making.

In order to determine whether potential differences in methylation were specific to individual lineages and if different level of regulation were observed as cells differentiated towards a lineage in which Sox2 expression is required, similar analyses were performed in mouse ES cells as they differentiate towards the osteogenic lineage (Chapter 3) or neural lineage (Chapter 4).

Bone marrow derived MSCs are the physiological precursors of osteoblasts. MSCs are shown to be readily differentiated *in vitro* towards the osteogenic lineage and they are currently in use in the clinic. As the protocol used for osteogenic differentiation of MSCs and ES cells in this study are similar, it was thought possible to compare and contrast DNA methylation changes occurring at this gene locus in embryonic and adult stem cell types. It was thus hoped that further insights could be gained on whether there are conserved gene regulatory differentiation systems, among different kinds of stem cells.

## **Chapter 2 - Methods and Materials**

## **2. Methods and Materials**

### **2.1. Chemicals, reagents and medium**

All medium, chemicals and reagent used in this study are listed in Appendix 7.1 detailing suppliers and catalogue numbers. The composition of various medium, solutions and reagents prepared for use in experiments are detailed in Appendix 7.2.

### **2.2. Cell Culture**

#### **2.2.1. Mouse ES cell culture**

A frozen aliquot of mouse embryonic stem cells - E14Tg2a cell line created from mouse strain 129/Ola (Hooper *et al.*, 1987) was taken out from liquid nitrogen and defrosted in water bath at 37°C and quickly resuspended in complete mES medium. Cells were centrifuged at 180g for 5 minutes and during this time a T-25 cell culture flask was gelatinized (0.1% (v/v) gelatine in 1X PBS). Cells were collected after centrifugation, resuspended in 5ml medium and plated in gelatinized flask and incubated at 37°C in 5% CO<sub>2</sub>. Medium was changed the next day and cells were passaged when cells had reached to about 70-80% confluence or after 48 hours at the latest.

For passaging of mES, medium was aspirated and cells washed with 1X PBS. 0.5ml of 0.25% trypsin-EDTA (TE) added per T-25 flask and incubated at 37°C for 4 minutes. TE activity was inhibited by addition of complete medium in cells which were then passed through 19G needle for no more than four times to disaggregate cell clumps. An aliquot of cell suspension

was taken (about 20 $\mu$ l) to count the cells. Meanwhile, cells were centrifuged and then resuspended in complete ES medium at a concentration of  $1 \times 10^6$  cells per ml. 1ml of cell suspension again seeded in to a new gelatinized T-25 in 5ml of ES medium and cells incubated to grow. This passaging of ES cells continued to get enough cells to set up osteogenic and neural differentiation besides making some aliquots to freeze cells in liquid nitrogen.

For freezing mES cells, cells were trypsinized, passed through needle, counted and resuspended in freezing medium at a concentration of  $4 \times 10^6$  cells/ml and quickly transferred to Styrofoam box containing many layers of tissue to protect cells from temperature shock and stored in -80°C freezer. After two or three days, cells were transferred to liquid nitrogen.

### **2.2.2. Embryo Body formation**

In order to differentiate mES cells towards the osteogenic and neural lineages, first mES were grown as embryo bodies (EB) in suspension in non-adherent bacterial culture dishes. For this purpose, cells growing in adherent monolayer were trypsinized and counted.  $4 \times 10^6$  cells were seeded in to 15ml EB medium (medium used was of same composition as complete ES medium except LIF) in non-adherent bacterial culture dishes (90mm) and incubated at 37°C in 5% CO<sub>2</sub> for three days. Bacterial dishes were occasionally shaken during EB formation to prevent their attachment with surface.

After three days EBs was disaggregated by trypsinization to set up osteogenic differentiation (section 2.2.3). For neural differentiation some of the petri dishes were allowed to remain in suspension for an additional day, and at day four all-*trans*-retinoic acid (0.5 $\mu$ M) was added in

the EB medium and incubated for a further four days to induce neural precursors formation for setting up neural differentiation (section 2.24).

### **2.2.3. Osteogenic Differentiation**

EBs were collected in 50ml falcon tube after three days of incubation in suspension and trypsinized. First EBs were collected after centrifugation, medium aspirated and added 2ml of trypsin-EDTA and incubated at 37°C in water bath. EB medium was then added to inhibit TE activity and cells passed through syringe to ensure single cell population. An aliquot was removed for counting and cells were collected by centrifugation at 180g for 5 minutes. Cells obtained after dissociation of EBs were plated in osteogenic medium (differentiation medium) at a concentration of  $5 \times 10^5$  cells in 3ml of medium per well of six-well tissue culture plate. Four plates were seeded to harvest cells at different time points for gene expression studies, DNA methylation analysis, alizarin red staining and quantification and immunofluorescence for protein markers i.e. 7, 14, 21 and 28 days respectively.

Similarly cells were seeded in control medium which lacks additional factors responsible for osteogenic induction i.e. dexamethasone, betaglycerol phosphate and ascorbic acid. All the controls and osteogenic treated cells were incubated in 5% CO<sub>2</sub> at 37°C and medium regularly changed every two days. Cells were harvested at day 7, 14, 21 and 28 days of differentiation for RNA and DNA extraction. Also two well of cells were fixed with 4% paraformaldehyde (w/v) for alizarin red staining and DNA quantification and one well for immunofluorescence staining (section 2.7.2) having sterile cover slips placed in them.

#### **2.2.4. Neural Differentiation**

At day eight EBs were collected, disaggregated and counted as described above for osteogenic differentiation (2.2.3).  $4 \times 10^6$  cells were plated in 3ml of N2/F12 medium per well of six-well tissue culture plate. Medium was changed after 2hrs, 24 hrs and 48 hrs from plating. Then N2 medium was replaced with neurobasal medium supplemented with B27 and cells were grown for 28 days, harvesting samples at day 12, 21 and 28 for RNA, DNA extraction and immunofluorescence staining (section 2.7.2).

#### **2.2.5. Mouse MSC culture and osteogenic differentiation**

D1 – a mouse mesenchymal stem cells line (Diduch *et al.*, 1993) was retrieved from liquid nitrogen and defrosted in water bath at 37°C and quickly resuspended in MSC medium. Cells were centrifuged at 180g for 5 minutes, collected after centrifugation, resuspended in 5ml medium and plated in T25 tissue culture flask and incubated at 37°C in 5% CO<sub>2</sub>. Medium was changed the next day and cells were passaged when reached to about 80-85% confluence.

For passaging of MSCs, same protocol was used as described above for ESC (section 2.2.1) except that cells were not passed through syringe. This passaging of MSCs continued to get enough cells to set up osteogenic differentiation besides making some aliquots to cryopreserve. Then MSCs were plated in four tissue culture plates (six-well plates) in the same way as described above for ESC (section 2.2.3) to set up osteogenic differentiation and harvest cells at specified time points for RNA, DNA and markers protein analysis.

## **2.3.Molecular Analysis**

### **2.3.1. Reverse Transcription PCR**

#### **2.3.1.1.RNA Extraction**

mES cells were collected after trypsinization, washed with 1X PBS and resuspended in TRI-reagent, 5ml per  $10^8$  cells. After 5 minutes incubation at room temperature, chloroform (0.2ml per ml of TRI reagent) was added to cell lysate and then centrifuged at 12000g in refrigerated centrifuge at 4°C for 15 minutes. The supernatant was transferred to a fresh RNase free tubes and RNA grade isopropanol (0.5ml per ml of TRI reagent) added and left at -20°C for an hour. After centrifugation at 12000g for 30 minutes at 4°C, the aqueous phase was collected in new tube and RNA pellet was washed with 70% ethanol (v/v) and resuspended in RNA grade water. Quantification was then performed by taking absorbance at 260nm using Nanodrop (Thermo scientific).

After RNA extraction, DNase treatment was done to remove contaminating DNA. 200U of DNase added to RNA solution (2µg) with 40U of RNasin and incubated at 15°C for 45 minutes. Then 1:1 mixed phenol-chloroform was added (120µl/100µl of RNA) and centrifuged at 13000g at 4°C for 5 minutes to remove degraded DNA. Aqueous phase was transferred to a fresh RNA grade tube and two volumes of chloroform added and again centrifuged and supernatant collected in new tube. 3M sodium acetate was added (1/10<sup>th</sup> volume of supernatant) with 2 volumes of 100% ethanol to precipitate RNA and centrifuged at 13000g for 5 minutes at 4°C. Supernatant was discarded and pellet washed with 70%

ethanol (v/v), air dried and finally RNA resuspended in water and concentration was measured using Nanodrop.

#### **2.3.1.2.Reverse Transcription**

RNA extracted from mES cells at different time points during osteogenic and neural differentiation was reverse transcribed to cDNA for gene expression analysis. cDNA was synthesized using Expand RT from Roche according to their protocol with slight modifications with random primers. First 100ng/μl RNA was incubated with random primers for 10 minutes at 70°C and then after cooling at room temperature reverse transcriptase, RNasin, dNTPs and enzyme buffer were added and left at 42°C for 1 hour. Reaction was stopped by placing tubes in ice and then stored at -20°C. This cDNA was then used as template for PCR using different gene specific primers.

#### **2.3.1.3.Primers**

Primer pairs were designed using published genome sequences available on NCBI website and later checked for primer dimer formation, hairpin structures, and cross reactivity using Mac Vector software (MacVector Inc.). All primers were being synthesized from Eurofins MWG Operon and then optimized using temperature gradients and altering other PCR parameters sequentially. List of primer pairs and their optimal annealing and amplification conditions is given in Appendix 7.3 and regions of genes being amplified and primer optimization figures are shown in Appendix 7.4.

#### **2.3.1.4. Polymerase Chain Reaction (PCR) on cDNA**

PCR was carried out in 15µl reaction volume which contained 1X Thermopol buffer, 250mM dNTPs, 25µM forward and reverse primers and 0.5U/µl Thermopol Taq Polymerase (NEB). PCR cycle used for amplification was: 95°C for 3 minutes, 40 cycles of 95°C for 30 seconds, annealing temperature (optimal annealing temperatures vary for different primer pairs, Appendix 3) for 30 seconds, 72°C for 30 seconds; a final extension step at 72°C for 5 minutes.

#### **2.3.1.5. Agarose gel electrophoresis**

Amplified DNA subsequent to PCR was loaded on to 2% (w/v) agarose gel after mixing with 6X (final concentration 1X) Orange G (0.15% Orange G, 60% glycerol, 60mM EDTA) loading dye. The gel was run at constant voltage based on size of gel tank in 1X TAE (tris-acetate EDTA) buffer. DNA was stained with ethidium bromide (added in agarose solution prior to solidification at a final concentration of 1.5µg/ml) and following electrophoresis visualization and analysis was performed using Gel documentation system of FujiFilms.

### **2.3.2. Methylation sensitive PCR**

#### **2.3.2.1. DNA Extraction**

mES cells were collected after trypsinization, washed with 1X PBS and resuspended in 2ml of cell lysis buffer and incubated at 37°C overnight. DNA was extracted following the cell lysis method (Sambrook *et al.*, 1989). DNA was extracted from the cell lysate by

phenol/chloroform extraction using phase lock tubes (Qiagen). First phase lock tubes were prepared by centrifugation at 1500g for 2 minutes at room temperature. Then cell lysate was loaded onto phase lock tubes and subsequently equal volume of 1:1 phenol-chloroform added and centrifuged at 1500g for 5 minutes. Without removing the aqueous phase, phenol-chloroform extraction was repeated and finally 2 volumes of chloroform added to the cell lysate and centrifuged. The aqueous phase obtained after this step was transferred to a new 15ml falcon tube and DNA precipitated using 1/10<sup>th</sup> volume of 4M NaCl and two volumes of absolute ethanol. Precipitated DNA was washed with 70% ethanol (v/v) and resuspended in appropriate amount of Tris-EDTA (TE) buffer pH 8.0 and stored at -20°C.

#### **2.3.2.2. Restriction Digest of genomic DNA**

1000ng genomic DNA was digested with *MspI* and *HpaII* using the buffer supplied in a 50µl total reaction volume with 40Units of enzyme. The digestion was performed at 37°C and stopped by heat inactivation of enzyme as recommended by supplier. Afterwards samples were cleaned using phenol-choloroform as described in section 2.3.2.1 for DNA extraction with one exception that DNA was precipitated with 3M sodium acetate instead of sodium chloride. Samples were centrifuged at 13000rpm for 30 minutes, supernatant removed and DNA pellets washed with 70% ethanol (v/v) and air dried. DNA pellets were then resuspended in 50µl of deionised water and quantified using Nanodrop. Then 50ng was used for polymerase chain reaction (PCR) or based on quantification of amount of DNA present template volume altered accordingly.

PCR on genomic DNA was carried out as described in section 2.3.1.4 with annealing temperatures chosen according to primer pair being used. Primer pairs used and their optimal conditions are described in Appendix 3.

PCR products were visualized by running on 2% agarose gels (w/v) as described in section 2.3.1.5 and then photographed using FujiFilms (LAS 4000) and quantified using AIDA image analysis software.

### **2.3.3. Bisulphite Conversion of genomic DNA**

EZ DNA Methylation-Gold kit from Zymo research was used for bisulphite conversion of genomic DNA for methylation analysis following their protocol. First CT conversion reagent was prepared by adding 900 $\mu$ l water, 300 $\mu$ l M dilution buffer and 50 $\mu$ l dissolving buffer to solid reagent, all supplied with the kit and mixed at room temperature for 10 minutes to dissolve conversion reagent. Then 130 $\mu$ l of this CT conversion reagent was added to 20 $\mu$ l (1000ng) genomic DNA extracted from differentiation time points and samples were placed in PCR machine using cycling parameters recommended by manufacturer. After bisulphite treatment, samples were loaded on Zymo spin column, washed and desulphonated using reagents and protocol provided with kit. Finally treated DNA was eluted in eppendorf using deionized water and quantified using Nanodrop. This was then used for PCR using primers specific for regulatory regions of Sox2 and amplified product excised from agarose using Qiagen gel extraction kit and reagents following their protocol. After extraction amplified product was quantified using Nanodrop and Sanger-sequencing was outsourced to Source Biosciences by providing samples and appropriate sequencing primers according to their

guidelines. DNA sequences were analysed using Chromas software (Technelysium Pty Ltd.) and methylation analysis was done using BiQ analyser (Bock *et al.*, 2005).

## **2.4. Biochemical Analysis**

### **2.4.1. Alizarin Red Staining and Quantification**

Alizarin red staining was performed on cells after osteogenic differentiation (section 2.2.3) using this method (Gregory *et al.*, 2004). Following fixation with 4% (w/v) Paraformaldehyde (PFA) cells were stored in Phosphate buffer saline (PBS) pH 7.4 at 4°C. To perform the staining PBS was removed from the fixed monolayers of cells and cells washed three times 5 minutes each with distilled water at room temperature with shaking. After washing enough 40mM alizarin red dye solution (about 1ml) was added to each well of the plate to cover the cells and then left for 20 minutes at room temperature with shaking to develop the colour. Then the unincorporated dye was removed by thoroughly washing wells with water four times with shaking for 5 minutes before taking pictures under the microscope.

For quantification of dye incorporated into the cells 800µl acetic acid (10% v/v) was added to each well and incubated at room temperature while shaking for 30 minutes. After incubation, monolayer was scraped from plates with the help of cell scraper and transferred to 1.5ml eppendorf tube with 10% acetic acid. Tubes were then vigorously vortexed for 10 seconds and heated at 85°C for 10 minutes. After heating tubes were incubated in ice for 5 minutes to allow them to cool and then centrifugation was done at 15000g for 30 minutes. An aliquot of supernatant (about 500µl) was then taken in new eppendorf tube and pH tested so as to be in

the range of 4.1-4.5. Then 150µl of supernatant was transferred in three wells of a 96 well plate for each time point and absorbance was measured using Tecan (infinite M200) plate reader at 405nm. Standards of alizarin red were prepared in the range of 1000µM to 7.8µM by sequential two fold dilutions and read in the same plate at 405nm to create a standard curve for quantification of alizarin red amount extracted from stained monolayers.

#### **2.4.2. DNA Quantification**

In order to normalize the amount of alizarin red dye extracted from cells to cell count, DNA was quantified at each time point using method described here (Rago *et al.*, 1990). PBS was removed from fixed monolayers and plates were subjected to three freeze/thaw cycles at -80°C for about 30 minutes each. Subsequent to this freeze/thawing, cells were scratched from plates and transferred to 1.5ml eppendorf with TNE buffer and centrifuged at 1000rpm for 5 minutes to get rid of cell debris. An aliquot of 75µl supernatant was then transferred to three wells of a 96 well plate and 75µl of Hoechst stain added. Samples were then read using Tecan plate reader with excitation 360nm/Emission 460nm. DNA standards were prepared using mouse genomic DNA in the range of 10000pg to 78.12pg with two fold dilution in TNE buffer and standard curve generated to quantify DNA.

#### **2.4.3. Immunocytochemistry**

To perform immunocytochemistry experiments, cells were seeded on cover slips which were placed in the wells of a six well plate (four cover slips per well) while setting up differentiation (section 2.2.3, 2.2.4. 2.2.5). Cultured cells were fixed on cover slips by addition of 4% (w/v) paraformaldehyde at room temperature and then kept in PBS pH 7.4 at

4°C. In order to stain the cells for the presence of proteins of interest using fluorescent labelled antibody, cells were washed 10 minutes with PBS containing 0.1% Tween-20 (v/v) with shaking to make them permeable. Cells on cover slips were then blocked using block solution (PBS, 0.1% (v/v) Tween-20, 0.2% (v/v) serum) at room temperature for 1 hour and then incubated with primary antibody diluted in blocking solution over night at 4°C. Next day cover slips were washed with PBS containing Tween-20 three times for 15 minutes each with shaking and then incubated with secondary antibody for one hour diluted in same block as that of primary antibody in dark at room temperature. From this point on wards, care was taken to minimize exposure to light. After incubation with secondary antibodies, again cover slips were washed with PBS containing Tween-20 three times for 15 minutes each and finally mounted on glass slide using Vectra shield. Cover slides were kept in dark at 4°C until observation and then cells were visualized using Nikon Eclipse 90i fluorescent microscope and images were taken using Volocity 3D image analysis software (PerkinElmer).

**Chapter 3 - DNA methylation analysis of Sox2 regulatory regions in  
mouse embryonic stem cells (E14Tg2a) cultured in osteoblast  
differentiation medium**

### **3.1.Introduction**

As explained in section 1.2, mouse ES cells are now used as a model to study developmental events and understand molecular regulation of varied differentiation pathways. This study aimed (section 1.6) to understand if DNA methylation, a major epigenetic mechanism of gene regulation, of Sox2 regulatory regions in embryonic stem cells gets altered as cells differentiate to osteogenic and neural lineages derived from germinal ectoderm and mesoderm respectively. This chapter summarizes the findings of experiments about mouse embryonic stem cells grown in medium known to promote differentiation to osteoblasts (bone cells). Neural differentiation results are detailed in chapter 4.

#### **3.1.1. Bone Formation/Osteogenesis**

Bone is a specialized connective tissue under constant formation and resorption by osteoblasts and osteoclasts respectively throughout the life span of a vertebrate to maintain bone mass and calcium homeostasis (Aubin and Triffitt, 2002). Bone tissues are made up of hydroxyapatite crystals and an extracellular matrix consisting of type I collagen, osteocalcin, osteopontin, bone sialoprotein and proteoglycans (Young *et al.*, 1992; Robey *et al.*, 1993). Osteoblasts are cells that secrete and deposit most of these proteins, participate in the formation of hydroxyapatite crystals and have been shown to display high alkaline phosphatase activity besides responding to osteotropic hormones and cytokines (Mundlos and Olson, 1997).

In mammals bone tissue has been reported to form through two independent pathways: intramembranous ossification and endochondral ossification. During intramembranous

ossification, osteoblasts differentiate directly from mesenchymal cells (detailed in Figure 3.1).

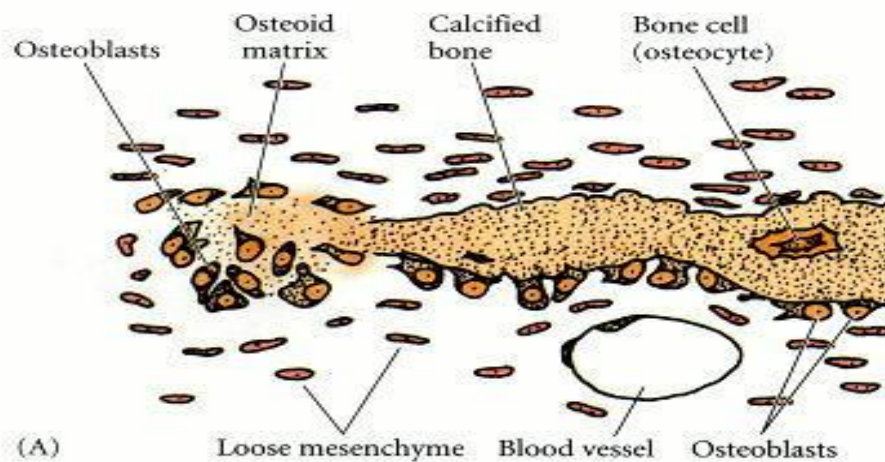


Figure 3.1: Schematic diagram of intramembranous ossification. (A) Mesenchymal cells condense to produce osteoblasts, which deposit osteoid matrix. These osteoblasts become arrayed along the calcified region of the matrix. Osteoblasts that are trapped within the bone matrix become osteocytes. (Text and figure taken from Gilbert, 2000)

In endochondral ossification, chondrocytes arise first from mesenchymal condensations and form a cartilaginous template. This template is subsequently mineralized by osteoblasts derived from surrounding mesenchymal stem cells once hypertrophic chondrocytes mature. The matrix around hypertrophic chondrocytes becomes calcified and invaded by blood vessels (shown in Figure 3.2). Bone marrow forms after the deposition of a bone matrix and then osteoclasts differentiation proceeds (Karsenty, 1999).

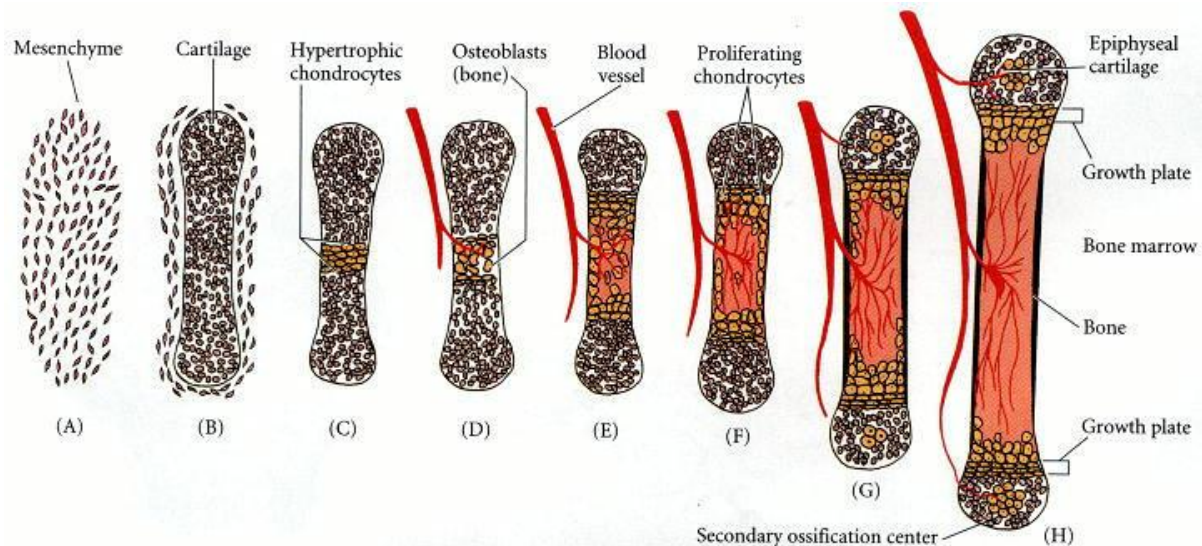


Figure 3.2: Schematic diagram of endochondral ossification. (A, B) Mesenchymal cells condense and differentiate into chondrocytes to form the cartilaginous model of the bone. (C) Chondrocytes in the center of the shaft undergo hypertrophy and apoptosis while they change and mineralize their extracellular matrix. Their deaths allow blood vessels to enter. (D, E) Blood vessels bring in osteoblasts, which bind to the degenerating cartilaginous matrix and deposit bone matrix. (F-H) Bone formation and growth consist of ordered arrays of proliferating, hypertrophic, and mineralizing chondrocytes. Secondary ossification centers also form as blood vessels enter near the tips of the bone. (Figure and its description are taken from Gilbert, 2000)

Mesenchymal stem cells or stromal stem cells are physiological precursors of osteoblasts and by definition are bone marrow derived fibroblasts that can differentiate in osteoblastic, adipogenic and chondrocytic lineages given the suitable stimuli (Pittenger *et al.*, 1999). The process of bone formation is under complex regulatory control of myriad of signalling cascades such as fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs), Wnt, Notch and, transcription factors but the exact molecular framework of genetic regulation is still elusive (Kozhevnikova *et al.*, 2008; Karsenty *et al.*, 2009).

Some of the key transcription factors regulating conversion of mesenchymal stem cells to mature osteoblasts and their interactions with each other are shown in Figure 3.3.

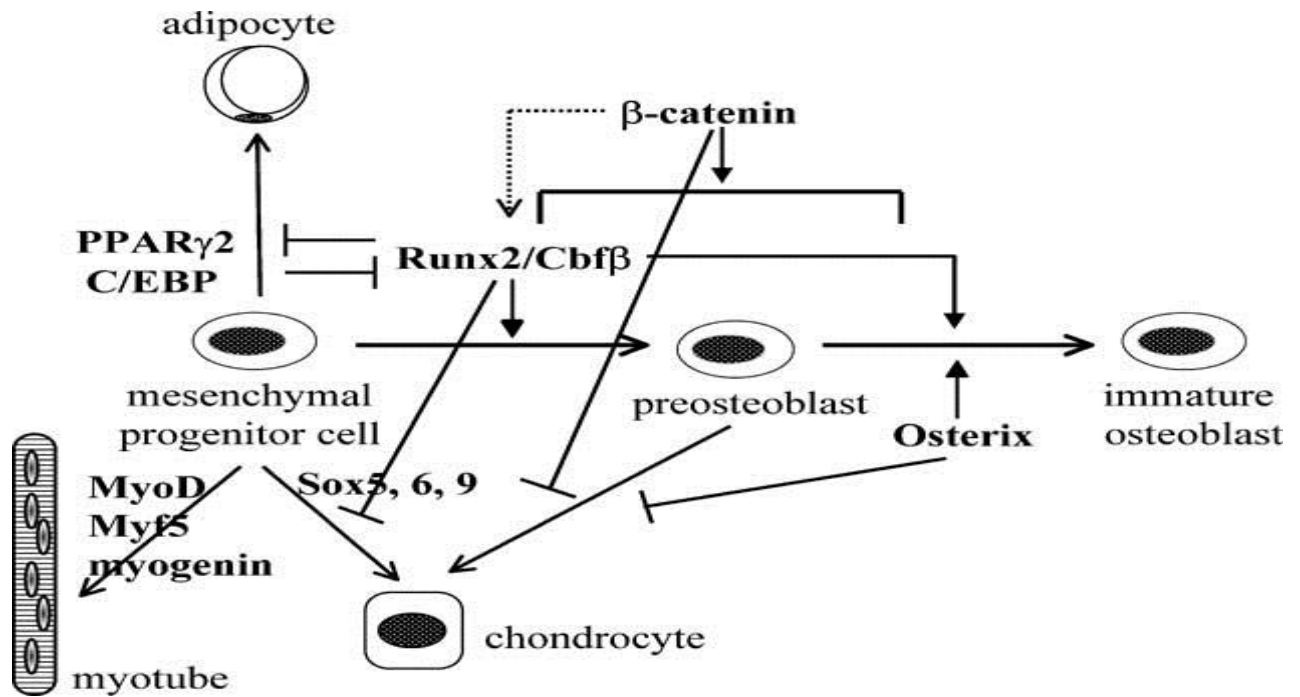


Figure 3.3: Determination of osteoblastic lineage by transcription factors. The differentiation of common mesenchymal progenitor cells into each skeletal component cells is determined by different transcription factors. In osteoblast differentiation, Runx2 directs mesenchymal progenitor cells to preosteoblasts, inhibiting adipocyte and chondrocyte differentiation. Runx2,  $\beta$ -catenin, and Osterix direct preosteoblasts to immature osteoblasts that express bone matrix protein genes and completely eliminating the potential to differentiate into chondrocytes (Text and figure from Komori, 2006).

### 3.1.2. ES cells as a model to study osteogenesis

Musculoskeletal and osteodegenerative disorders have been well documented as major sufferings of vast majority of aging population but still effective therapies to a large extent are not available (Alfred *et al.*, 2010). Embryonic stem cells are harvested from inner cell mass of embryo and have been shown to develop into any cell type *in vitro* making them as a possible source for transplantation and other clinical applications (Smith, 1998). The ability of embryonic stem cells to renew themselves indefinitely and still retaining the multilineage differentiation potential gives them a competitive advantage over other stem cell types and hence can potentially serve as a limitless supply of any cell type.

Directed differentiation of ES cells to osteoblasts has been successfully attempted so far in either of two major ways: use of three dimensional spherical structures called embryoid bodies (EB) prior to culturing single cells in a defined culture milieu known to induce osteogenesis, and direct culture of dissociated ES cells in conditioned medium avoiding EB formation (Hwang *et al.*, 2008). Embryoid bodies are 3D near spherical structures containing cells of all three germinal layers namely ectoderm, mesoderm and endoderm and recapitulate the environment of early embryonic development (Itskovitz-Eldor *et al.*, 2000).

The major limitation in EB-mediated differentiation strategies is poor characterization of molecular and signalling networks governing the cell fate decisions and hence spontaneous differentiation to unwanted cell types may lower the efficiency in terms of yield and homogeneity (Hwang *et al.*, 2008). Co-culture and conditioned medium can be employed to overcome this bottleneck but then the risk of pathogen transmission and undefined composition of medium limits the use of this approach in clinical setting (Heng *et al.*, 2004). Also this can be more expensive than EB formation. Another strategy that has also been reported is through genetic manipulation for expression of some transcription factors known to induce osteogenesis such as osterix but that also involves potential risks associated with recombinant approaches if differentiated cells have to be used for treatment (Tai *et al.*, 2004).

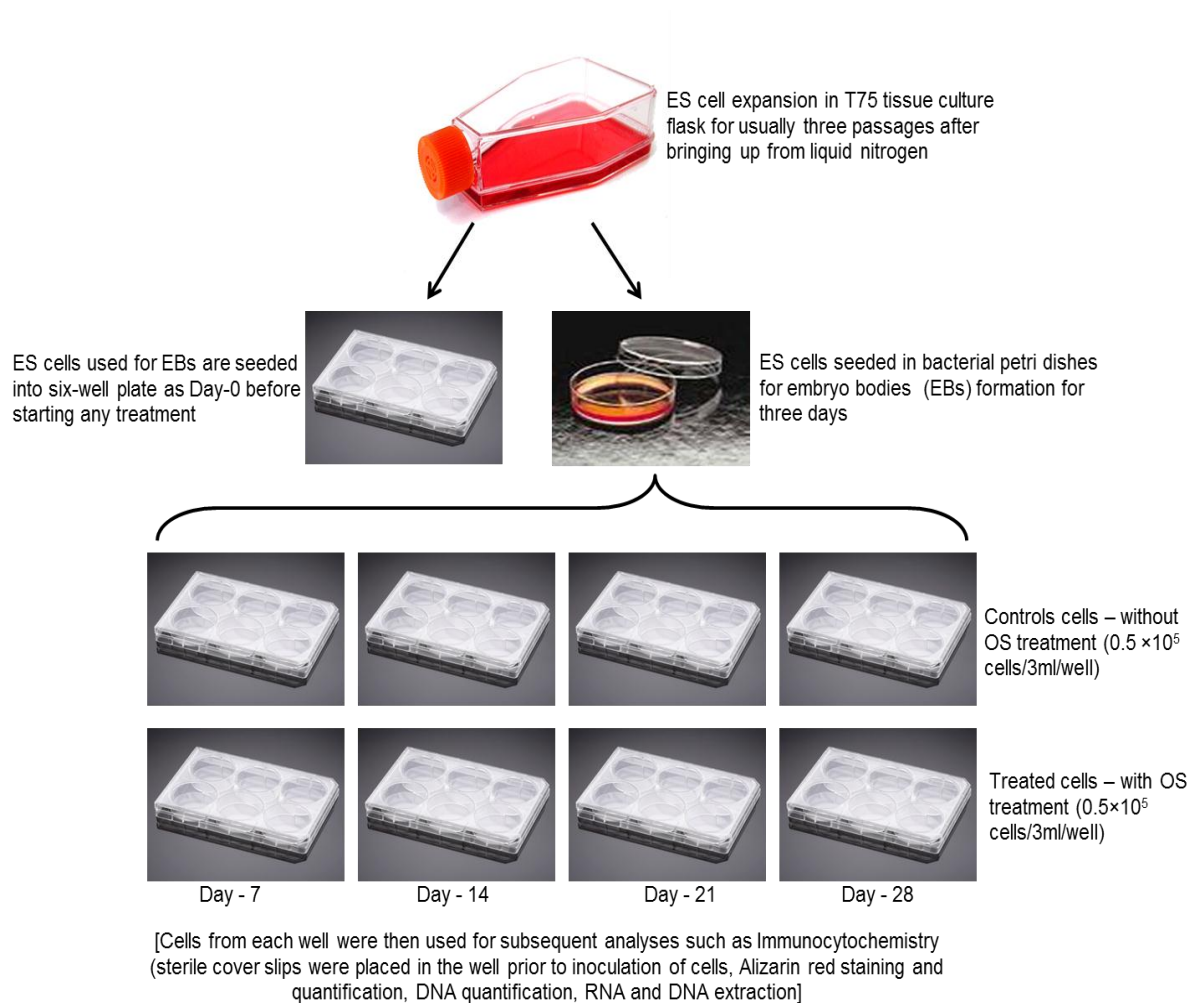
### **3.1.3. Sox2 in Osteogenesis**

There are only limited studies so far investigating the role of Sox2 in osteoblast lineage in particular. Sox2 has been shown to be expressed at low levels in osteoprogenitors and induced by FGF signalling and, favours their proliferation potential over terminal differentiation to osteoblasts by activating Wnt signalling pathway (Ambrosetti *et al.*, 2008; Mansukhani *et al.*,

2005). Some recent studies have shown experimentally that Sox2 is required for self-renewal of osteoblast precursors and inhibit their differentiation (Roy *et al.*, 2010).

In order to identify the mechanisms that could possibly contribute to down regulation of Sox2 in mature osteoblasts, two regulatory regions namely SRR1 and SRR2 were analysed for DNA methylation changes in this study. This is the first study looking at DNA methylation changes of Sox2 regulatory regions in osteoblast lineage. It was hoped that this information would help to better understand the regulatory role of Sox2 in osteogenic differentiation. Also as described above that MSCs (section 3.1.1) are physiological precursors of osteoblasts so a parallel set of experiments were carried out with MSCs keeping differentiation protocol and other experimental parameters identical to compare and contrast the molecular changes occurring in both cell types. The results of these experiments are detailed in chapter 5.

### 3.2. A schematic representaiton of experimental work plan



### **3.3.Results**

#### **3.3.1. Cell Culture**

##### **3.3.1.1.Revival and passage of E14Tg2A cells**

E14Tg2A (wild type embryonic stem cells line) were revived from liquid nitrogen and grown on gelatine-coated tissue culture flasks. They were passaged every other day by trypsinization until the majority of the cells in culture looked undifferentiated as described in methods section (2.2.1). The morphology of cells at different passages is shown in Figure 3.4.

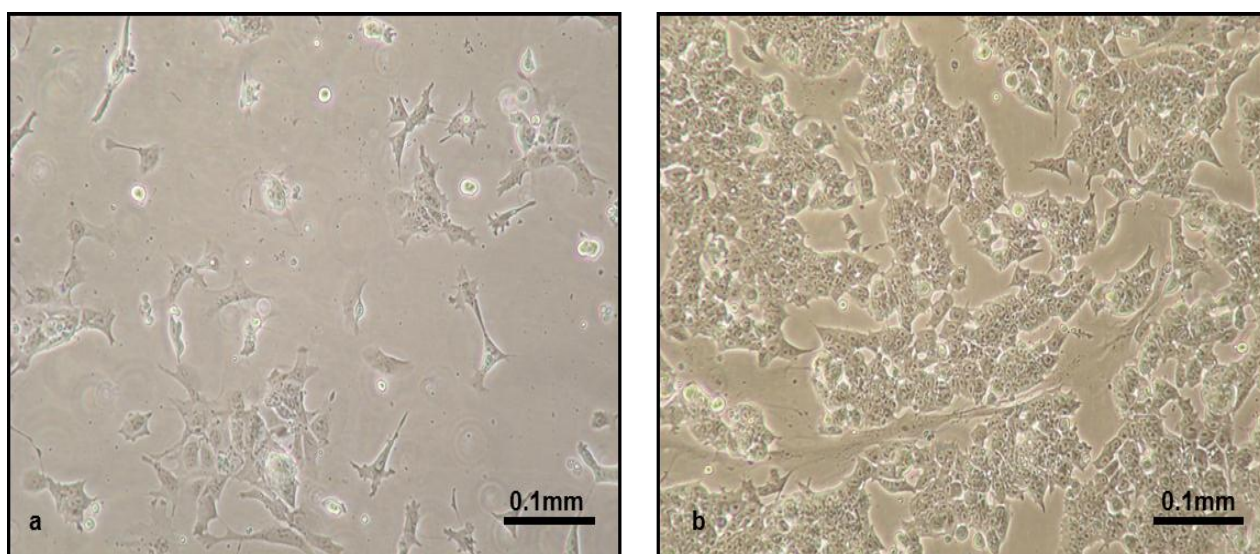


Figure 3.4: E14Tg2A growing on gelatinized flasks at different passages. (a) mES cells after bringing from liquid nitrogen. (b) mES cells with flatten and uniform morphology and then some were cryopreserved and some were used to set up differentiation (all pictures were taken at 10X magnification).

### **3.3.1.2. *In vitro* differentiation of mES cells to osteoblasts**

ES cells were first grown in gelatinized flasks in undifferentiated state and then allowed to form aggregates, i.e. embryoid bodies, in bacterial dishes (Figure 3.5) which were then disaggregated and plated in medium supplemented with osteogenic supplements as described in detail in methods section 2.2.2.

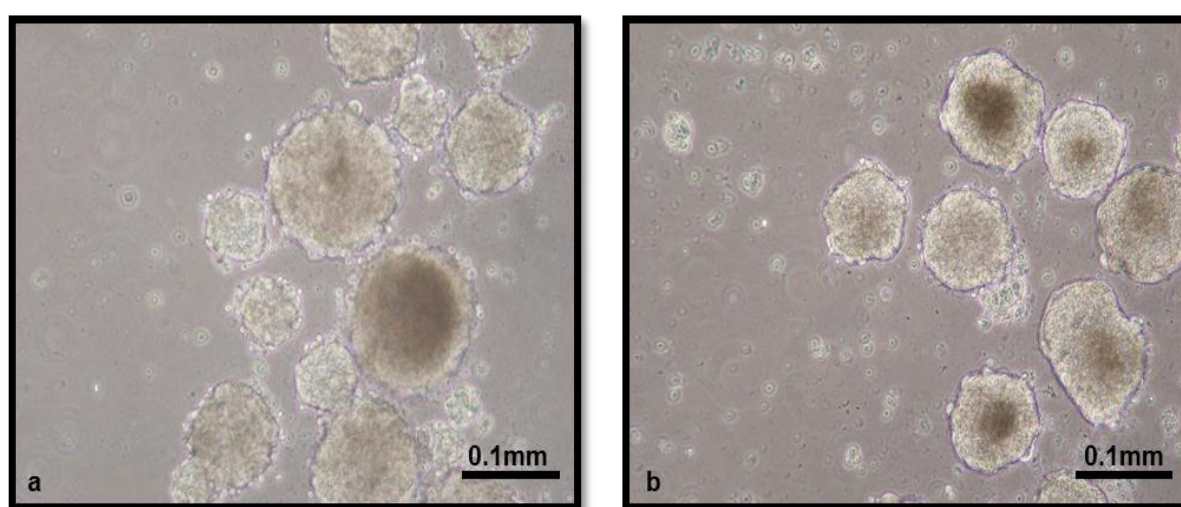


Figure 3.5: (a-b) E14tg2a cells at day-3 in EB medium forming EBs (all pictures were taken at 10X magnification).

Previous research work indicates that embryonic stem cells grown in presence of osteogenic supplements (OS) form colonies with mineral deposition. Mineralization can be observed as a black layer surrounding the cells by phase contrast microscopy when differentiating osteoblasts form calcified matrix and as shown in Figure 3.6 the majority of the cells by day-14 had started to form a dense matrix which was further confirmed by alizarin red staining, stain extraction and quantification. Alizarin red staining has been used historically to demonstrate the presence of calcium-rich deposits in histology for staining bones and skeleton (Puchtler *et al.*, 1969). The staining mechanism is thought to involve absorption of alizarin red molecule on calcium of calcium hydroxyapatite, a major component of calcified

bone by chelate formation (Moriguchi *et al.*, 2003). Use of Alizarin red dye has an added advantage in particular that, it can be later extracted from the entrapped cells and then can be used as a quantitative index to access the extent of mineralization (Gregory *et al.*, 2004).

OS supplements used in medium were ascorbic acid, beta glycerol phosphate and dexamethasone. Beta glycerol phosphate has been reported to act as a source of phosphate ions for *in vitro* mineralization and ascorbic acid is required for formation of collagenous matrix (Binderman *et al.*, 1986; Chentoufi *et al.*, 1993). As can be seen in Figure 3.6 and 3.7, the cell line E14tg2a when cultured under these conditions, displays a gradual differentiation towards the osteogenic lineage with increasing density of dark granules that stained red with Alizarin red dye. Cells grown in same medium but lacking osteogenic supplements i.e. beta glycerol phosphate, ascorbic acid and dexamethasone were taken as control and found to be of mixed morphology and to some extent showed mineral deposition towards the end of differentiation experiment which was further confirmed by alizarin red staining (Figure 3.7).

However this also need to be pointed that this medium composition may also lead to nonspecific mineral deposition which may not be cell mediated and so additional characterization for the presence of matrix associated proteins was also carried out to ascertain whether mineralization is cell mediated or nonspecific. Osteocalcin as discussed in section 3.4 is one of the major and matrix specific protein in osteoblasts. Gene expression analysis and immunocytochemistry were employed to stain for the expression of osteocalcin at the levels of RNA and protein in differentiating cultures and results are shown in Figures 3.22, 3.20 and 3.21 respectively. As is evident from these figures that expression of osteocalcin both at RNA and protein level coincided well with the pattern of Alizarin red

staining strengthening the observation that cells grown under these culture conditions might be mineralizing.

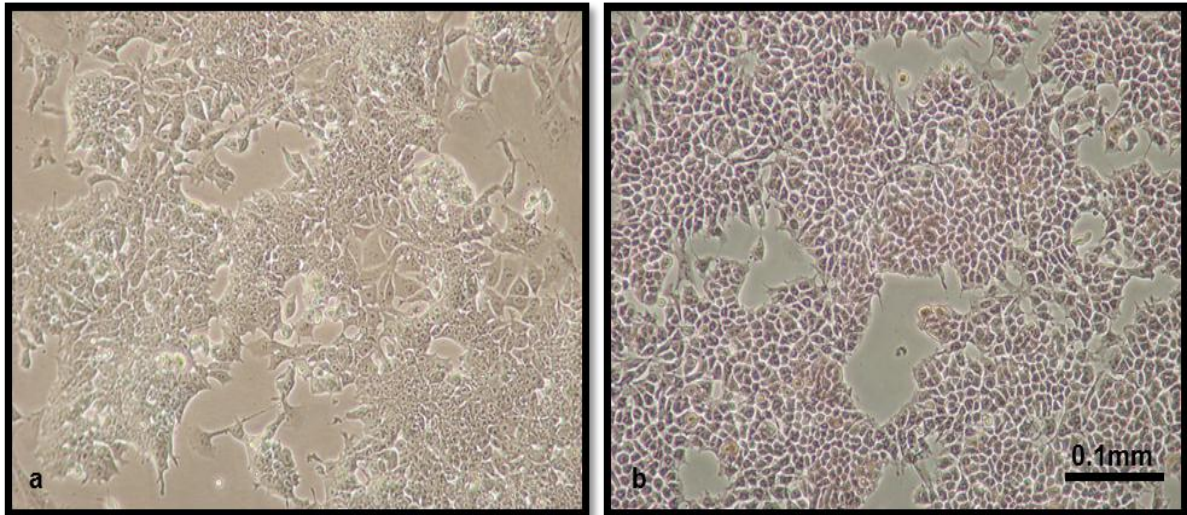


Figure 3.6: E14tg2a cells at day-0 before starting differentiation treatment (a) and stained with alizarin red dye (b) (all pictures were taken at 10X magnification).

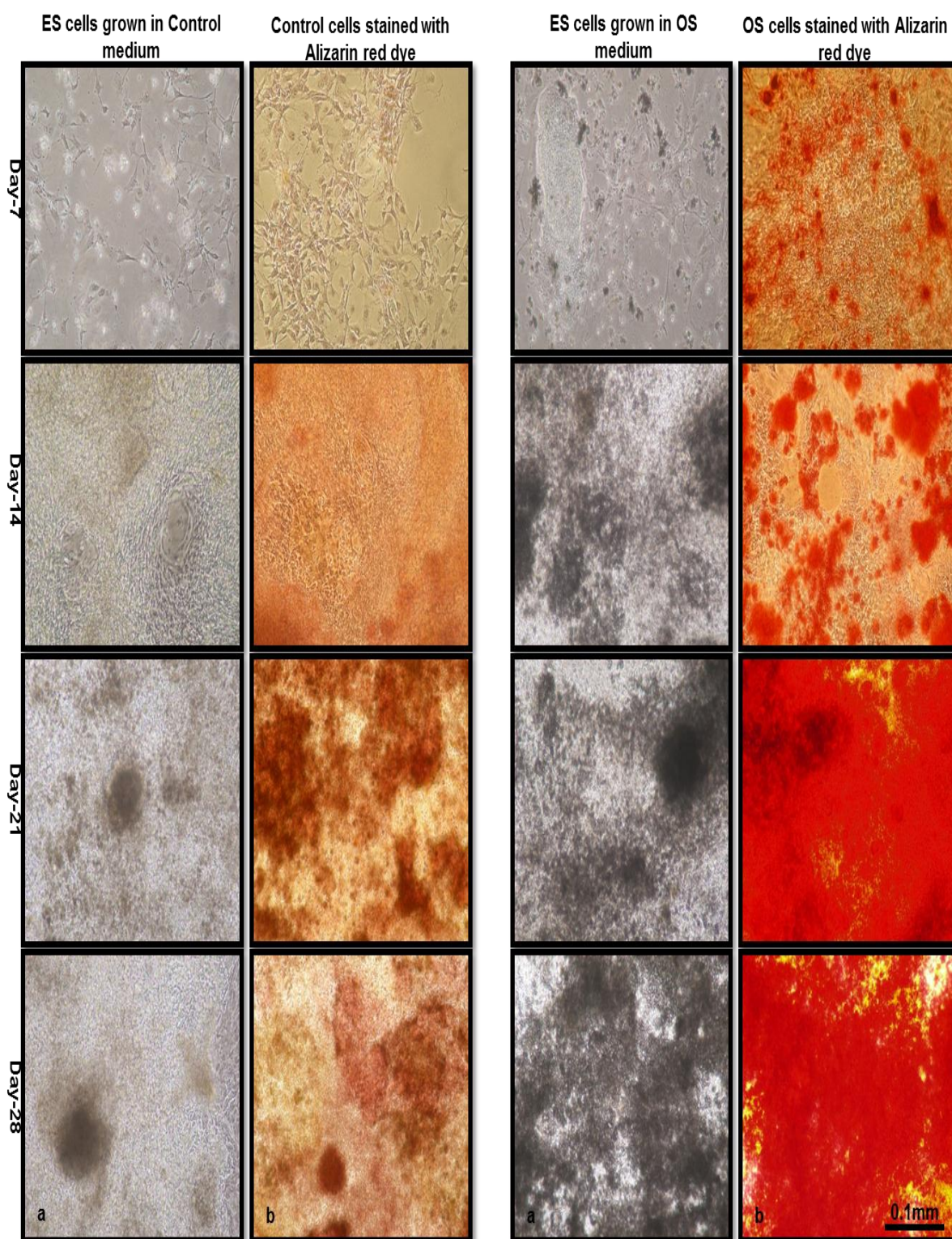


Figure 3.7: E14tg2a cells grown in medium supplemented without and with ascorbic acid, beta glycerol phosphate and dexamethasone i.e. control and OS respectively and analysed by microscopy (a) and alizarin red staining (b). Mineral deposition can be seen as densely dark areas as cells differentiate (a) and they stained dark red with alizarin red (b) (All pictures were taken at 10X magnification). It has been detailed earlier in section 3.3.1.2 that such a staining could also be possible due to non-specific calcium accumulation in culture and hence more data is presented in section 3.3.3 and 3.3.4 to support these results.

### 3.3.2. Alizarin red dye extraction and quantification

Alizarin red dye was extracted after staining of cells and quantified as described in detail in chapter 2 (section 2.4.1) and normalized to cell count (section 2.4.2). Briefly the amount of alizarin red incorporated by cells after staining was calculated using a standard curve (Figure 3.8). This amount of alizarin red was then normalized to cell count of that well measured by DNA quantification (Figure 3.9). Since technical limitation did not permit to perform both quantification assays using the same well, two wells of six-well plates were seeded at the time of setting up differentiation experiment with equal number of cells and treated alike in every respect except the quantification assay itself.

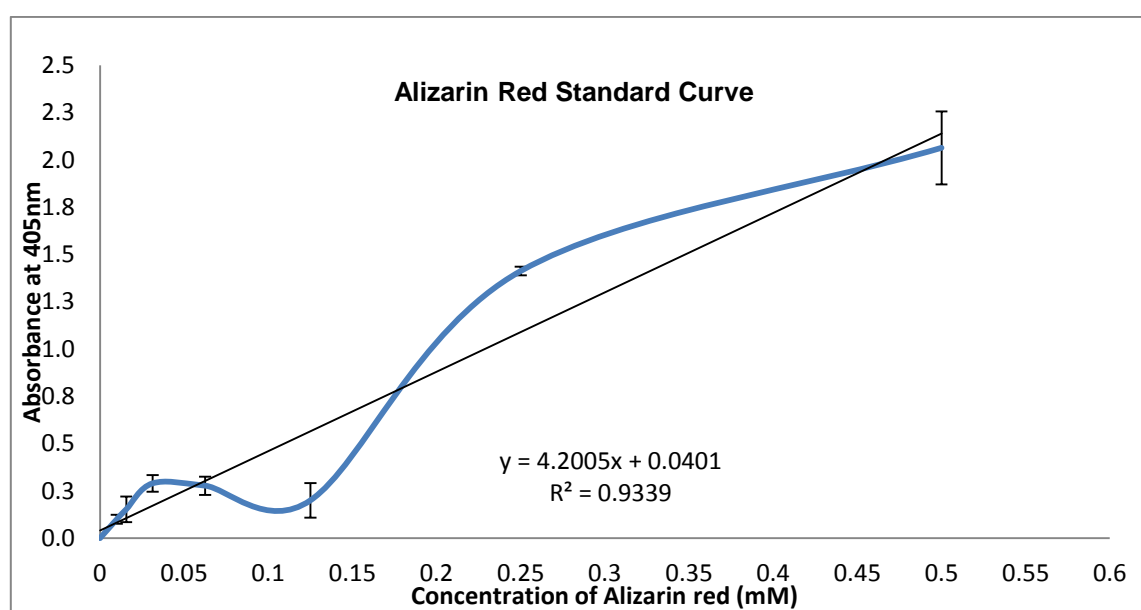


Figure 3.8: Alizarin red standard curve generated to calculate the amount of alizarin red dye extracted from cells (data is collected from two plates and each analysed in triplicates so mean $\pm$ SEM with n=2)

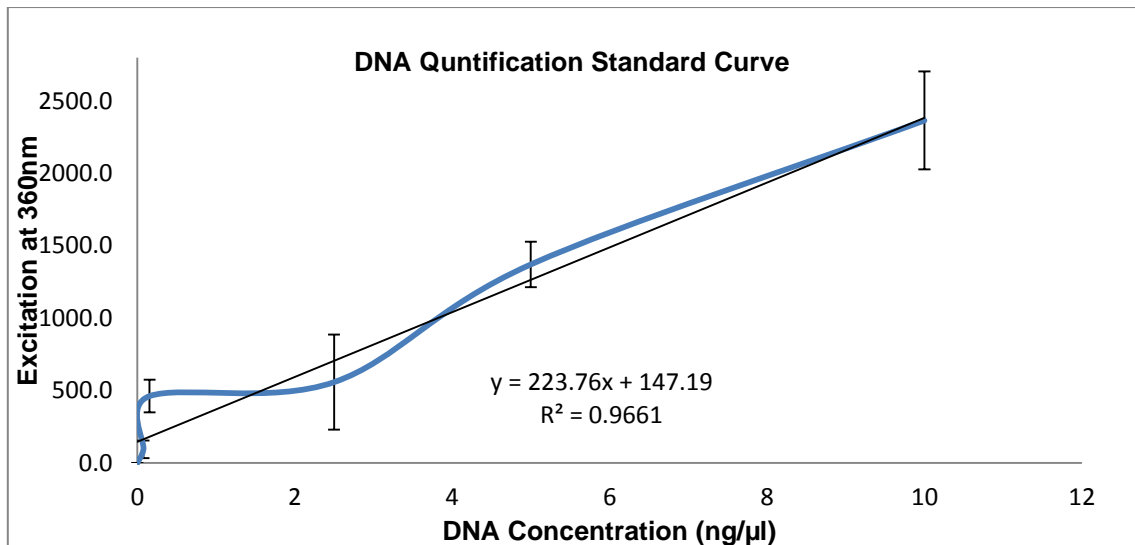


Figure 3.9: DNA calibration curve generated to normalize the amount of alizarin red dye relative to cell contents of plates (data is collected from two plates and each analysed in triplicates so mean±SEM with n=2)

Cells grown in medium containing osteogenic supplements showed a marked increase in amount of alizarin red dye extracted in comparison to cells grown in medium devoid of them after two weeks of culturing as shown in Figure 3.10.

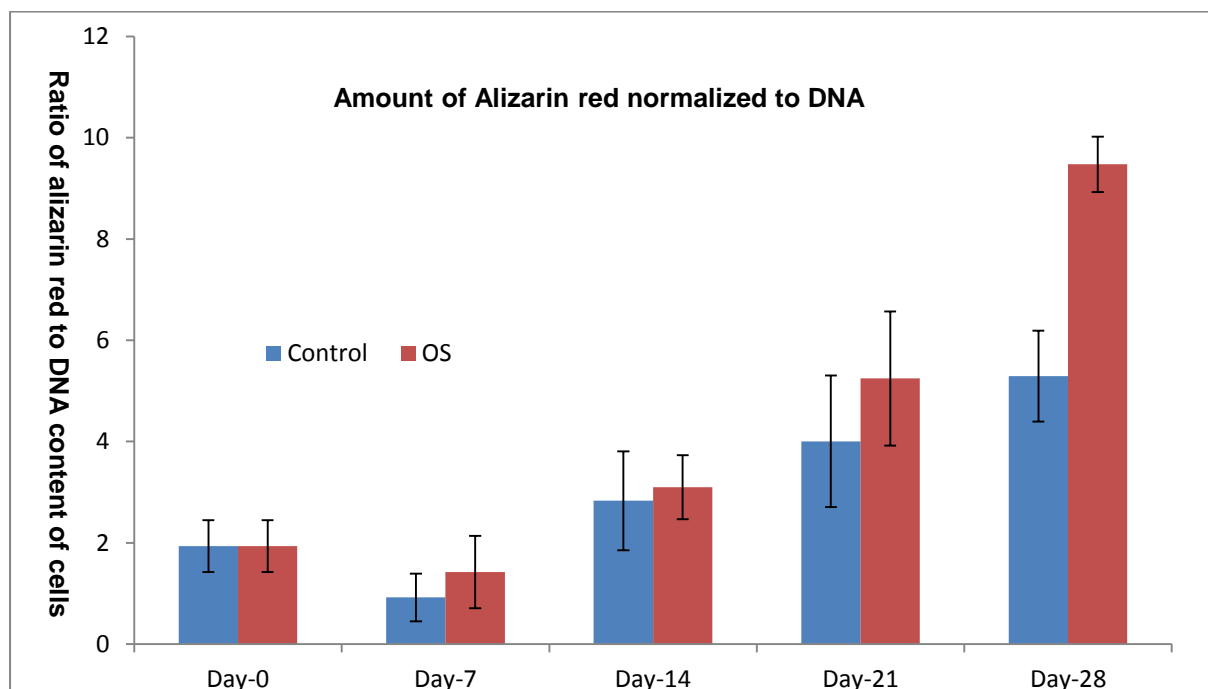


Figure 3.10: Mineral deposition was detected by Alizarin red dye and then dye was acid extracted and quantified using a colourimetric assay. Data are collected from two plates, each analysed in triplicates and expressed as mean±SEM (n=2). There seems to be a marked difference in amount of alizarin red extracted from treated and untreated cells.

It needs to be emphasized here from statistical point of view that this particular assay was carried out twice in triplicates for time-course samples obtained from two independent biological replicates because only one well of 6-well plate was seeded with cells to perform Alizarin red staining when differentiation experiment was set up for the first time. As explained earlier that technically it was not possible to carry out both alizarin red staining and DNA quantification assays using one well from multi-well plate, hence this was corrected in subsequent experiments and data collated and documented here.

### **3.3.3. Immunocytochemistry**

Immunocytochemistry was performed using antibodies against Sox2, Sox9, Osterix and Osteocalcin proteins to detect the localization and expression of these markers (antibody supplier details are in appendix 7.1). Mouse ES cells - E14tg2a (used in this study), mouse NSCs from lateral ventricle (kindly prepared and given by Stephanie Strouhbecker, a fellow student working with Dr Virginie Sottile), primary mouse calvaria and primary mouse osteoblasts cells (kindly given by Omar Qutachi, a fellow student working with Dr Lee Buttery) were used as positive control for Sox2, Sox9, Osterix and Osteocalcin antibodies respectively. And mouse primary calvaria cells were taken as negative control for Sox2 antibody and mouse ES (E14tg2a) were used as negative controls for Sox9, Osterix and osteocalcin respectively (Figures 3.11-3.18). In addition all these cells were also stained with primary antibody only (Figure 3.21), secondary antibody only (Figure 3.21) and no antibody to account for nonspecific and/or background staining( Figure 3.19-3.20).

Almost all of the cells in undifferentiated ESC culture were found to express Sox2 protein. Then only small population of the cells were found to express Sox2 protein at day-7, 14 and

21 in control cells and at day-28 no signal was detected (Figure 3.22). Similarly cells grown in medium containing OS supplements were found to express Sox2 at day-7 and day-14 and then no signal was detected as shown in Figure 3.23.

Sox9 protein expression was not observed in ESCs at day-0. Cells grown in medium lacking OS supplements started to show Sox9 protein expression at day-7 which appeared to persist until day-28 (Figure 3.24). While cells grown in medium containing OS supplements were found to express Sox9 at day-7 which increased with time until day-21 and then fewer cells were found positive a day-28. Some representative fields are shown in Figure 3.25. Sox9 expressing cells were more in number in OS-treated culture than non-treated cells.

Osterix protein was not found to be expressed at any time points chosen for analysis in cells grown with and without OS promoting factors as shown in Figure 3.26 and Figure 3.27.

Osteocalcin was not found to be expressed by ES cells at day-0. Cells were found be expressing osteocalcin starting from day-7 until day-28 grown in both kinds of medium with and without osteogenic supplements where signal in treated cells was significantly stronger than control population as can be seen in Figure 3.28 and 3.29.

An important consideration regarding all the figures shown on next few pages from Figure 3.11-3.21 is that Day-0 refers to undifferentiated embryonic stem cells (E14tg2a) before subjecting them to any differentiation treatment and is the common experimental starting point before seeding cells derived from EBs into either control medium or OS medium from Day-7 to Day-28 (schematics of experimental design are elaborated in section 3.2).

## Controls for Antibodies

### 3.3.3.1. Sox2 Positive

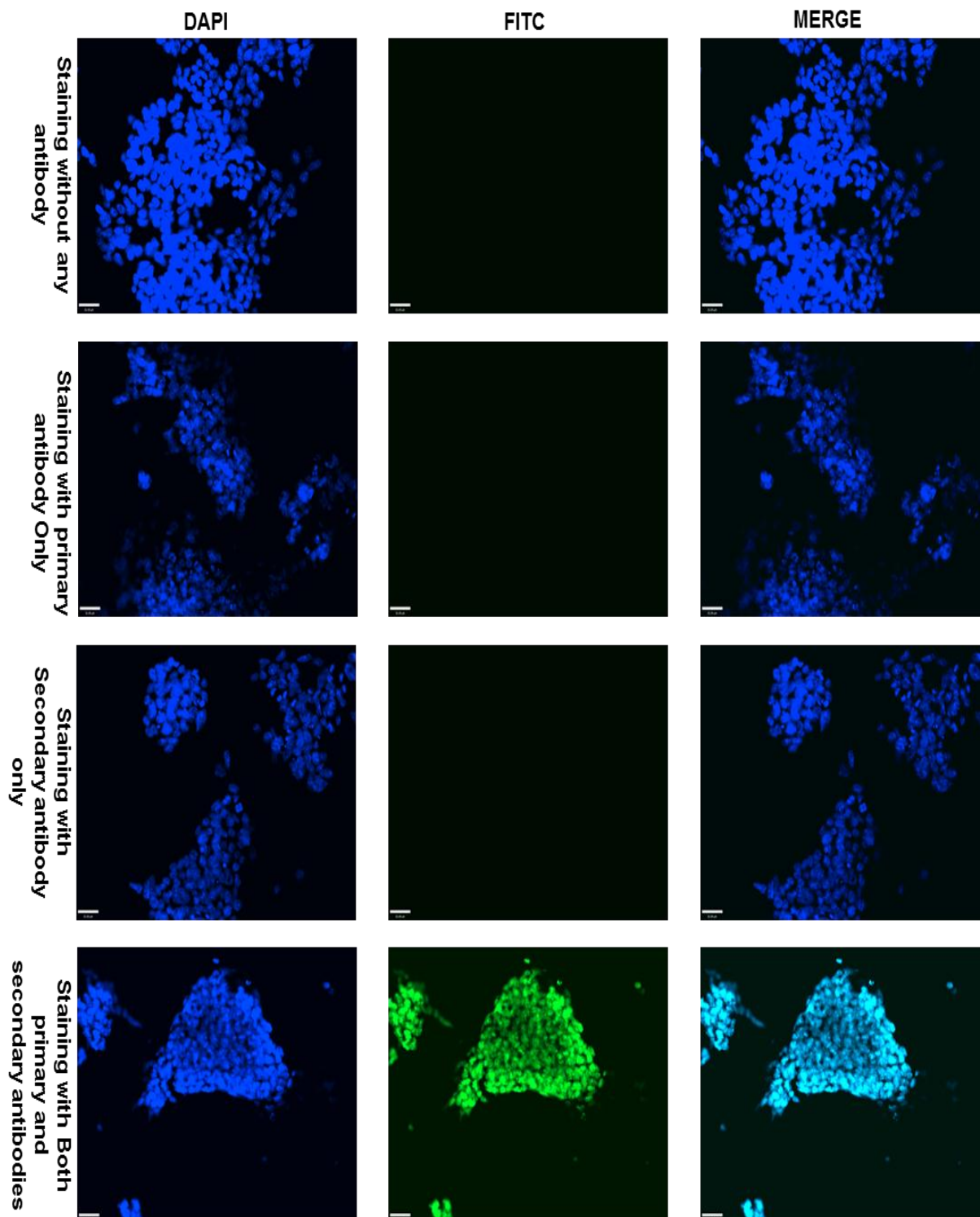


Figure 3.11: Mouse ES (E14tg2a) cells were used as positive control for Sox2 expression. ES cells were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Sox2). Only ES cells stained with both primary and secondary antibodies showed positive signal (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

### 3.3.3.2. Sox2 Negative

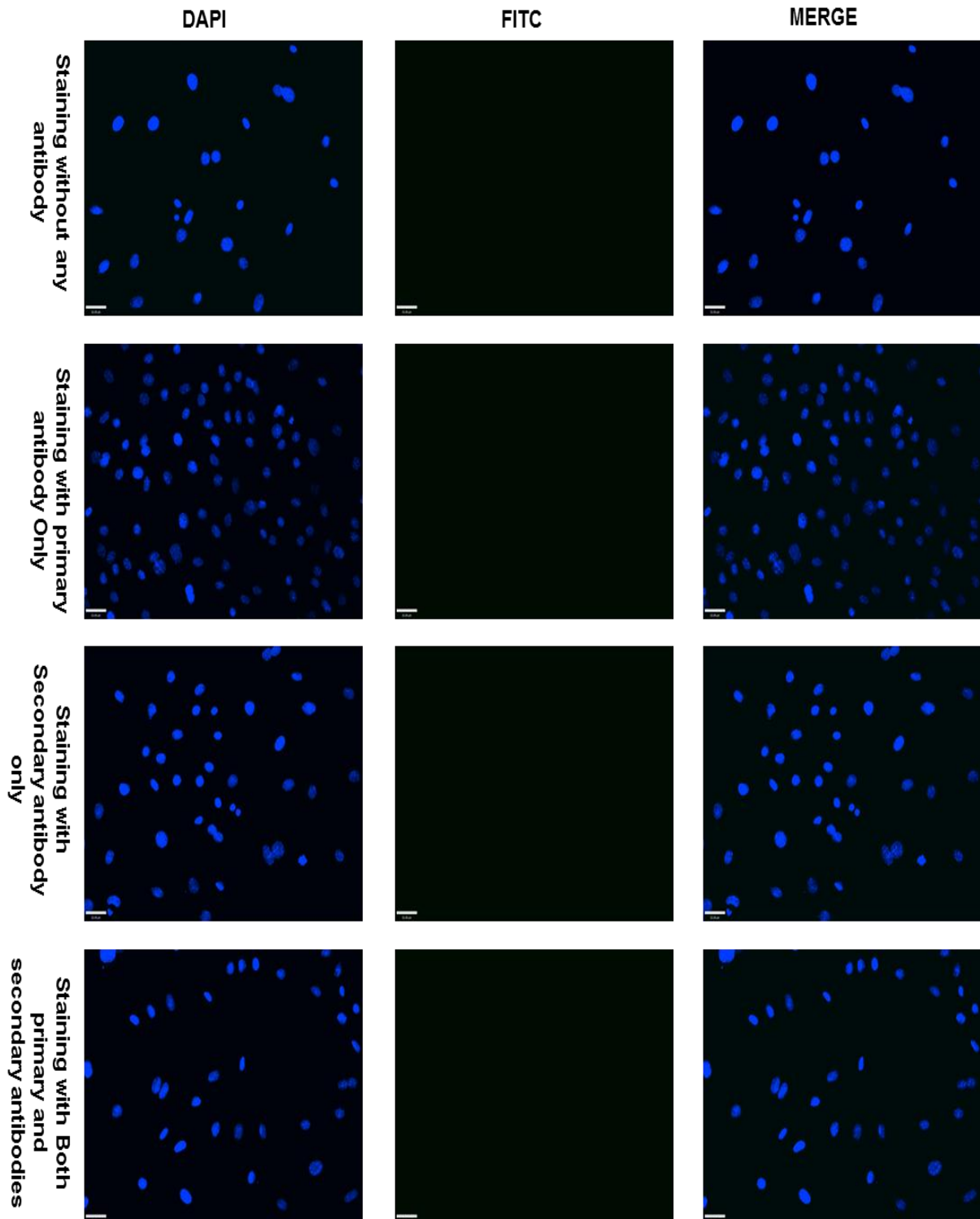


Figure 3.12: Primary mouse calvaria cells (kindly given by a colleague Omar Qutachi from Dr Lee Buttery research group) were used as negative control for Sox2 expression. Calvaria cells were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Sox2). No signal was detected (All pictures were taken at 20X magnification and scale bar is 32µm).

### 3.3.3.3. Sox9 Positive

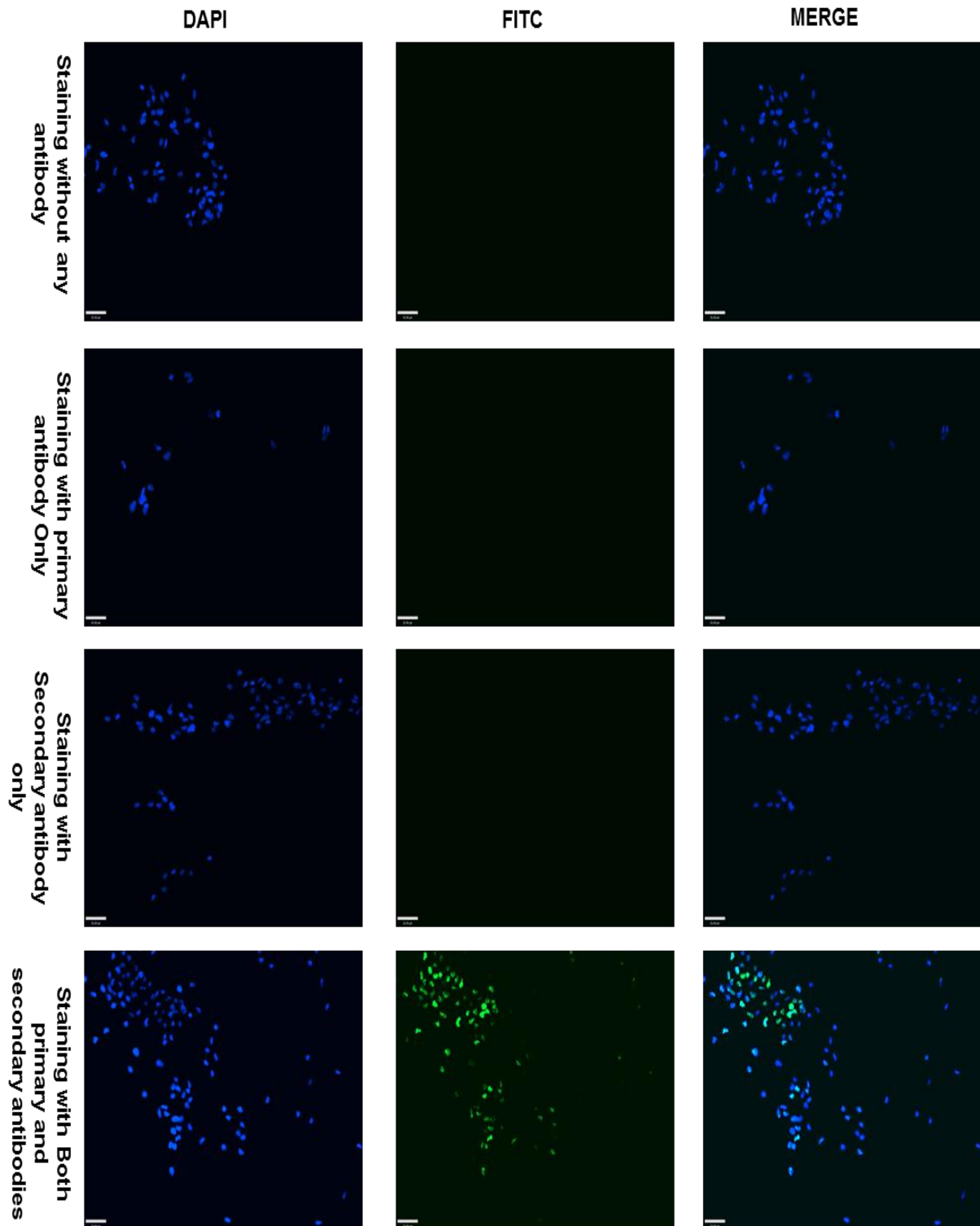


Figure 3.13: Mouse NSC cells isolated from lateral ventricle (kindly given by fellow research student Stephanie Strouhbecker from Dr Virginie Sottile research group) were used as positive control for Sox9 expression. NSCs were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Sox9). Only NSCs stained with both primary and secondary antibodies showed positive signal (All pictures were taken at 20X magnification and scale bar is 32µm).

### 3.3.3.4. Sox9 Negative

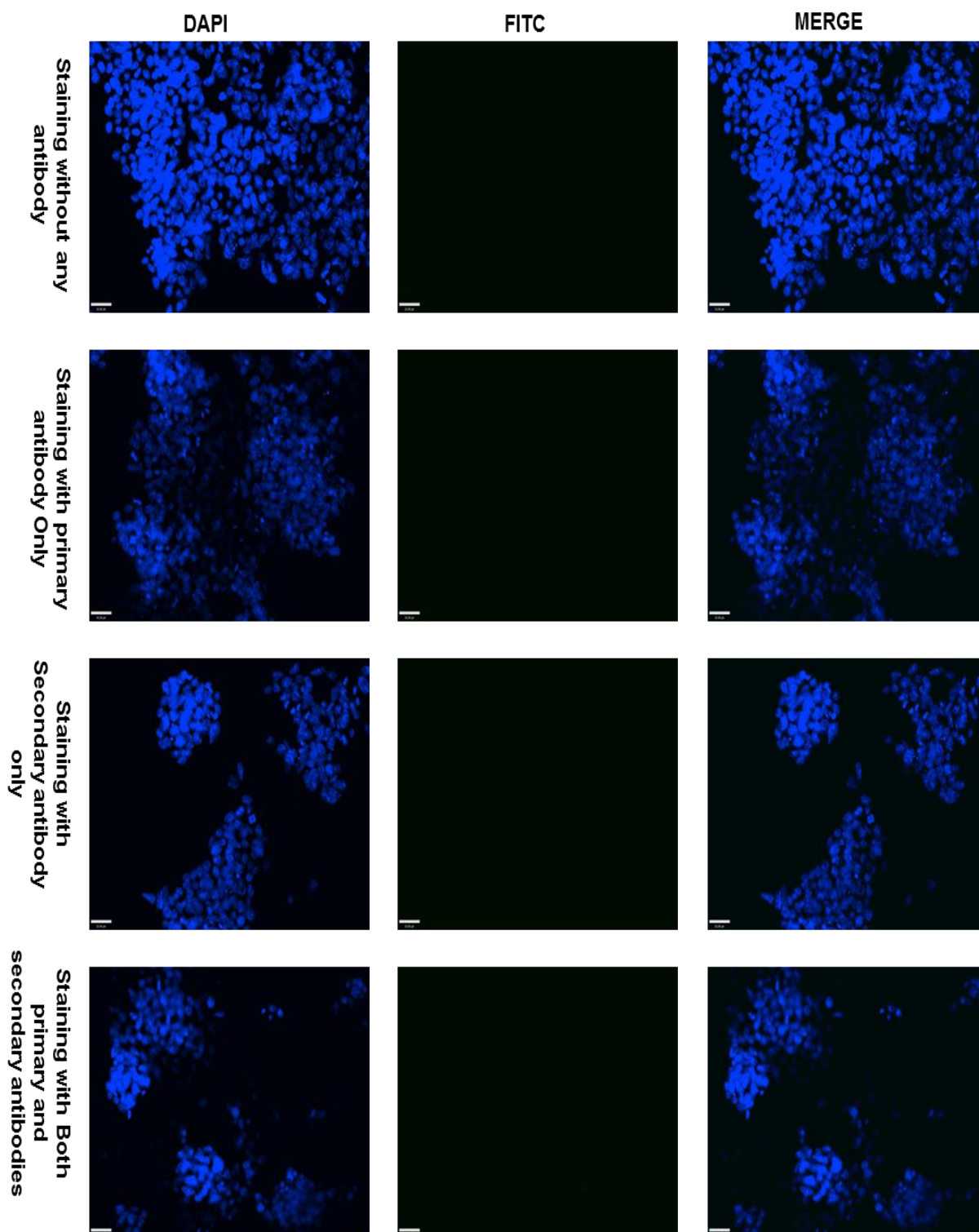


Figure 3.14: Mouse ES (E14tg2a) cells were used as negative control for Sox9 expression. ES were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Sox9). No positive signal was observed (All pictures were taken at 20X magnification and scale bar is 32µm).

### 3.3.3.5. Osterix Positive

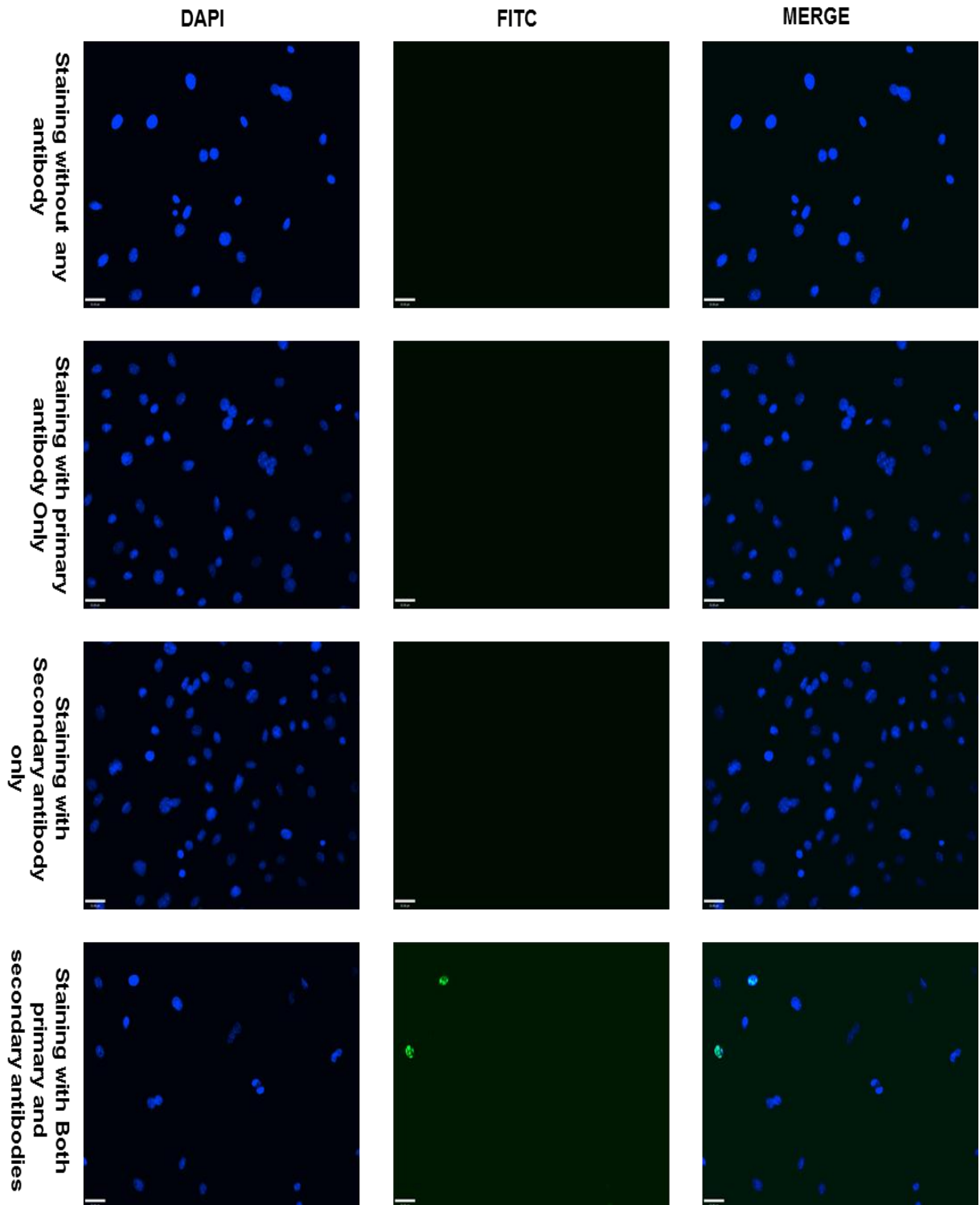


Figure 3.15: Mouse calvaria cells were used as a positive control for Osterix expression. Calvaria cells were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Sox2). Only few cells were found to be positively stained (All pictures were taken at 20X magnification and scale bar is 32µm).

### 3.3.3.6. Osterix Negative

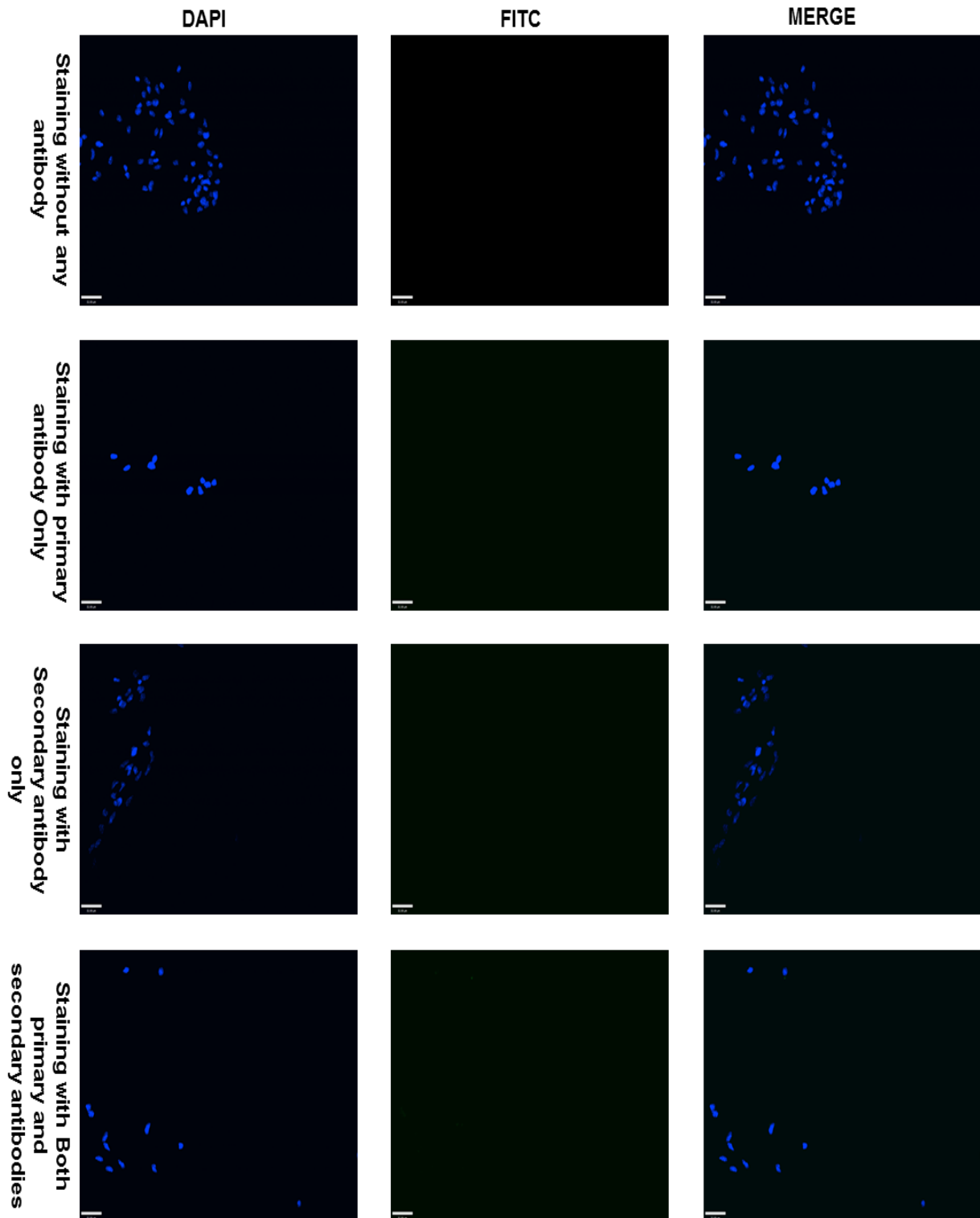


Figure 3.16: Mouse NSC (from lateral ventricle) cells were used as negative control for Osterix expression. NSCs were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Sox9). No signal was detected in any of the stained cells (All pictures were taken at 20X magnification and scale bar is 32µm).

### 3.3.3.7. Osteocalcin Positive

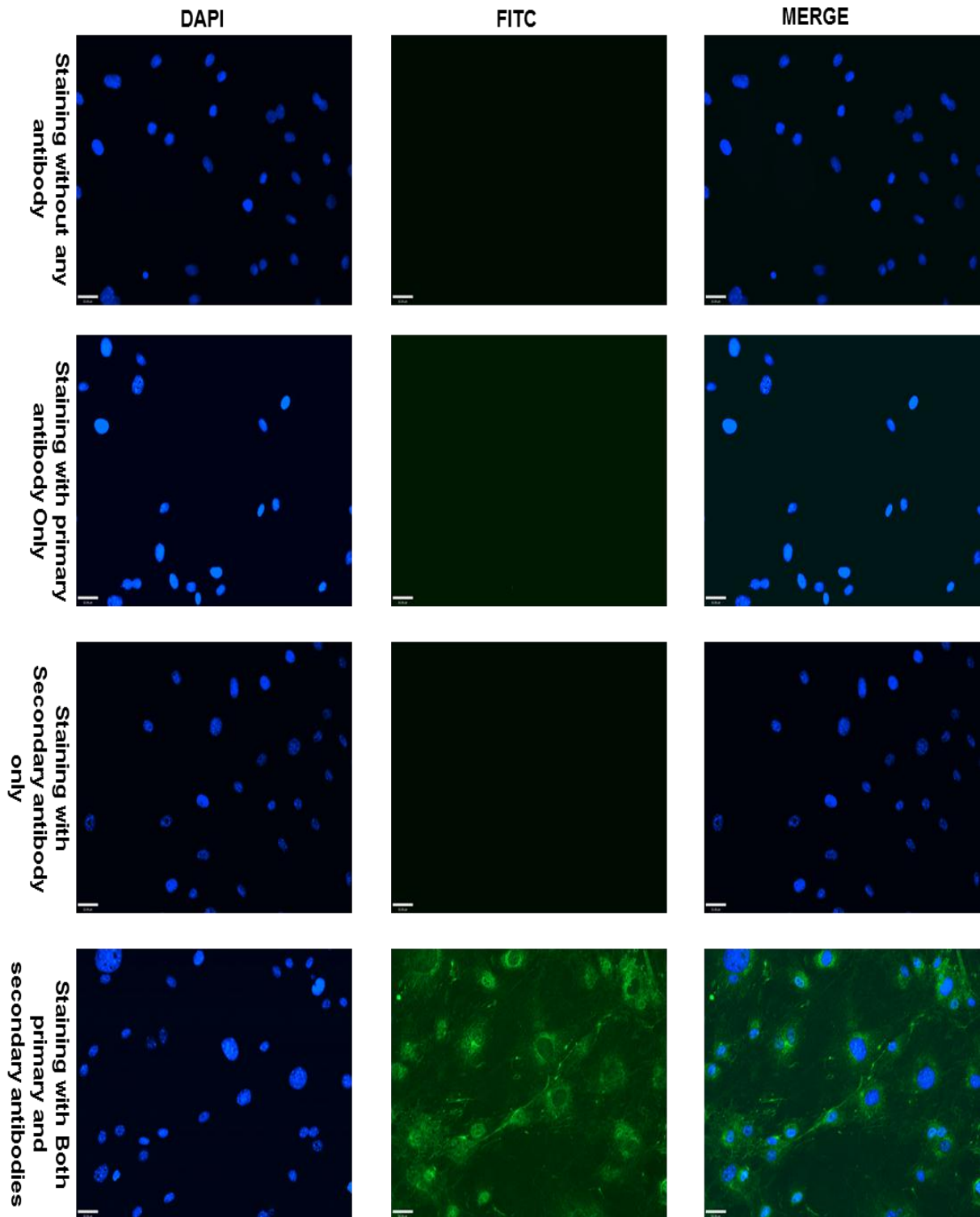


Figure 3.17: Primary mouse osteoblast cells (kindly given by a colleague Omar Qutachi) were used as a positive control for Osteocalcin expression. Osteoblast cells were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Sox2). Cells showed a cytosolic expression without any background (All pictures were taken at 20X magnification and scale bar is 32µm).

### 3.3.3.8. Osteocalcin Negative

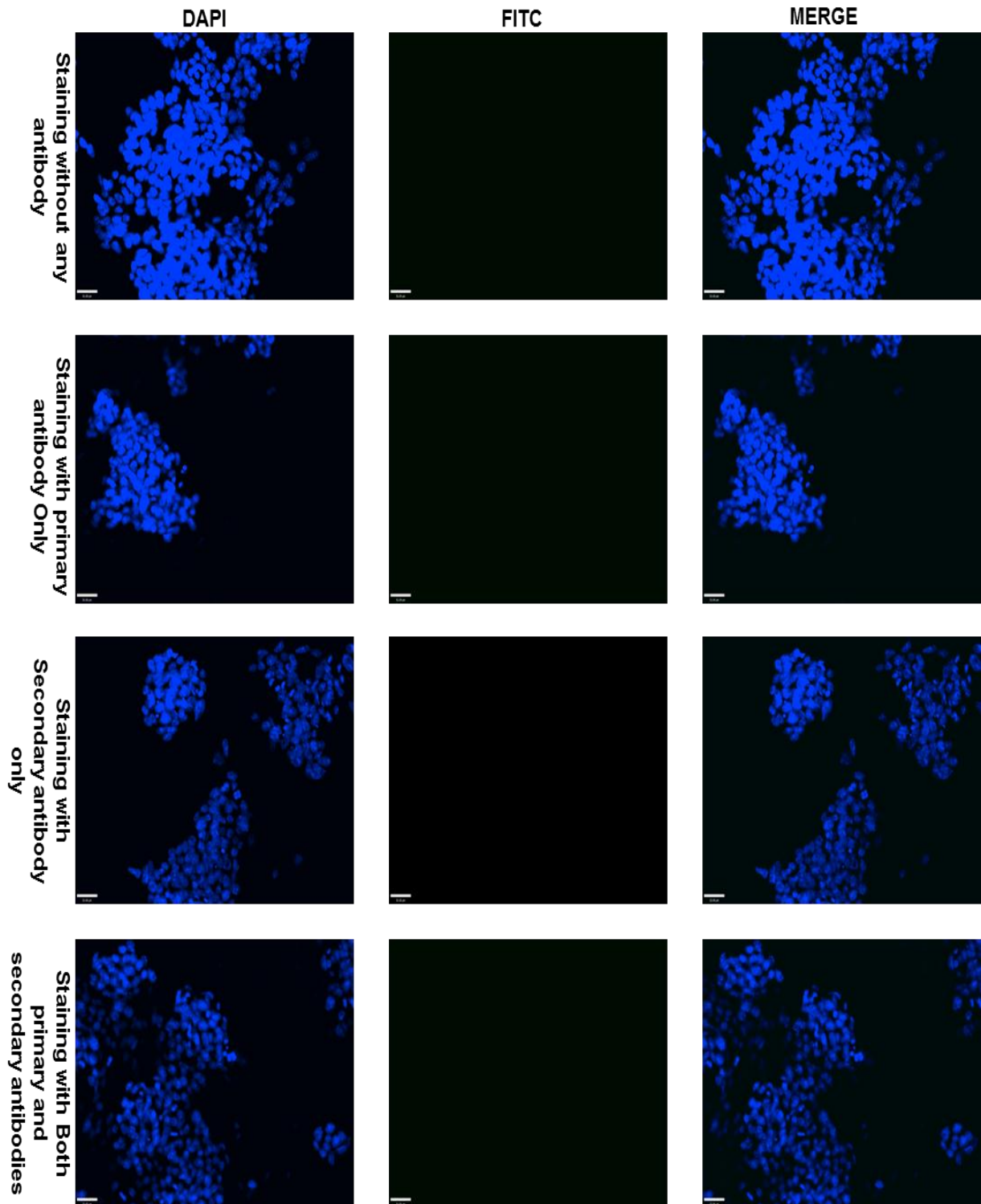


Figure 3.18: Mouse ES (E14tg2a) cells were used as negative control for Osteocalcin expression. ES were stained without any antibody, primary antibody only, secondary antibody only and both primary and secondary antibodies to account for auto fluorescence and/or nonspecific staining (Dapi was used to stain the nucleus and FITC conjugated secondary antibody against primary antibody Osteocalcin). No positive signal was observed (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

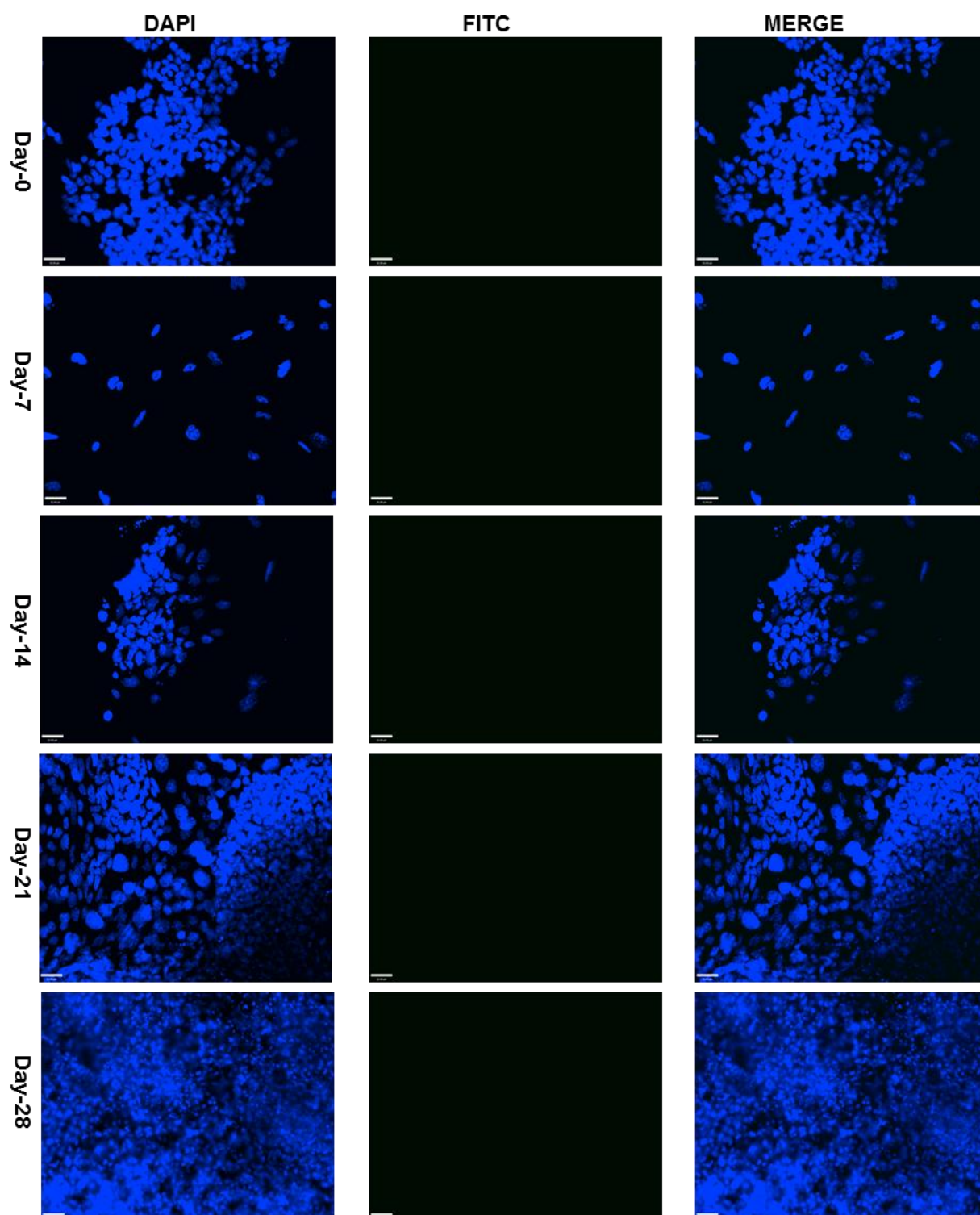


Figure 3.19: Mouse ES (E14tg2a) cells grown in control medium were stained without primary and secondary antibodies to account for auto fluorescence and/or background staining (Dapi was used to stain the nucleus). No fluorescence was observed (All pictures were taken at 20X magnification and scale bar is 32μm).

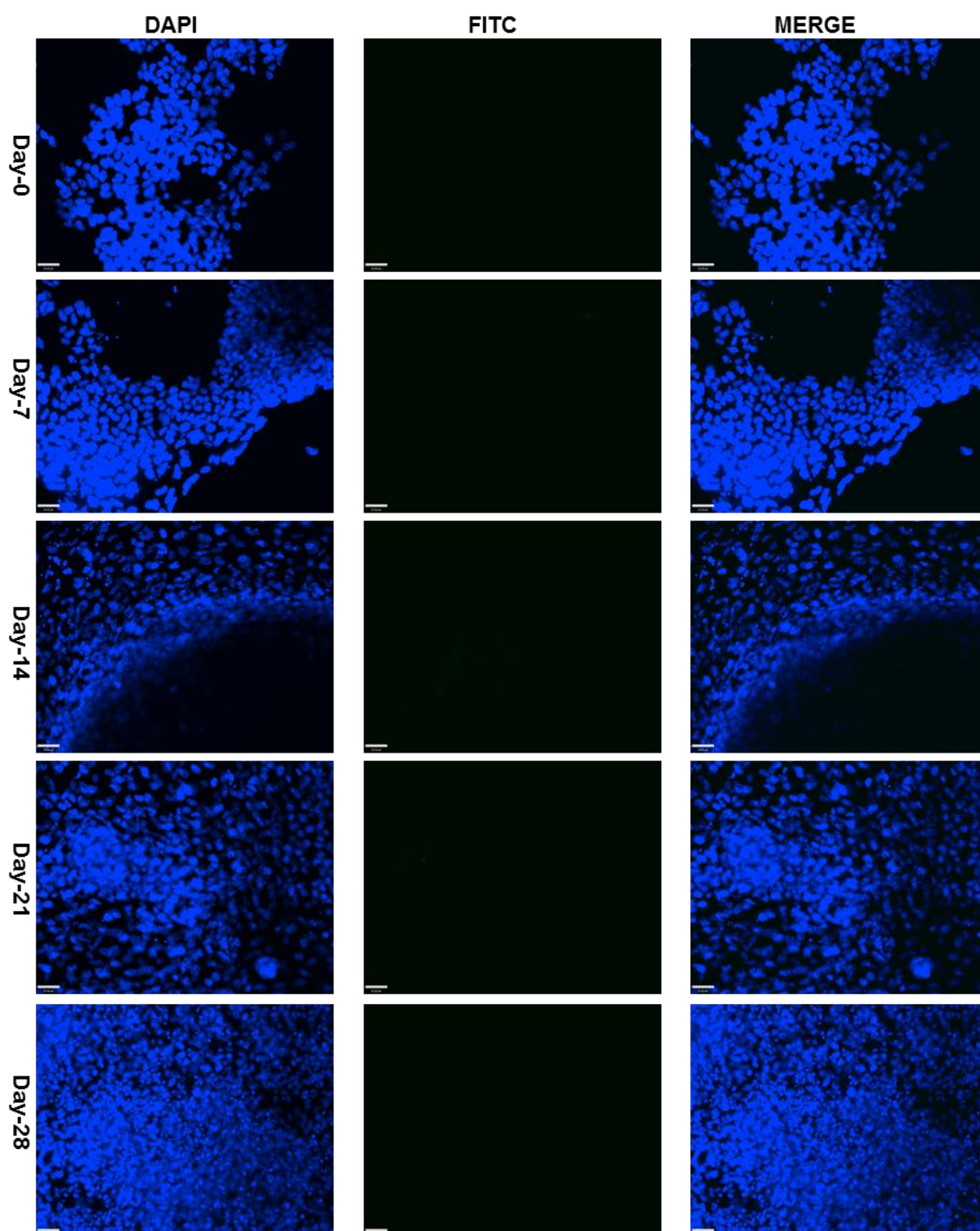


Figure 3.20: Mouse ES (E14tg2a) cells grown in OS medium were stained without primary and secondary antibodies to account for auto fluorescence and/or background staining (Dapi was used to stain the nucleus). No fluorescence was observed (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

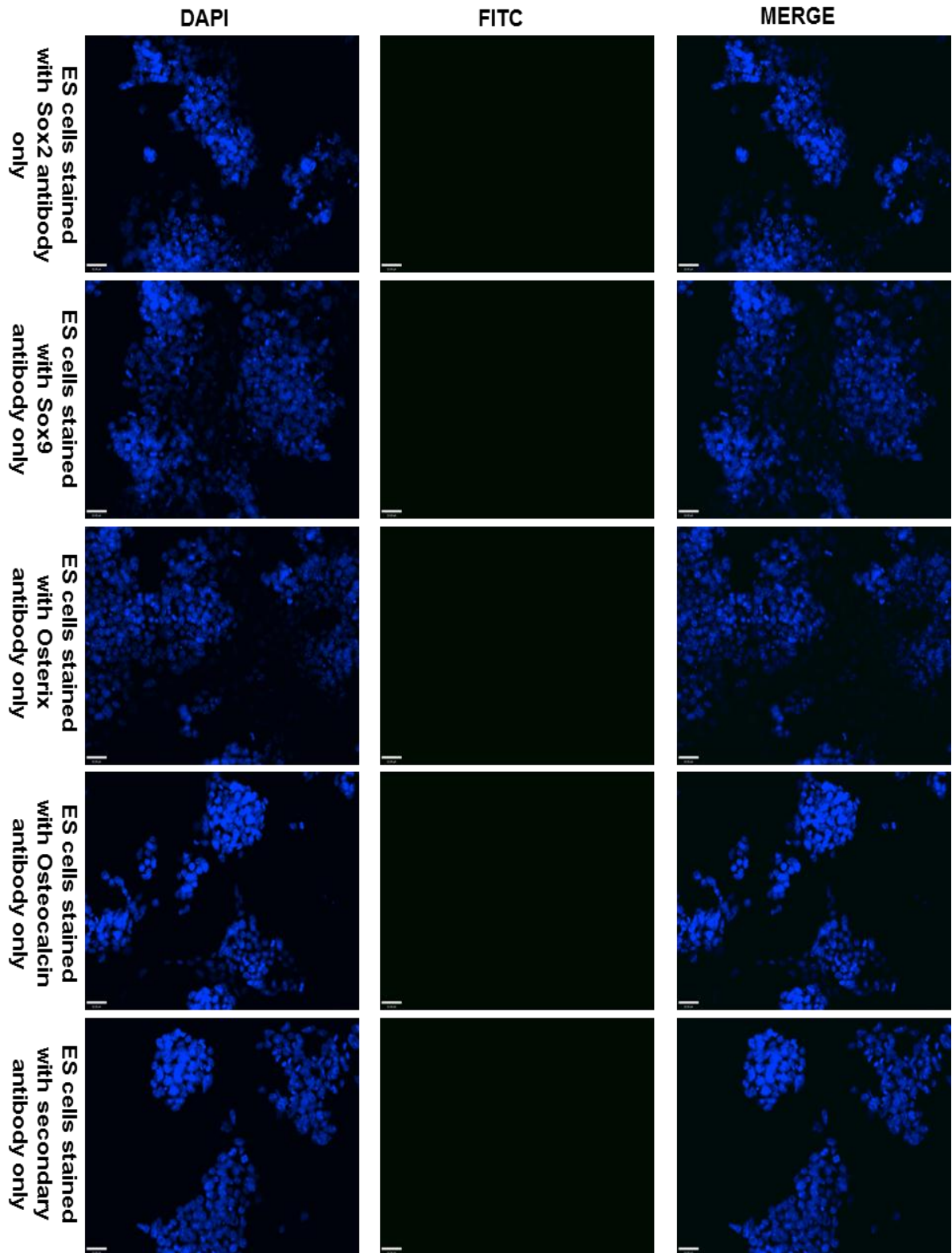


Figure 3.21: Mouse ES (E14tg2a) cells were stained with primary antibodies only and secondary antibody only to account for nonspecific fluorescence and/or background staining (Dapi was used to stain the nucleus). No fluorescence was observed (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

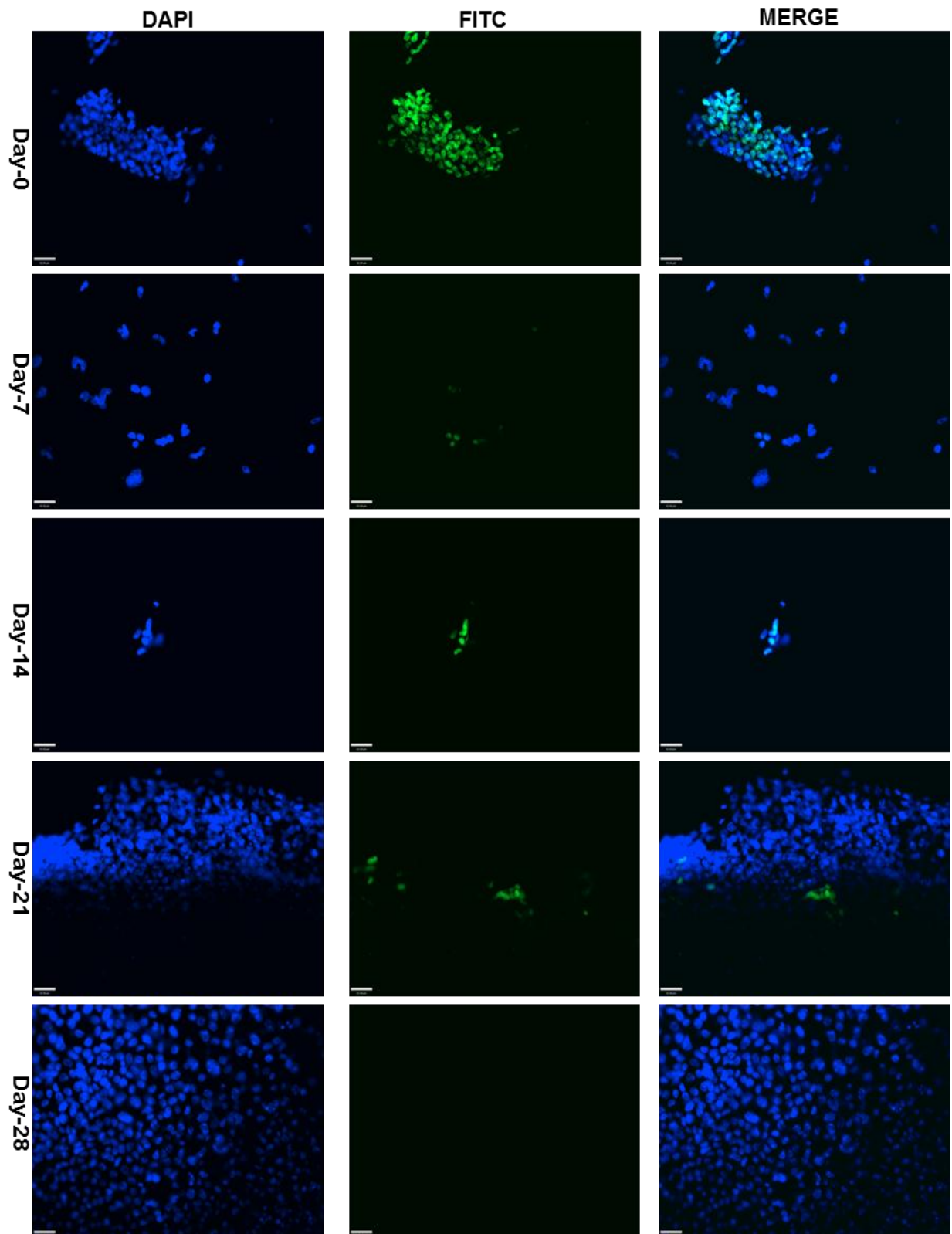


Figure 3.22: ESCs grown in medium lacking osteogenic supplements stained using antibody against Sox2 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox2) and found to be expressive at day-0 with a decreased level at day-7, day-14 and day-21 in cells. No signal for Sox2 was observed at day-28 (All pictures were taken at 20X magnification).

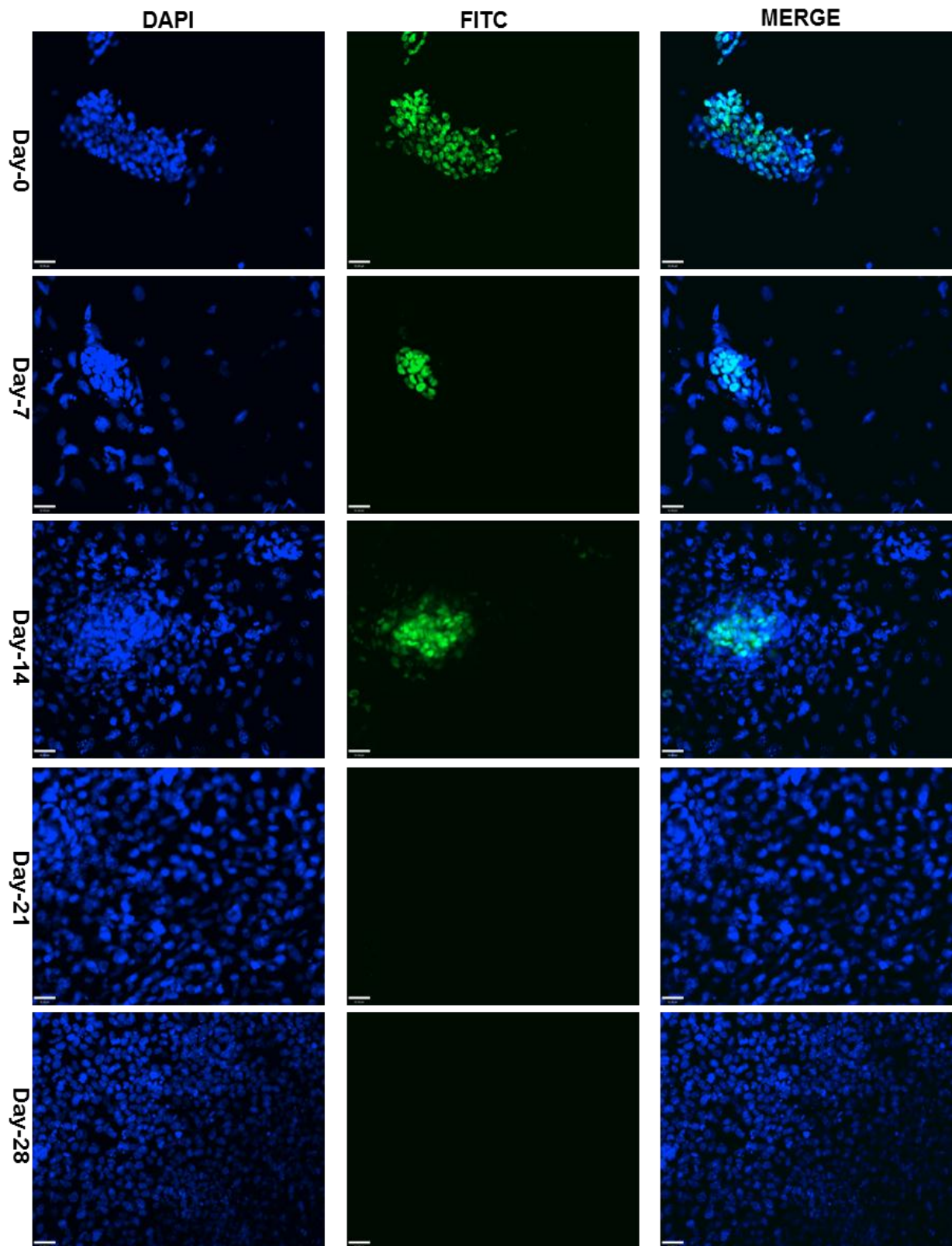


Figure 3.23: ESCs grown in medium containing osteogenic supplements stained using antibody against Sox2 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox2) and found to be expressive at day-0 with a decreased level at day-7 and day-14 in cells. No signal for Sox2 was observed at day-21 and day-28 (All pictures were taken at 20X magnification).

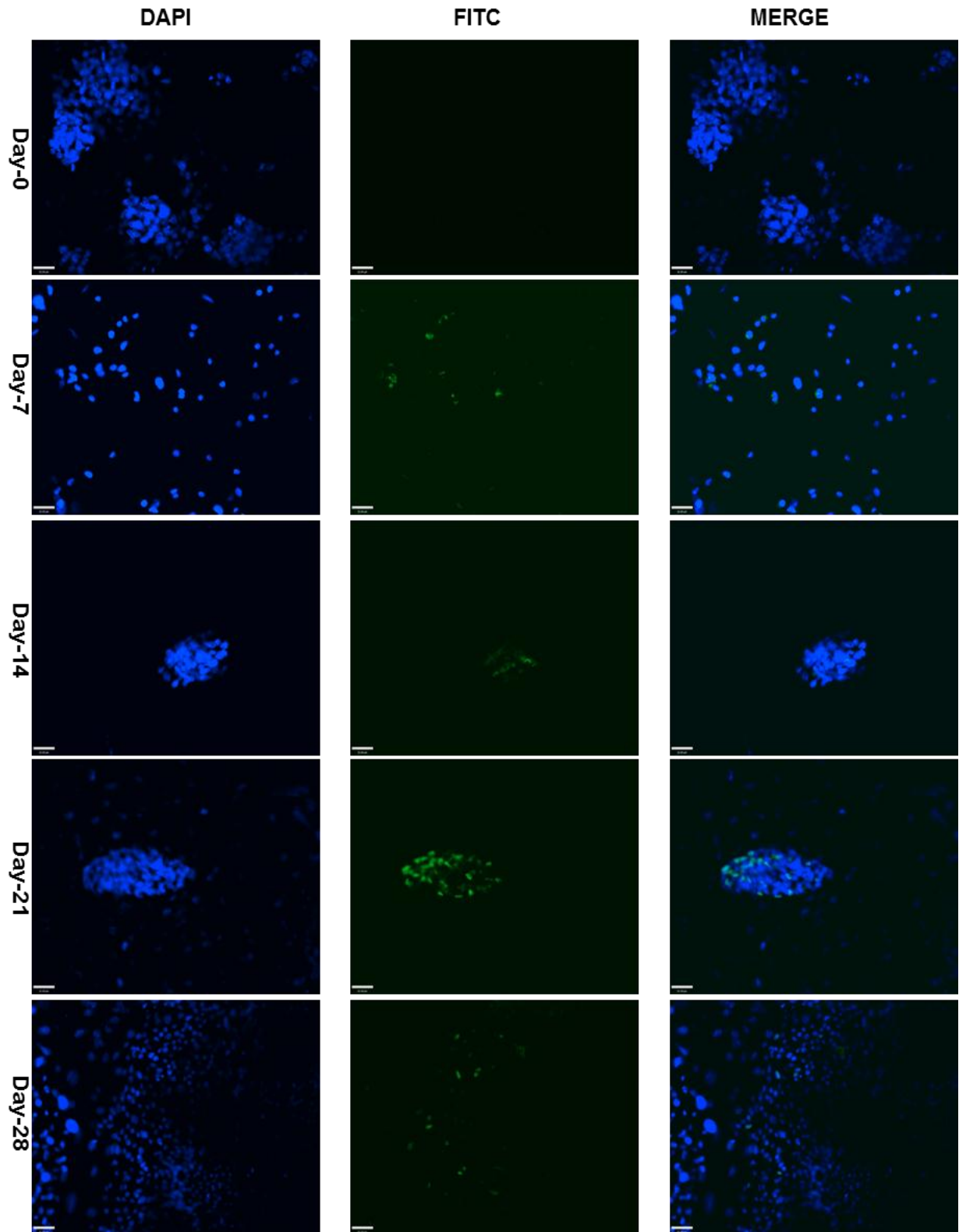


Figure 3.24: ESCs grown in medium lacking osteogenic supplements stained using antibody against Sox9 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox9). Expression of Sox9 was observed at all time points except at day-0 (All pictures were taken at 20X magnification). More cells appear to express Sox9 at day-21 than day-28.

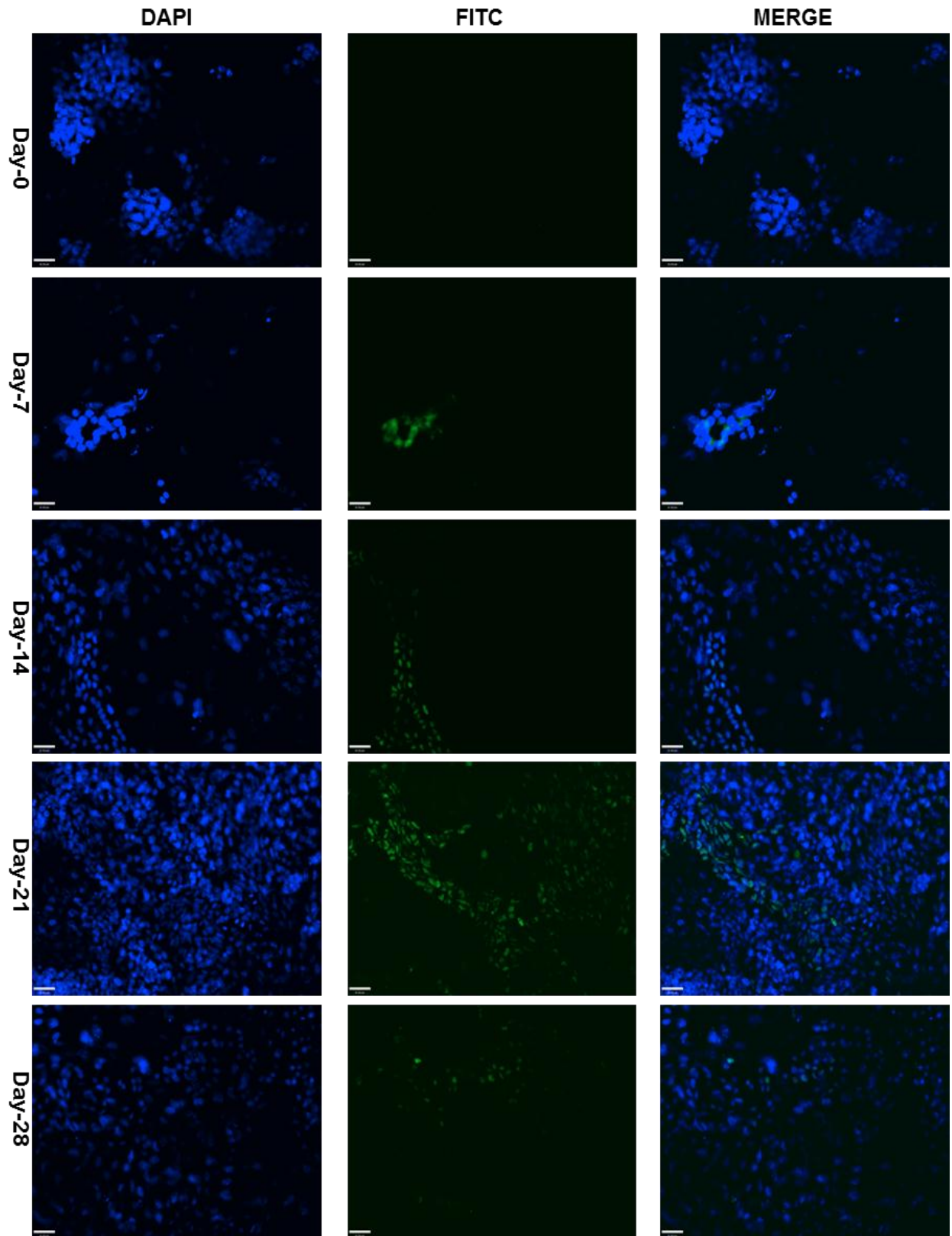


Figure 3.25: ESCs grown in medium containing osteogenic supplements stained using antibody against Sox9 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox9). Expression of Sox9 was observed at all time points except at day-0 (All pictures were taken at 20X magnification). It appears that number of Sox9 positive cells increased up to day-21 and then decreased at day-28.

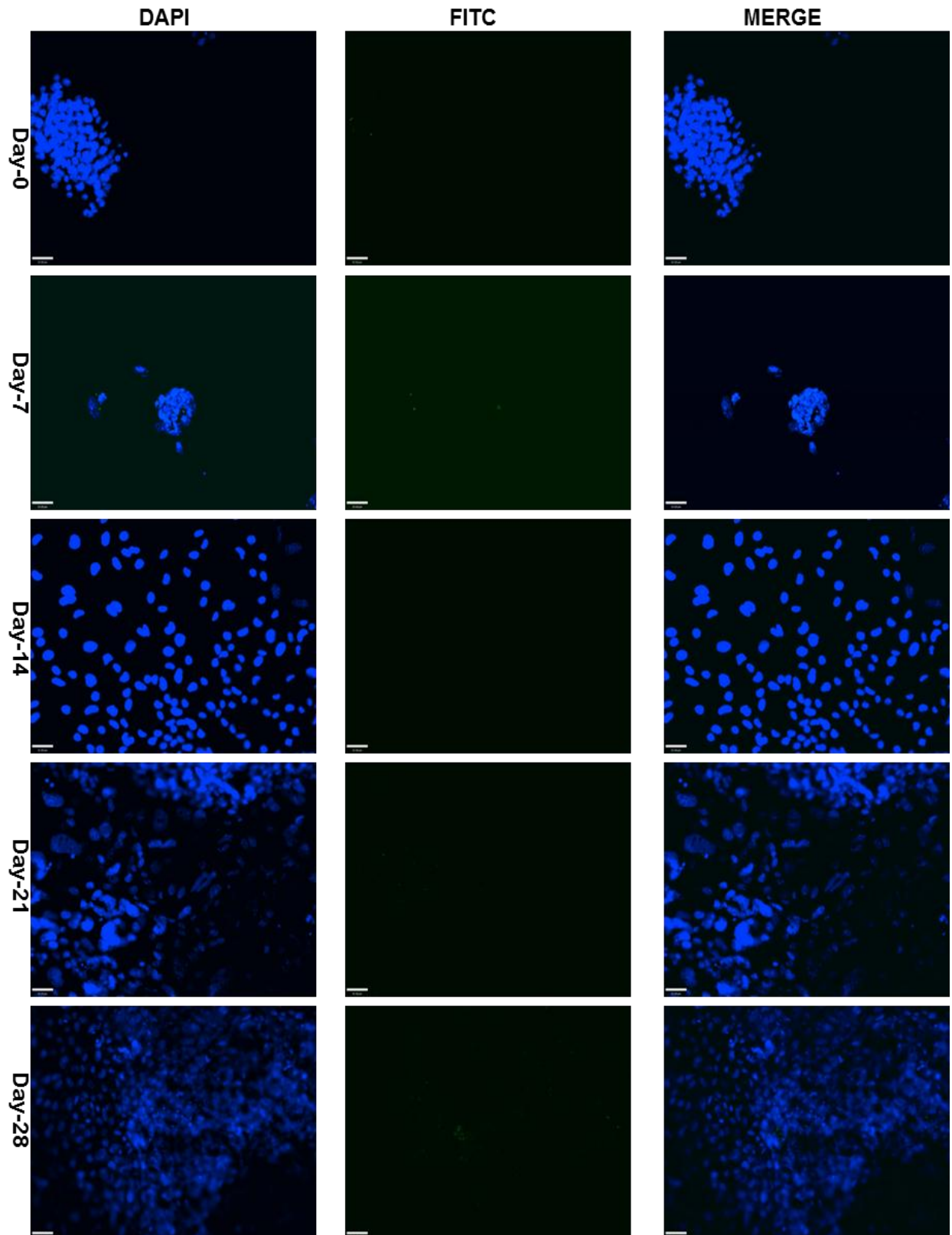


Figure 3.26: ESCs grown in medium lacking osteogenic supplements stained using antibody against Osterix protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osterix). No expression was observed at any time points selected for analysis (All pictures were taken at 20X magnification).

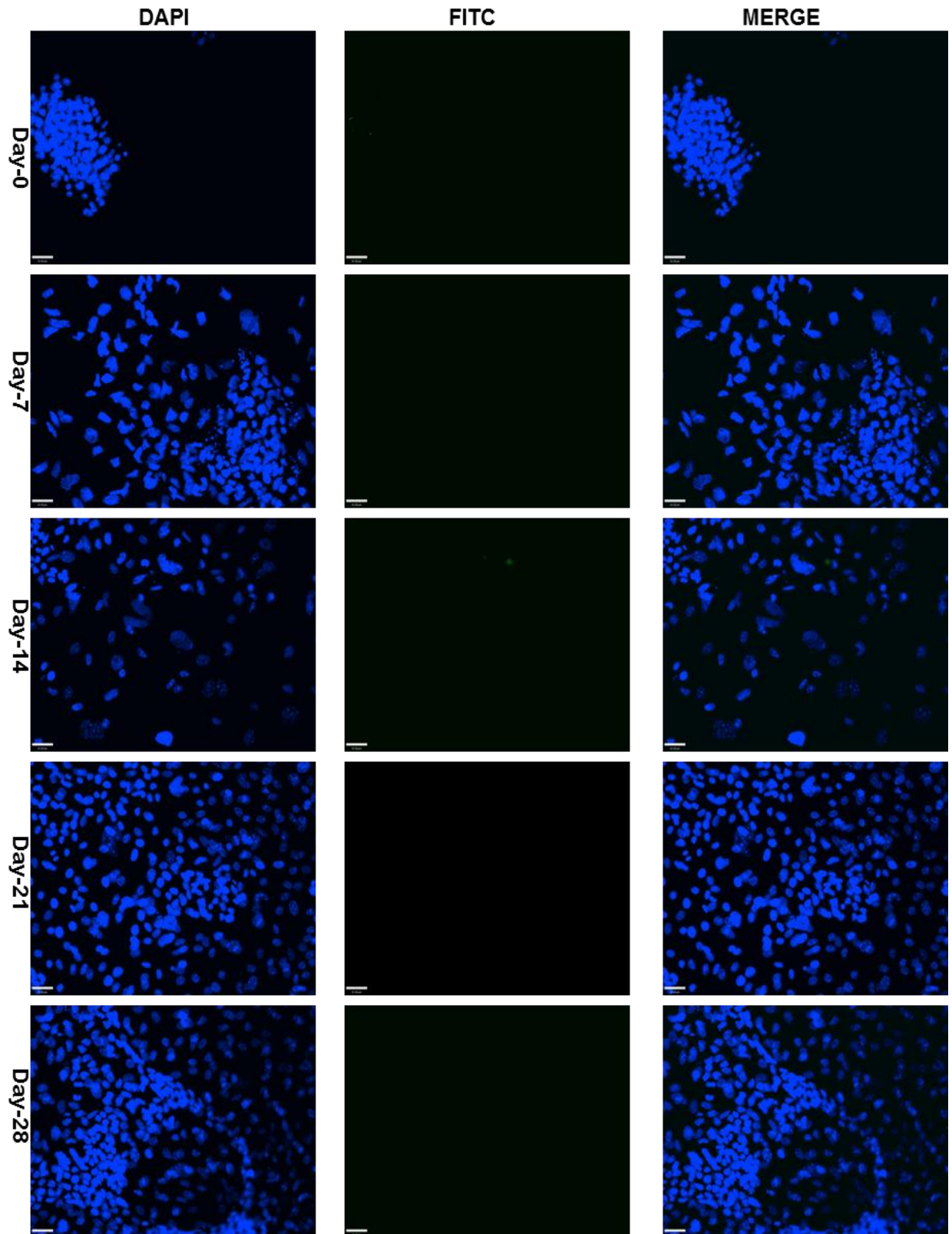


Figure 3.27: ESCs grown in medium containing oestrogenic supplements stained using antibody against Osterix protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osterix). No expression was observed at any time points selected for analysis (All pictures were taken at 20X magnification).

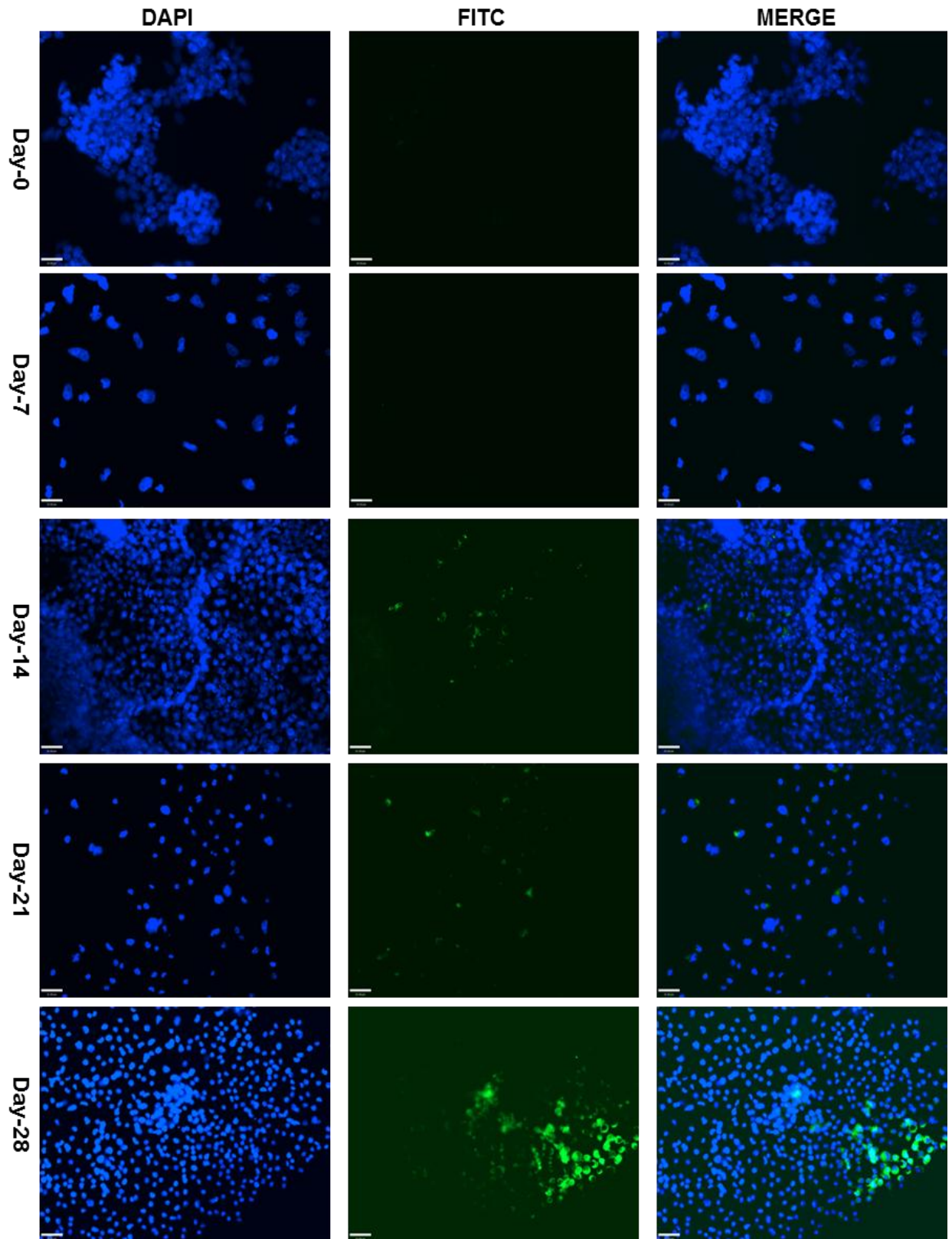


Figure 3.28: ESCs grown in medium lacking osteogenic supplements stained using antibody against Osteocalcin protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osterix). With the exception of day-0 and day-7, all time points showed cytosolic expression which increased with time (All pictures were taken at 20X magnification).

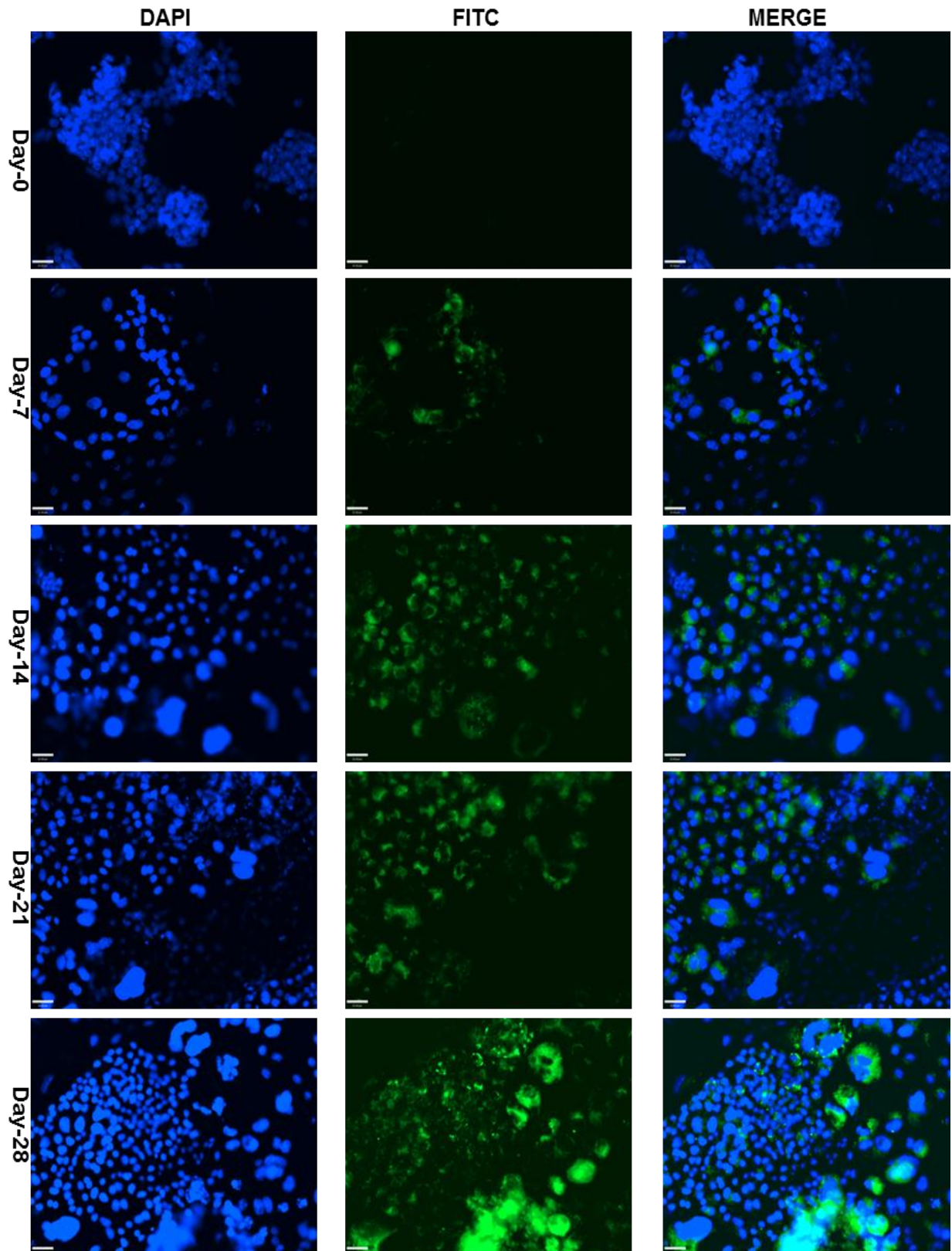


Figure 3.29: ESCs grown in medium containing osteogenic supplements stained using antibody against Osteocalcin protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osterix). With the exception of day-0 all time points showed cytosolic expression of osteocalcin which increased with time (All pictures were taken at 20X magnification).

### **3.3.4. Molecular Analysis**

#### **3.3.4.1. Gene Expression Analysis by reverse transcription PCR (RT-PCR)**

Gene expression studies of Oct4, Nanog and Sox2 as marker of pluripotency and Sox9, Alkaline phosphatase, Runx2, Osterix, Osteopontin and Osteocalcin as osteo-specific markers were carried out on random primed cDNAs. cDNA was prepared using RNA extracted from ES cells undergoing differentiation at different time points as shown in Figure 3.22. A house keeping gene GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) was used as endogenous control. GAPDH was selected as invariable control because this differentiation protocol is based on previous studies which had also employed the same for normalizing gene expression analysis data (Buttery *et al.*, 2001; Bourne *et al.*, 2004). In addition other separate studies carried out to specifically compare different housekeeping genes in differentiating mouse embryonic stem cells to select for best reference gene have also shown that GAPDH is more reliable choice as normalization control over other reference genes such as HPRT and beta tubulin (Murphy *et al.*, 2002; Willems *et al.*, 2006). It appears from Figure 3.30 that GAPDH expression levels did not remain same throughout the differentiation time course which could be more likely due to amount of input used for PCR reaction. All gene products after PCR reaction were loaded on to ethidium bromide containing agarose gels and intensity of DNA bands were then calculated using AIDA, an image analysis software (raytest, Germany). Semi-quantitative gene expression analysis was carried out by normalizing the DNA band intensities values to GAPDH and ratio of product intensities plotted against days of treatment to obtain an expression profile in differentiating cultures and shown in Figures 3.31-3.39.

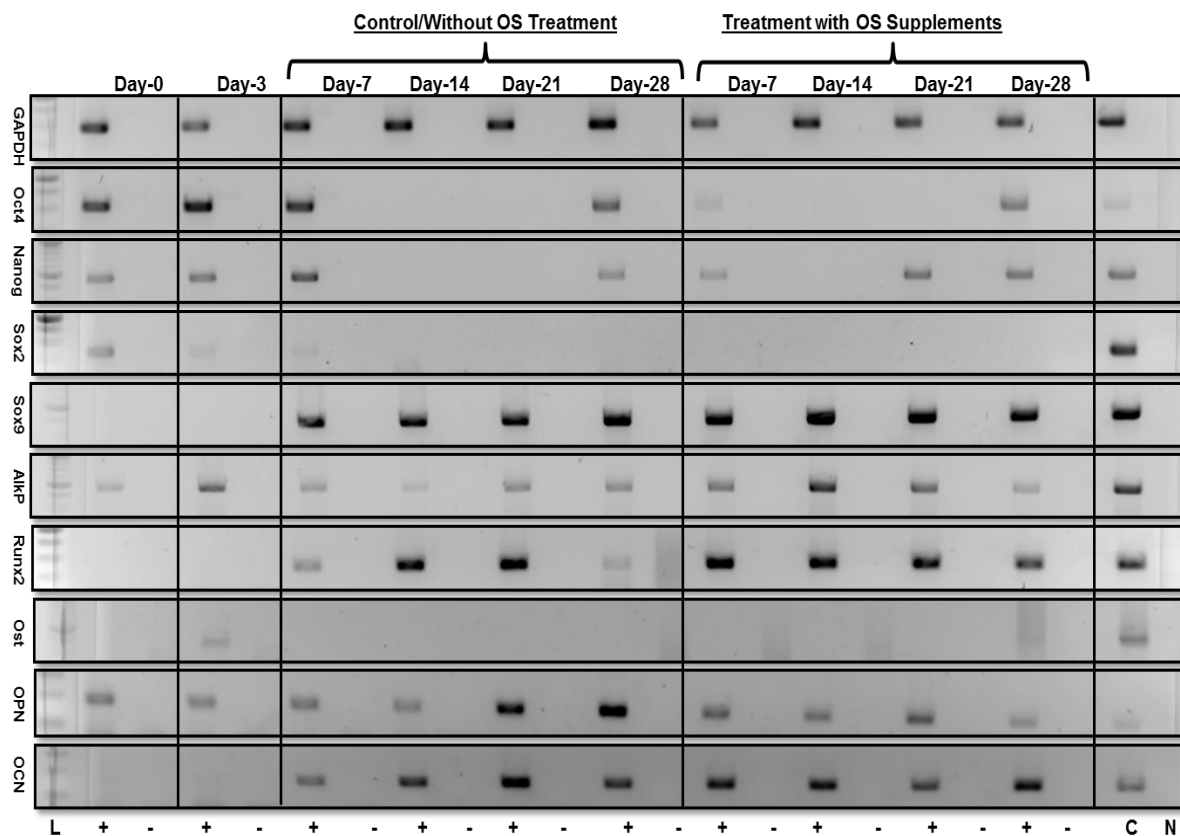


Figure 3.30: Gene expression analysis using random primed cDNAs of ESC osteogenic time-course. This analysis was carried out in triplicates for three independent biological replicates (n=3) and semi-quantitative analysis is shown below (Figures 3.31-3.39). Note: + and – refers to RT and –RT respectively whereas C is Positive control, N is PCR Negative and L is 100bp DNA Ladder.

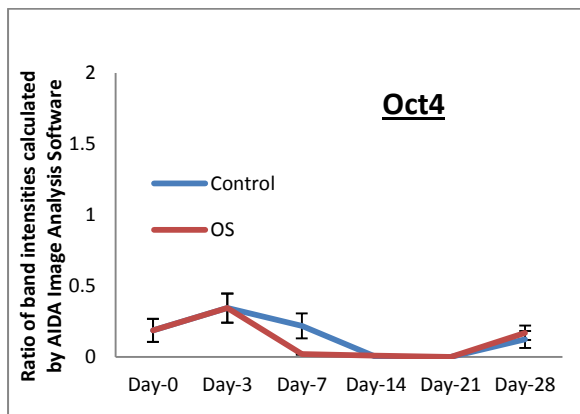


Figure 3.31: Semi-quantitative gene expression analysis showing expression of Oct4 in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean $\pm$ SEM.

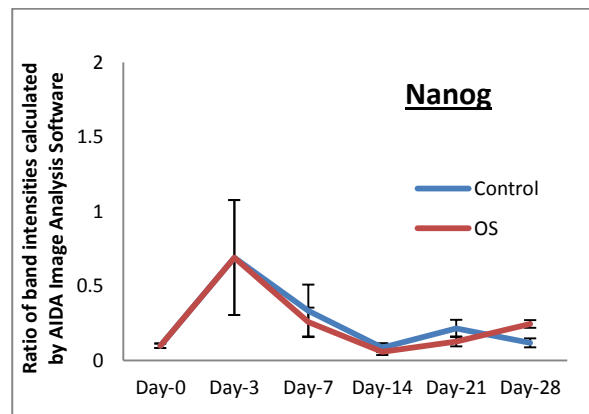


Figure 3.32: Semi-quantitative gene expression analysis showing expression of Nanog in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean $\pm$ SEM.

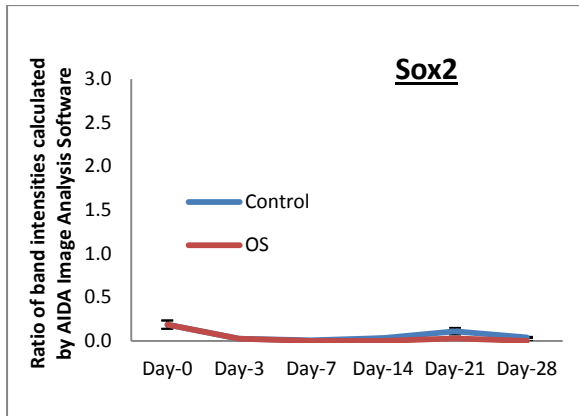


Figure 3.33: Semi-quantitative gene expression analysis showing expression of Sox2 in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean $\pm$ SEM.

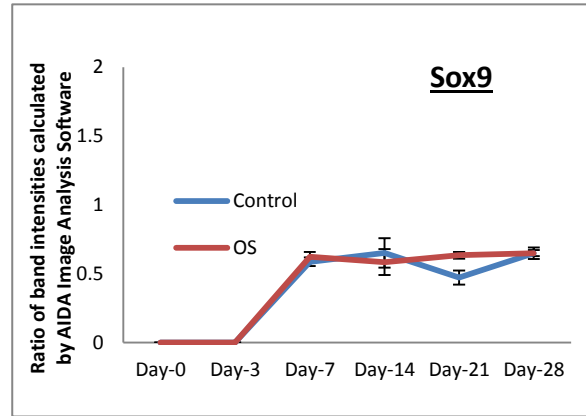


Figure 3.34: Semi-quantitative gene expression analysis showing expression of Sox9 in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean $\pm$ SEM.

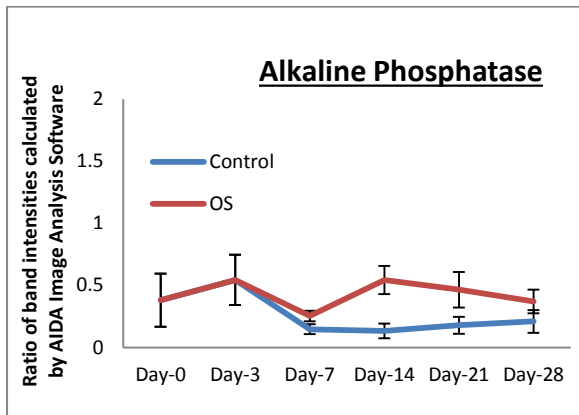


Figure 3.35: Semi-quantitative gene expression analysis showing expression of Alkaline Phosphatase in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean $\pm$ SEM.

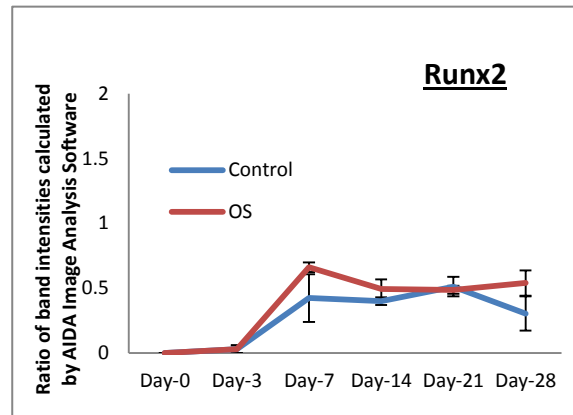


Figure 3.36: Semi-quantitative gene expression analysis showing expression of Runx2 in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean $\pm$ SEM.

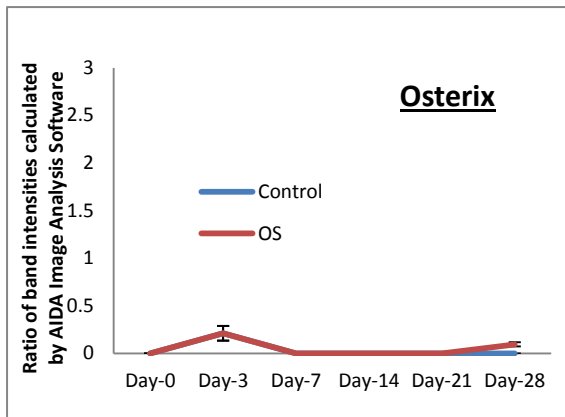


Figure 3.37: Semi-quantitative gene expression analysis showing expression of Osterix in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean $\pm$ SEM.

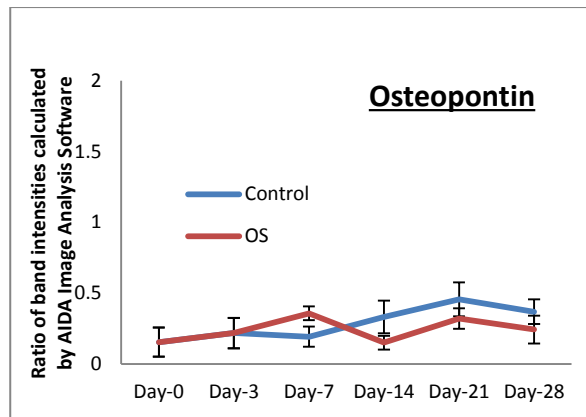


Figure 3.38: Semi-quantitative gene expression analysis showing expression of Osteopontin in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean $\pm$ SEM.

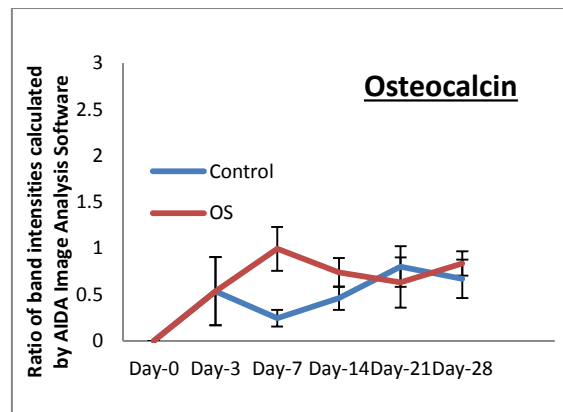


Figure 3.39: Semi-quantitative gene expression analysis showing expression of Osteocalcin in mouse embryonic stem cells grown in Control and OS medium for 28 days. Results are presented as mean $\pm$ SEM.

**Oct4** was found to be strongly expressed in undifferentiated cells and then subsequently becomes down regulated as cells differentiate and seemed to up regulate again after day-21. In both control and OS treated cells the pattern of RNA expression seemed to be the same with a sharp reduction in level of expression after day-7 (levels beyond the resolution of this assay at day-14 and day-21) but seemingly up regulated at day-28 (Figures 3.30 and 3.31).

**Nanog** displayed a pattern of expression similar to Oct 4 in control cells, with high detectable levels in day3 and day7, no expression in day14 and day 21 cells and expression back on at day 28. Similarly, in OS-treated cells expression of Nanog is lost after day 7 as observed in control cells, but resumed at day21 and maintained in day28 cells (Figures 3.30 and 3.32).

**Sox2** expression was detected in undifferentiated E14Tg2A (day-0) and found to be down regulated in both control and OS cells after day-3 (Figures 3.30 and 3.33). Though Sox2 protein was found to be present in cells grown in control medium at day-7, day-14 and day-21 with fewer cells being positive past day-0 (Figure 3.22). Sox2 protein was not detected after day-14 in OS-treated population (Figure 3.23), and this correlates with lack of RNA detection at these time points by the RT-PCR (see discussion section 3.4 for further details).

**Sox9** expression at RNA level was observed by RT-PCR throughout the differentiation time course in E14Tg2A grown in medium with and without OS supplements (Figures 3.30 and 3.34) except day-0 and day-3. These results are in agreement with those obtained by immunofluorescence where Sox9 protein was also found to be present at all time-points starting from day-7 to day-28 in both treated and untreated cells (Figures 3.24 and 3.25).

**Alkaline phosphatase** was found to be present at all time-point examined with significant up regulation in second week of differentiation in OS treated cells as shown in Figures 3.30 and 3.35.

**Runx2** expression was undetectable at day 0 and day 3 of differentiation and was found to be peaking up after two weeks of differentiation in both control and OS treated cell populations and then it appeared to be down regulated after day-21 (Figures 3.30 and 3.36).

**Osterix** expression was not detected at earlier time points in E14Tg2A growing in both kinds of medium, containing and lacking OS supplements as shown in Figures 3.30 and 3.37 though a faint signal was observed at day-3 and very little at day-28 in OS cells. As previously described, osterix protein was not found to be present at any time point during differentiation of ES cells (Figure 3.26 and 3.27).

**Osteopontin** was found to be present in undifferentiated as well as differentiated cells. There appeared to be a marked increase in expression at day-21 and day-28 in control cells whereas observed to be down regulating in OS cells past day-21 as is shown in Figures 3.30 and 3.38.

**Osteocalcin** expression was first observed at day-7 in OS differentiated cells and then found at all time-points (Figures 3.30 and 3.39). This correlates well with osteocalcin protein expression detected by immunofluorescence as shown earlier in Figure 3.28 and 3.29. Protein expression was found to be enhanced at late stages of differentiation i.e. at day-21 and day-28 in both control and OS cells. OS-treated cells were found to be expressing more osteocalcin protein than non-treated cell populations.

#### **3.3.4.2. Methylation sensitive PCR (MS-PCR) for methylation analysis**

In order to determine whether DNA methylation could play a role in regulating expression of Sox2 as E14Tg2A cells grow in medium prompting osteogenic differentiation, two regulatory regions of Sox2 (SRR1 and SRR2) were analysed by methylation sensitive PCR. For this first, genomic DNA is digested with *MspI* and *HpaII* restriction enzymes which are

isoschizomers but *MspI* activity is not affected by DNA methylation at its target site. *HpaII* is unable to cleave its target sequence when it is methylated. This difference can be exploited to investigate the methylation status of the sequence in question, as a fragment containing *Msp* sites should never be amplified following digestion with *MspI*, but can be amplified after *HpaII* digestions if all the sites are methylated. Three PCR reactions were run in parallel for these three set of samples using primers specific for either control sequences, SRR1 and SRR2. Uncut (U) refers to mock digested genomic DNA which has not received any enzymatic treatment, M refers to genomic DNA digested with *MspI* and H is for genomic DNA digested with *HpaII* restriction enzyme (Figures 3.40-3.43).

To determine the quality of DNA, efficiency and success of restriction reaction, controls were included in PCR reaction that can selectively amplify the regions of digested DNA without any *MspI/HpaII* sites, sites that are always unmethylated and sites that are always methylated. For this purpose a region of DNA is selectively amplified using mAprt primers which have been reported to contain *MspI/HpaII* sites but remain unmethylated (Macleod *et al.*, 1994). And a sequence of known methylation status namely insulin like growth factor receptor differentially methylated region 2 (Igf2R-DMR2) from mice was selected as a control since this region becomes methylated during oogenesis and remains methylated (Feil *et al.*, 1994).

As can be seen in Figure 3.40 that region of mAprt known to be unmethylated did not amplify in both *MspI* and *HpaII* digested DNA samples at any time point in both OS-treated and not treated cell populations. This indicates that digestion is not partial and any product obtained with primers specific for Sox2 regulatory regions would be due to their methylation.

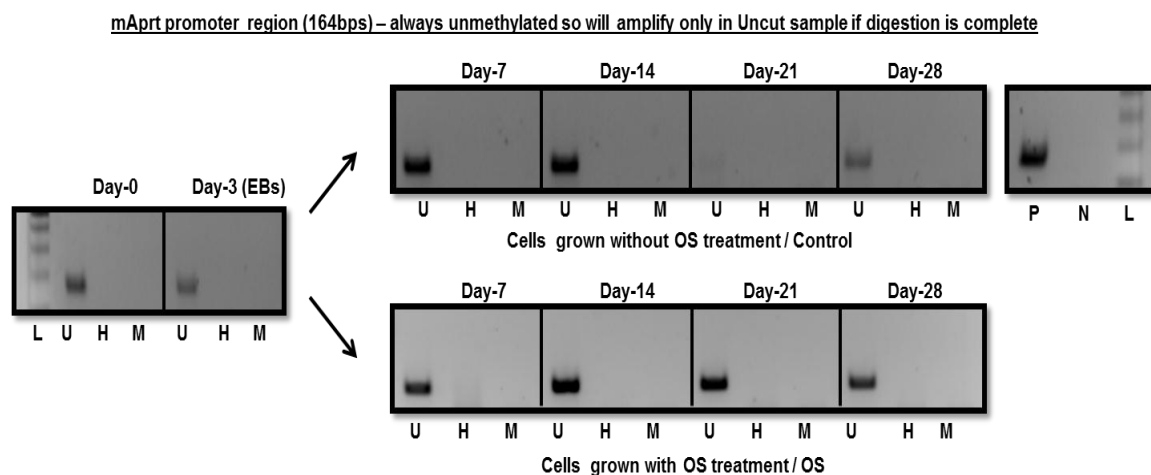


Figure 3.40: mAprt promoter region used as control and showing that no amplified product was observed in *MspI* and *HpaII* digested samples so digestion is complete.

Figure 3.41 shows the amplified product form mIgf2R region in *HpaII* digested samples and not in corresponding *MspI* digested samples. This also indicates that any product with primers specific to Sox2 regulatory regions in *HpaII* digested DNA samples would be due to methylation at *Msp* sites and not due to incomplete digestion.

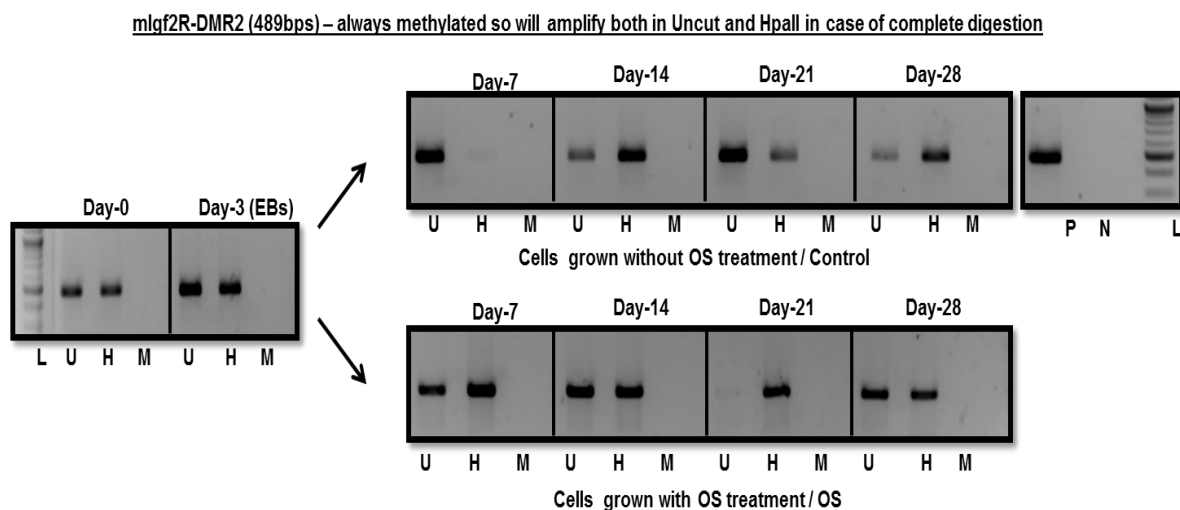


Figure 3.41: mIgf2R-DMR2 region used as control and showing that no amplified product was observed in *MspI* digested samples so digestion is complete.

SRR1 and SRR2 as already detailed in section 1.5.3 are two evolutionary conserved regulatory regions of Sox2. Both of these regions i.e. SRR1 and SRR2 were found to be

methyated at all time-points analysed in both non-treated and OS-treated cell populations except day-0 and day-3 as shown in Figure 3.42 and Figure 3.43.

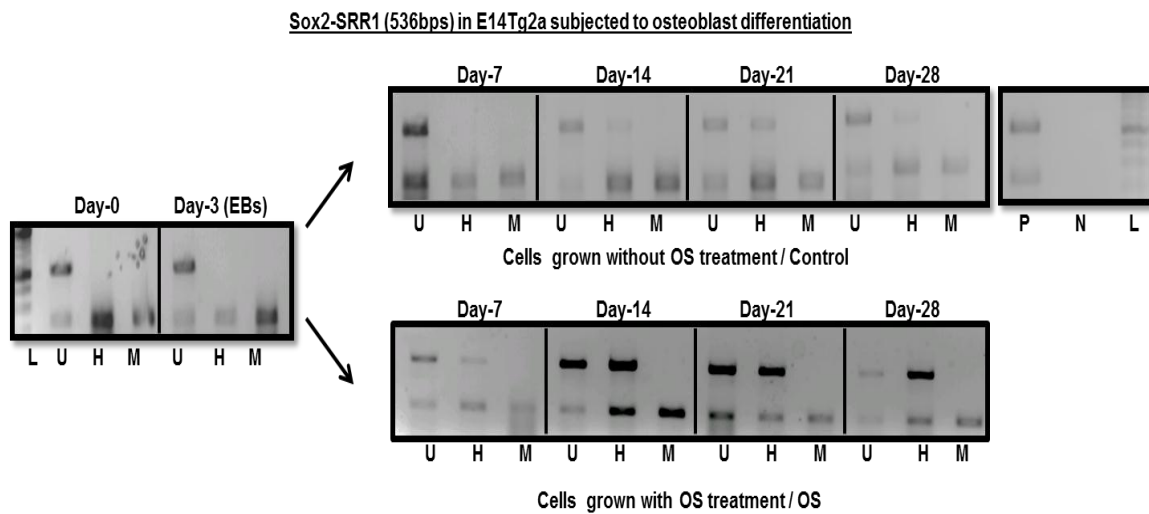


Figure 3.42: SRR1 region of Sox2 was found to be methylated at all time-points examined by MS-PCR (day-7 of control could be due to less amount of DNA input in PCR reaction)

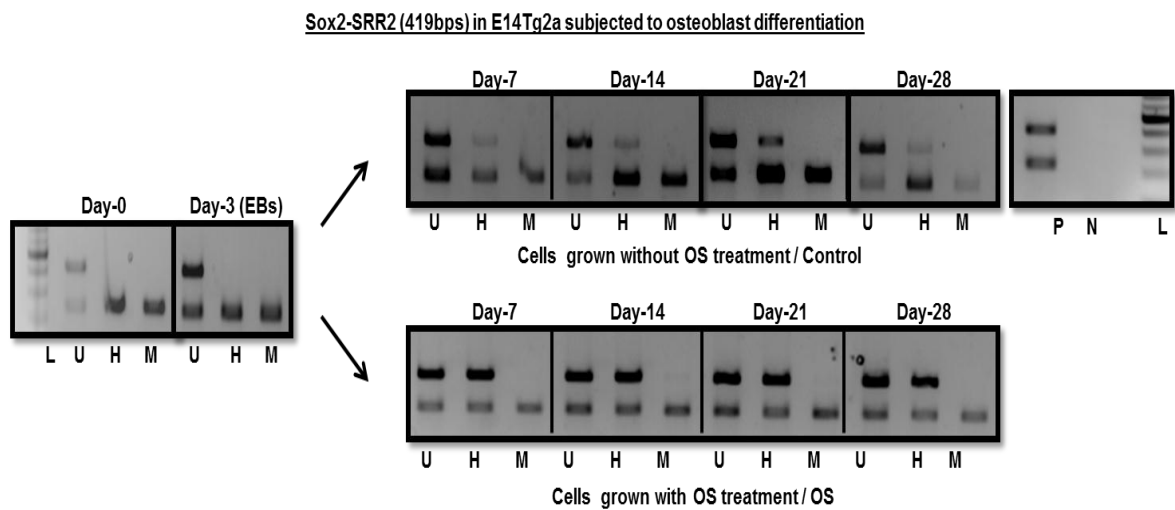


Figure 3.43: SRR2 region of Sox2 was found to be methylated at all time-points examined by MS-PCR (Key: U – Uncut Sample of DNA; M – *MspI* digested DNA; H - *HpaII* digested DNA; P – Positive Control; N – PCR Negative Control; L – 100bp DNA Ladder)

### 3.3.4.3. Bisulphite Sequencing of SRR2 region

In order to further confirm and analyse the methylation status at each individual CpG dinucleotide in the SRR1 and SRR2 region of Sox2, DNA collected at each differentiation

time point was treated with bisulphite, then PCR amplified and sequenced. Unfortunately SRR1 did not yield good quality sequence results and due to limitations of time only the region of SRR2 which gave readable sequence data was further analysed.

The region analysed by sequencing contained three CpG dinucleotide which were found to be completely unmethylated at day-0, day-7, day-14, day-21 and day-28 in both population of cells i.e. control and OS as shown in Figure 3.44 (see section 3.4 for detailed explanation). This data was obtained by direct sequencing of amplified product using DNA prepared from cells collected from two independent biological replicates (sequencing chromatograms are given in Appendix 7.5).

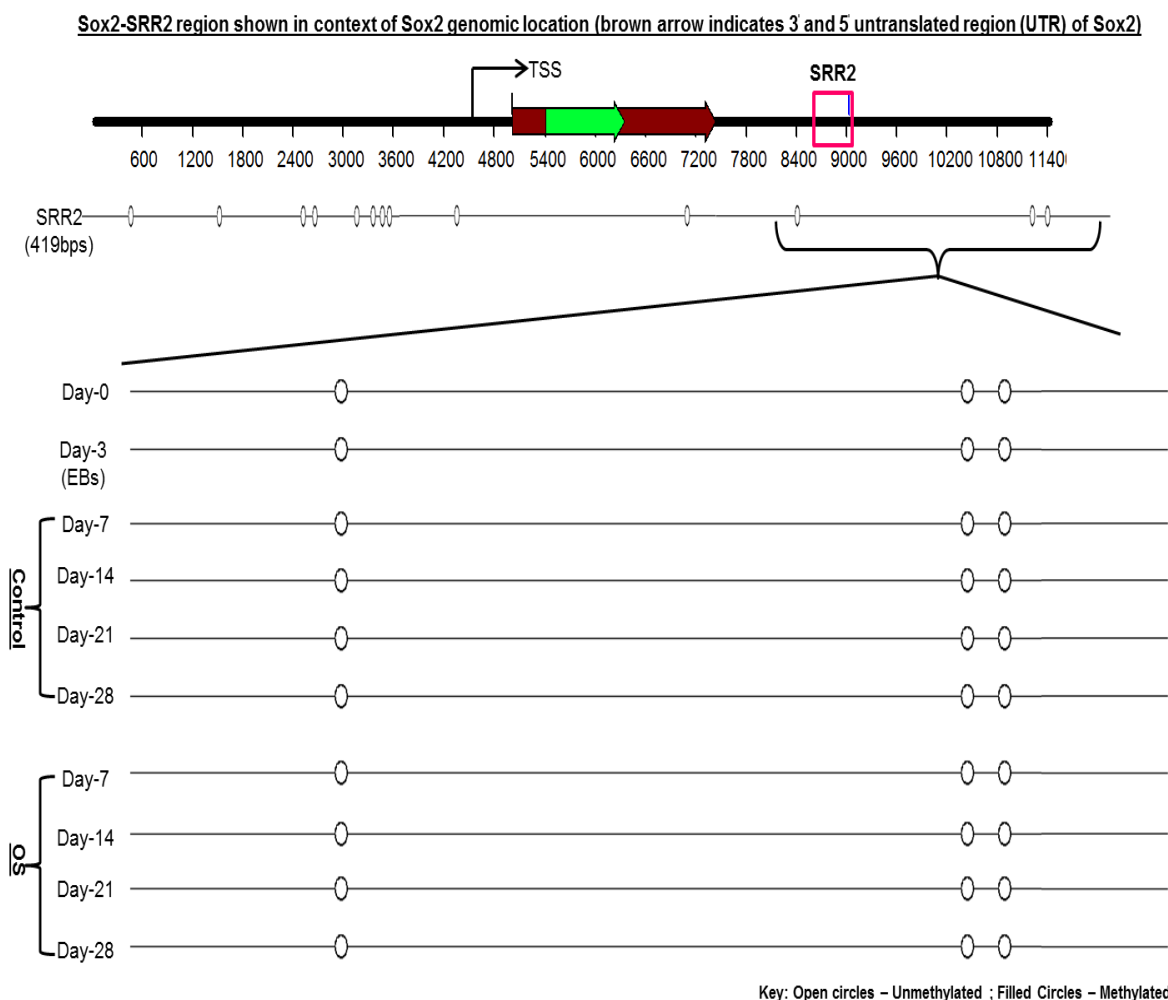


Figure 3.44: Lollipop diagram showing DNA methylation status of CpG dinucleotides in SRR2 region of Sox2. These three sites analysed by direct DNA sequencing of amplified product of SRR2 region showed no methylation at any of time-points chosen for analysis.

### **3.4. Discussion**

The work presented in this chapter described the differentiation of mouse embryonic stem cells (E14Tg2A) into osteoblast through the formation of embryoid bodies in medium supplemented with factors known to induce osteogenesis (Buttery *et al.*, 2001). Different studies attempting *in vitro* osteogenesis have used mineralization capacity of cells undergoing differentiation and expression of osteoblast specific marker at RNA and protein level to characterize the osteoblasts (Duplomb *et al.*, 2007).

So the cells grown in osteoblast differentiation medium were first analysed by staining with Alizarin red dye which has been historically used to characterize the calcified mineral depositions in bone matrix (Gregory *et al.*, 2004). The cells grown in osteogenic differentiation medium showed mineralization in a time dependent manner i.e. increased with culture time as is evident in Figure 3.7 (microscopy) and Figure 3.10 (quantitative analysis).

Osteoblast differentiation is a multistep complex process under the control of a myriad of transcription factors and signalling pathways (Lian *et al.*, 2006). The different stages of bone development are proliferation of osteoprogenitors, deposition of extracellular matrix and mineralization (Aubin, 1998). So the expression pattern of markers associated with osteoblasts is organized temporally and sequentially (Nieden *et al.*, 2003). Type I collagen, Alk-P and osteonectin have been reported to appear at the end of proliferation phase; osteopontin followed by bone sialoprotein and Runx2 at the start of mineralization phase and finally osteocalcin at high level during mineralization and they can have varying times of onset in culture dependent on cell line and methods of differentiation (Duplomb *et al.*, 2007).

Runx2 related transcription factor 2 (Runx2 / Cbfa1 / Osf2) is thought to be a central gene coordinating signals from BMP and Wnt pathways for cellular commitment and osteogenesis (Lian *et al.*, 2006). It is one of the earlier markers and required for the formation of preosteoblast and Runx2 null mutant have been reported to show no endochondral and membranous bone formation (Komori *et al.*, 2006). Down regulation of Runx2 has been shown to be associated with chondrocyte commitment (Lengner *et al.*, 2005). As reported by earlier studies, Runx2 mRNA was detectable at earlier stages and remained present throughout the differentiation time course peaking at day-14 in OS cells and then down regulating compared to control/non treated cells (Kawaguchi *et al.*, 2005; Hwang *et al.*, 2008). As shown in Figure 3.30, similar pattern of RNA expression was observed in this study where reduction in expression level was seen after day-21 in both OS-treated and non-treated cell populations.

Osterix is osteoblast specific transcription factor acting downstream of Runx2 and lack of Osterix has been shown to associate with no bone formation (Nakashima *et al.*, 2002). It has been shown to be up regulated at later stages of differentiation by an early study (Woll and Bronson, 2006). Here in this experiment no osterix expression was detected at any point of time course either at RNA level or protein level. As cells lacking osterix would not form bone and fail to express osteoblast specific marker genes, it could be possible that PCR reaction was not efficient enough, RNA transcript was not abundant and/or short lived and could not be detected at time points chosen for analysis. As for protein expression, it is possible that antibody used needed further optimization to detect osterix protein since known cell line expressing osterix was not available to use as a control and verify.

Sox9 has been reported to act on Collagen II (col2a1) to ensue chondrogenesis and persistent expression of this has been reported to block osteogenesis (Mori-Akiyama *et al.*, 2003). Here Sox9 expression was found to be induced in cells grown in medium with and without osteogenic supplements at day-7 and RNA observed to be lasted until day-28. Sox9 protein was also observed to be present at these time points with majority of cells positive at day-21 in OS cells and then only few retained expression of that at day-28. It could mean that all of the cells in culture are not undergoing differentiation and some progenitor type cells are still present.

Alkaline phosphatase has been reported to be expressed in large amounts in osteoblasts (Zernik *et al.*, 1990) and is an established marker for mineralization and osteoblast characterization (Benayahu *et al.*, 1989). Also it's an established marker of undifferentiated ES cells (Phillips *et al.*, 2001). Alk-P was found to be peaking up in expression at day-14 in OS cells compared to control and then down regulated as very faint product can be seen at day-28.

Osteopontin is a glycoposphoprotein that binds with calcified matrices to provide adhesion and secreted by osteoblasts at early stage of development (McKee and Nanci, 1996). It has been shown to express at the start of mineralization phase during bone formation and found to be upregulated in cultures upon induction with osteogenic supplements (Nieden *et al.*, 2003; Kawaguchi *et al.*, 2005). Here OPN expression was found to be down regulated after day-21 in OS cells compared to control cells.

Osteocalcin is considered to be an osteoblast-specific gene (Lian *et al.*, 1989) and its expression is associated with matrix synthesis and mineralization (Ryoo *et al.*, 1997; Nieden

*et al.*, 2003). OCN was strongly up regulated after day-14 in OS cells compared to control cells both at RNA level and protein level.

Oct4, Nanog and Sox2 were included as markers of pluripotency and it has been well established now that down regulation of these factors leads to the differentiation of embryonic stem cells (Boyer *et al.*, 2006). Oct 4 is down regulated when stem cells differentiate during gastrulation and subsequently it becomes localized to germ cell lineage (Yeom *et al.*, 1996). Furthermore target gene deletion experiments of Oct4 have resulted in failure of fetal development and differentiation to trophectoderm lineage (Nicholas *et al.*, 1998).

Lack of Nanog in ES cells favours differentiation towards endoderm lineage (Chambers *et al.*, 2003). Nanog has been reported to inhibit BMP induced mesoderm differentiation of ES cells by interacting with Smad1 pathway (Suzuki *et al.*, 2006). Sox2 has been reported to repress osteoblast differentiation by down regulating Wnt target genes through FGF signalling (Mansukhani *et al.*, 2005). Also Sox2 has been reported to act as repressor of osteopontin gene which is a middle stage marker of osteoblasts (Botquin *et al.*, 1998). Sox2 has been shown to be present in osteoprogenitors stimulating their proliferation over differentiation (Basu-Roy *et al.*, 2010).

As expected all three genes showed significant down regulation upon differentiation at day-3 stage but seemed to be again up regulated after day-21. It is possible that only RNA is present in cells while no functional protein is there. Post transcriptional gene silencing involving long non coding RNAs and small interfering RNAs is now considered important regulatory phenomenon particularly with reference to their role in embryonic stem cell differentiation

(Tay *et al.*, 2008; Dinger *et al.*, 2008). Sox2 protein was seen until day-14 in OS treated cells while no expression was detected at RNA level past day-3 which could be due to transcript having short half-life than protein. It has been shown by some previous studies that mRNA stability is related to molecular function of encoded protein and transcription factor mRNAs are fast degrading transcripts compared to other transcripts (Yang *et al.*, 2003).

Taken together this data suggests that cells are undergoing differentiation as markers associated with osteoblast phenotype are upregulating after two weeks in culture i.e. Sox9, Alk-P, Runx2 and osteocalcin at around the third week with down regulation of pluripotency-related markers particularly, Sox2, after day-14 which would otherwise has been known to favour the maintenance of osteoprogenitors over differentiated cell types. Sox9 expression was seen until day-28 at both RNA and protein level which could mean that not all of the cells are terminally differentiating and some progenitors like cells still exist in culture. Since Sox9 expressing cells can still differentiate to either chondrocytes or osteoblasts. Given the fact that cells are indeed differentiating, it is a good model for looking at down regulation of Sox2 as ES cells differentiate.

Methylation of DNA cytosine residues at the carbon 5 position ( $5^{\text{me}}\text{C}$ ) is a predominant epigenetic mark in many eukaryotes found in the sequence context of CpG and most often leads to silencing of a gene if found at a promoter sequence. However, increased CpG DNA methylation is associated with gene bodies of actively transcribed regions in mammals and plants (Laird, 2010). Two regulatory regions of Sox2 (SRR1 and SRR2) chosen for DNA methylation analysis were found to be methylated when examined by MS-PCR at all time-points except day-0 and day-3. As this technique only tests the CpGs within *Msp* restriction

enzyme recognition site, bisulphite sequencing analysis was also carried out to better understand the extent to which methylation occurred within these regions.

Different approaches can be used to study DNA methylation depending on the objective of analysis i.e. genome wide/global or locus specific/single gene (Laird, 2010; Shen and Waterland, 2007). Methylation-sensitive restriction enzymes are primarily used to identify the regions of DNA methylation in chromosome and then depending on goal of analysis either affinity enrichment strategies (MeDIP based) are used or bisulphite conversion is chosen (Laird, 2010). Methylated immunoprecipitation (MeDIP) allows the efficient enrichment of methylated DNA and assay depends on the use of an antibody specific for methylated cytosines to immunocapture methylated genomic fragments (Weber *et al.*, 2005). Methylation profiles obtained by MeDIP approach are not base-pair specific even when combined with arrays (MeDIP-ChIP) or next-generation sequencing (MeDIP-seq) since resolution is restricted by the size of sonicated DNA fragments (Chavez *et al.*, 2010; Laird, 2010). Hence MeDIP based approaches are chosen when the objective is to create genome-wide methylation profiles. Bisulphite conversion and sequencing is the sensitive and preferred method of choice to resolve DNA methylation at single base-pair level (Laird, 2010). This study aimed to screen DNA methylation status at each individual CpG site in SRR1 and SRR2 regions of Sox2, hence methylation-sensitive restriction enzymes were used as a first step to screen for presence or absence of DNA methylation and then bisulphite conversion was used to study each CpG site.

Unfortunately the whole regions of both SRR1 and SRR2 could not be sequenced as direct DNA sequencing attempts after bisulphite treatment were unsuccessful and time limitation did not allow attempting cloning of the fragment and subsequent clonal analysis. Only a part

of SRR2 region could be resolved at individual CpGs which fortunately contains the sequence which is core SRR2 sequence and contains an enhancer site which is under the control of Sox2/Oct4 complex (Tomioka *et al.*, 2002) and has been reported as neural stem cell specific enhancer in telencephalon (Miyagi *et al.*, 2006). Three CpGs located in this enhancer region in SRR2 were analysed by direct sequencing and they all were found to be unmethylated in both undifferentiated and differentiated cells as shown in Figure 3.44. This region has been reported to exert its enhancer activity in embryonic stem cells and neural stem cells but do not function when cells differentiate (Miyagi *et al.*, 2004).

So it would be of interest to profile DNA methylation status of these regions in other differentiation pathways too. This study has also looked into DNA methylation status of SRR2 region in ES cells when they were grown in neural differentiation medium and the next chapter (Chapter 4) summarizes the results of those analyses. This region has not been yet investigated for DNA methylation profiling in differentiating mouse embryonic stem cells particularly when they are grown in osteogenic differentiation cocktail. The data presented here points towards an interesting and important finding that this region could possibly get methylated at other CpG sites in SRR2 than those analysed here as cells undergo differentiation though still preliminary and need further work.

**Chapter 4 - DNA methylation analysis of Sox2 regulatory regions**  
**during *in vitro* differentiation of mouse embryonic stem cells in**  
**neurogenic medium**

## **4.1. Introduction**

This chapter summarizes the findings of experiments about mouse embryonic stem cells (E14tg2a) grown in media known to promote neural differentiation.

### **4.1.1. Neurogenesis**

Neurogenesis is an on-going and continuous process in the brain resulting in differentiation, maturation, localization and functional incorporation of new cells into neuronal networks from neural progenitors (Ming and Song, 2005). The adult brain and spinal cord contains a population of stem cells that can generate three major cell types of the central nervous system i.e. astrocytes, oligodendrocytes and neurons and are referred to as neural stem cells - NSCs (Clarke *et al.*, 2000). Neuron, are the functional component of the nervous system and are involved in information processing and transmission, while oligodendrocytes and astrocytes, collectively known as glia, are supportive cells required for nervous system maintenance (Zhao *et al.*, 2008).

NSCs have been shown to be spatially heterogeneous and temporally specified generating progressively restricted cell types (Merkle and Alvarez-Buylla, 2006). The most active regions of an adult brain in terms of neurogenesis are the sub-ventricular zone (SVZ) of the lateral ventricle in the olfactory bulb and subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Duan *et al.*, 2008). Figure 4.1 is a schematic illustration of NSCs localization in adult brain and different cell types originating from them.

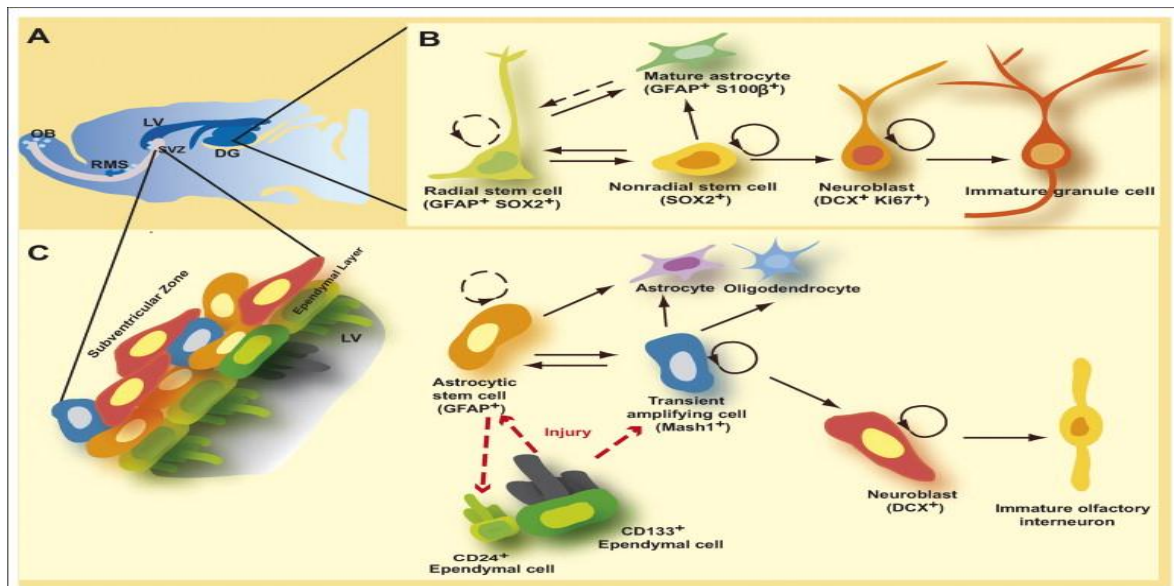


Figure 4.1. Models on the identities of potential quiescent neural stem cells in the adult brain. **(a)** Two neurogenic regions in the adult brain: the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (LV). **(b)** Potential lineage relationships in the adult SVZ. **(c)** Potential lineage relationships in the adult SGZ (text and figure from Duan *et al.*, 2008)

#### 4.1.2. ES cells as a model to study neurogenesis

Neurogenesis is a complex process and much of the current knowledge about vertebrate embryogenesis has been obtained through studies in animal embryos. But the heterogeneity of the cell types involved in neurogenesis and difficulties in obtaining sufficient number of cells for detailed molecular and signalling cascades analysis have limited their regional and temporal characterization (Suter and Karausa, 2008). Embryonic stem cells, being harvested from the inner cell mass of embryos and maintaining the multilineage development potential in cultures have become an attractive system to study gene functions and gene regulations during directed differentiation experiments and also understand developmental events (Murrey and Keller, 2008). Hence mechanistic characterization of complex regulatory networks involved in neurogenesis obtained using embryonic stem cell systems would aid in understanding normal brain development and eventually, to develop cell-based therapies for

central nervous system disorders that affect significant sections of population (Schwartz *et al.*, 2008).

ES cells are normally maintained in culture in an undifferentiated state by addition of a cytokine, leukaemia inhibitory factor (LIF) in culture media. When this LIF is removed from the culture medium, ES cells can be made to differentiate into different cell types (Smith, 2001). In the absence of LIF in suspension culture, ES cells form cellular aggregates called embryoid bodies (EBs) which have been shown to recapitulate events of early mammalian embryogenesis and form derivatives of all three germinal layers *in vitro* (Doetschman *et al.*, 1985; Guan *et al.*, 1999). EBs are initially (within 2-4 days) an outer layer of hypoblast like cells (extra embryonic visceral endoderm) surrounding an epiblast like core (shown schematically in Figure 4.2). At this stage EBs are able to generate derivatives of all three germ layers; definitive endoderm, mesoderm and ectoderm. Between day 6 and 8, the core undergoes cavitation and forms an inner epithelial layer where cells can be committed to definitive ectoderm which subsequently assumes a morphology resembling to neural tube (Coucouvanis and Martin, 1995; Rathjen and Rathjen, 2001; Rajthen *et al.*, 2002).

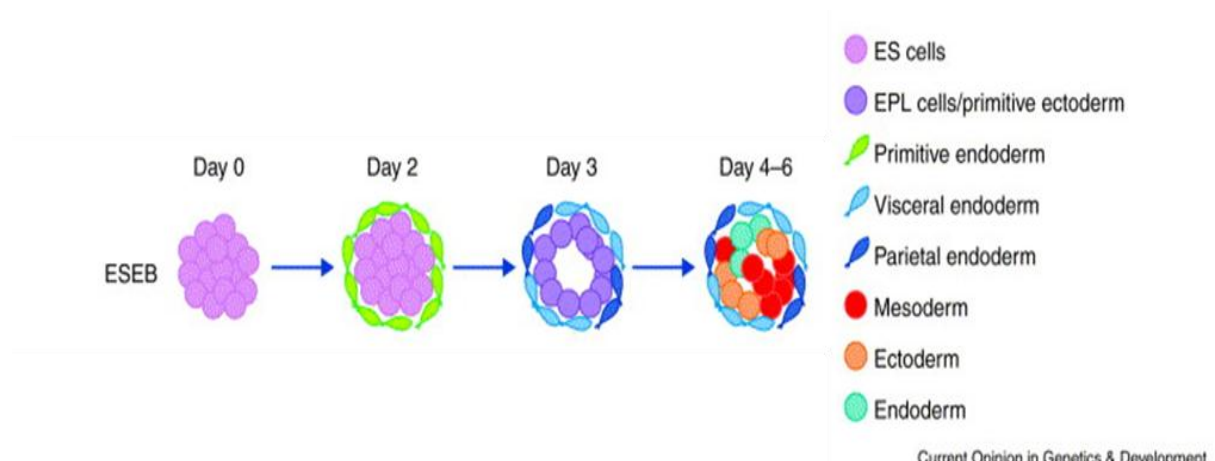


Figure 4.2: Schematic representation of ES cell differentiation into EBs (Rathjen and Rathjen, 2001).

Thus EB mediated differentiations potentially provide a model system for characterization of early embryogenesis and by using varying culture condition various cell types can be generated *in vitro* by lineage induction and/or lineage selection. However, lack of positional and organizational information coupled with poor characterization of molecular and signalling networks governing the cell fate decisions within EBs also lead to spontaneous differentiation to unwanted cell types resulting in heterogeneous cultures (Nishikawa *et al.*, 2007). This can be overcome by using optimal differentiation protocols using defined media supplemented with specific inductive factors and/or lineage selection if appropriate marker is available to achieve highly enriched desired cell types (Keller, 2005). Non EB approaches of directed ES differentiation in monolayer cultures using serum free, defined culture conditions have also been documented but with little success compared to EB mediated differentiation (Yin *et al.*, 2003; Tada *et al.*, 2005; Yasunaga *et al.*, 2005; Cai *et al.*, 2007). The cell types obtained through these directed differentiation experiments are routinely characterized and analysed by cellular morphology, cell surface markers, gene expression patterns, marker proteins and functional characterization of cell phenotypes (Wobus and Boheler, 2005).

Neural differentiation from ES cells has been documented using variety of protocols. Most of these protocols employ formation of EBs which are enriched using retinoic acid to form neural progenitors which can then be made to differentiate into any of neural lineages: neurons, astrocytes or oligodendrocytes using appropriate culture conditions (Stavridis and Smith, 2003; Cai and Grabel, 2007). Retinoic acid (RA) has been reported to influence nervous system development and maintenance (Maden, 2007) and has been shown to enhance neural gene expression and repress mesodermal specification (Bain *et al.*, 1996). Many differentiation protocols make use of RA at different stages of EB formation in varying concentrations depending on the neural subtype needed (Okada *et al.*, 2004).

Earlier studies reporting the *in vitro* differentiation of mouse embryonic stem cells to neural cell types applied RA at different time intervals to EBs and then subsequently plated them as intact aggregates in serum containing media using different attachment substrates (Bain *et al.*, 1995; Strubing *et al.*, 1995; Fraichard *et al.*, 1995; Finlay *et al.*, 1996). Later studies combined this approach of initial treatment of EBs with RA, disaggregated and plated them in serum free media where specific growth factors were added to favour differentiation towards a particular neural subtype and have reported to achieve purity up to 90% (Li *et al.*, 1998; Bible *et al.*, 2004; Bible *et al.*, 2007). Neural differentiation has also been reported in monolayer culture without EB formation and RA treatment (Ying *et al.*, 2003). Extent of neural differentiation was studied in every study by observing typical neuronal morphology using phase contrast microscopy, expression of neural specific genes and/or response of those cells to neurotransmitters by electrophysiology.

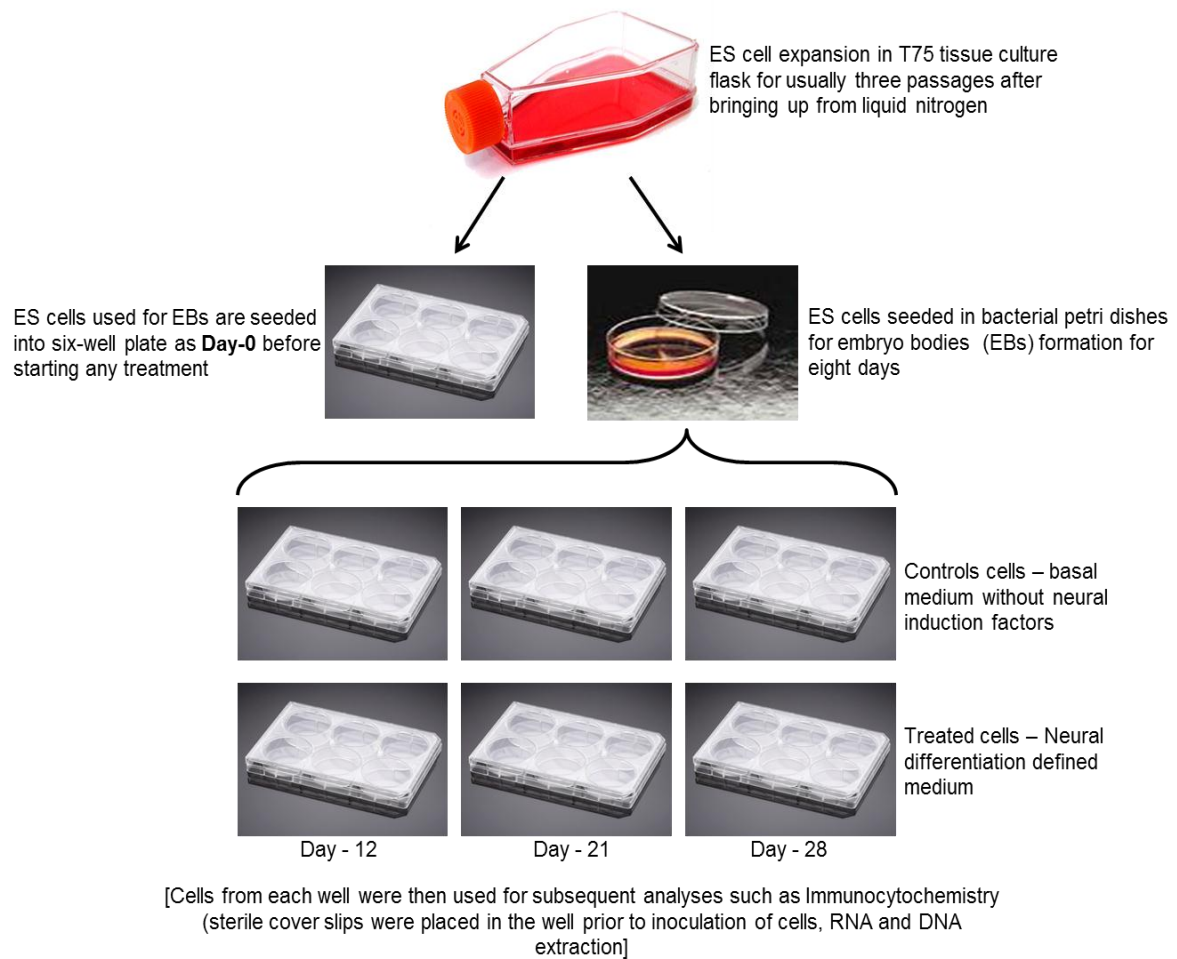
#### **4.1.3. Sox2 in Neurogenesis**

A number of transcription factors of the SOX family have been shown to regulate pathways common to all neural stem cells regardless of their origin and state in the brain. They are mainly members of the Sox B1 subclass (Sox1, Sox2, Sox3); Sox E subclass (Sox8, Sox9, Sox10) and Sox B2 subclass (Sox21) (Pevny and Placzek, 2005). Sox2 is reported to be expressed throughout the mouse embryogenesis during initial phases of development and subsequently becomes localized to the neural progenitors (Wood *et al.*, 1999). Loss of function of Sox2 has been shown to promote premature differentiation of neural progenitors to neuronal cell types (Graham *et al.*, 2003). At the same time, high expression of Sox2 has also been found in some post mitotic neurons of the thalamus, medial-dorsal striatum and

septum of adult brain implying its role in neuronal function and maintenance (Ferri *et al.*, 2004).

It has been shown that the expression of Sox2 is regulated by different enhancers during neurogenesis which are temporally and spatially specific and are significantly conserved among vertebrates (Kamachi *et al.*, 2009; Uchikawa *et al.*, 2003). This chapter summarizes the results of experiments aimed to look at DNA methylation changes of a regulatory region of Sox2 (SRR2) in ES cells grown in neural differentiate media. It was hoped that gaining an insight into regulatory mechanisms by which this enhancer act would possibly help to understand neural stem cell maintenance, differentiation and commitment in neurogenesis.

## 4.2. Experimental work plan



### **4.3. Results**

#### **4.3.1. In vitro Differentiation of mES cells towards neural lineage**

E14Tg2a cells were first grown in EB medium for 4 days and then for additional 4 days after adding all-trans-retinoic acid (RA) in medium using this protocol (Bibel *et al.*, 2007). It has been shown that addition of RA during EB formation in media promotes formation of neural progenitors. These EBs were then disaggregated and cells plated in control and neural differentiation medium and, allowed to grow for about 28 days. Cells plated in neural differentiation medium started to show typical spindle shaped neuronal morphology after 24 hours of plating. Neurites were then observed to extend from the cells and grew out over the differentiation time course (some representative pictures in Figure 4.3). By the end a dense network of neurons emerged and found to be distributed throughout the culture surface (Figure 4.3). In contrast, cells plated in control medium failed to grow and beyond day-14 no attached cells were observed in medium. Day-12, 21 and 28 were chosen for analysis (representing second, third and fourth week of culture) but the same analysis was not possible for cells seeded in control medium as no cells were found attached after day-14 (Figure 4.3 shows pictures of cells dying at day-12 and day-14).

#### **4.3.2. Characterization of neural differentiation by Immunocytochemistry**

Immunocytochemistry was performed using antibodies against Sox2 and Sox9 to detect the localization and expression of these two proteins associated with neuronal cell types. Almost all of the cells in undifferentiated ES cultures found to express Sox2 (Figure 4.5 day-0) whereas no Sox9 expression was detected (Figure 4.5 day-0). There was no expression of

either Sox2 or Sox9 in cells cultured in neural differentiation medium at day-12, 21 and 28 respectively (Figure 4.5 and 4.6). In order to account for auto fluorescence and/or nonspecific staining cells were fixed and stained in parallel set of experiment but without using primary and secondary antibodies (Figure 4.4).

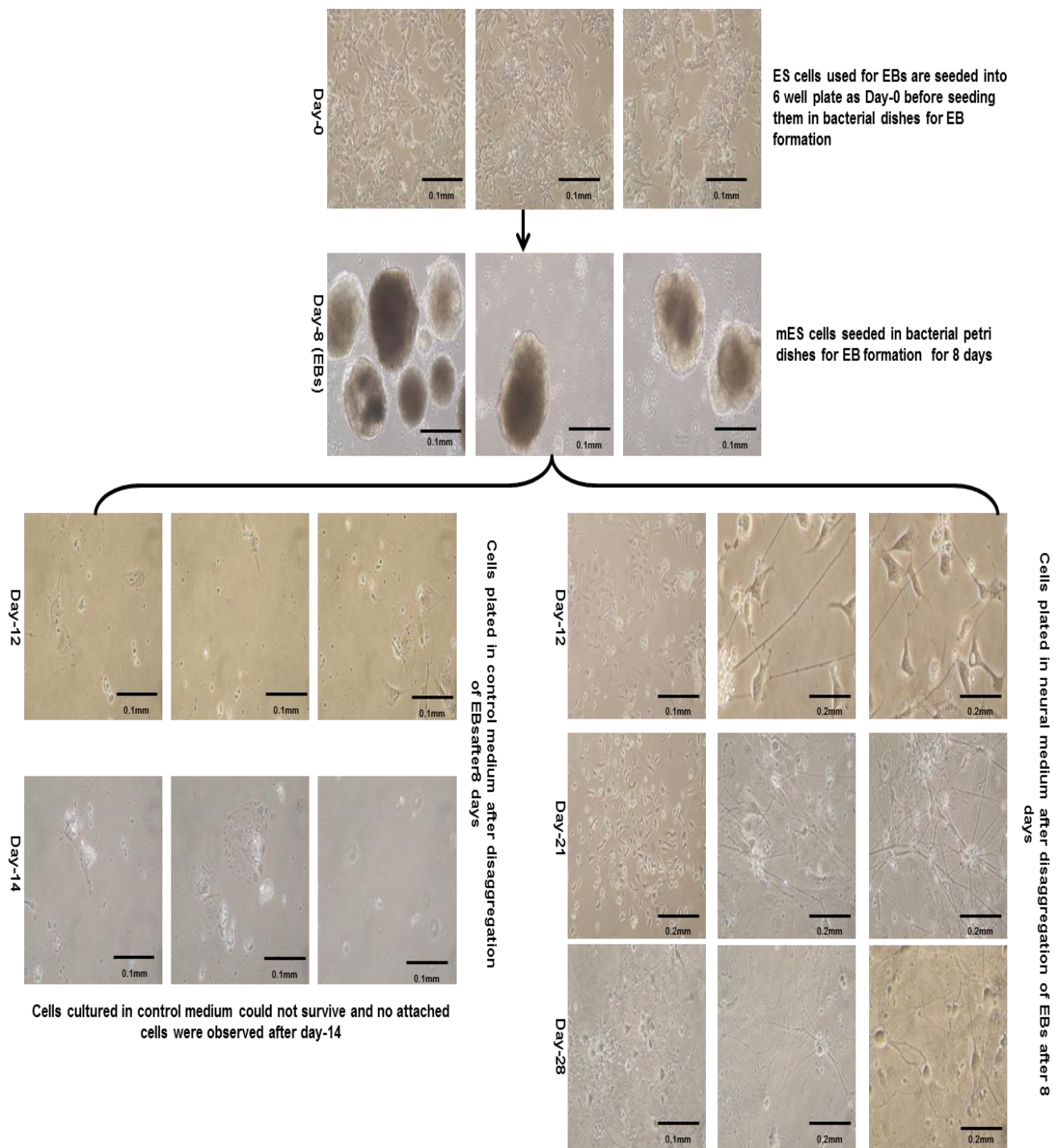


Figure 4.3: Some representative pictures of cells grown in neural differentiation medium at time-points chosen for analysis. mES cells were first expanded in complete ES media and then seeded in bacterial culture dishes for EB formation. Cells were also seeded at this stage in 6 well plate as Day-0 (starting population of ES cells for subsequent biochemical and molecular analysis). EBs were dissociated after 8 days and cells plated in medium containing and lacking neural supplements. Cells subjected to grow in control medium died while those seeded in neural medium grew and showed typical neuronal morphology under microscope.

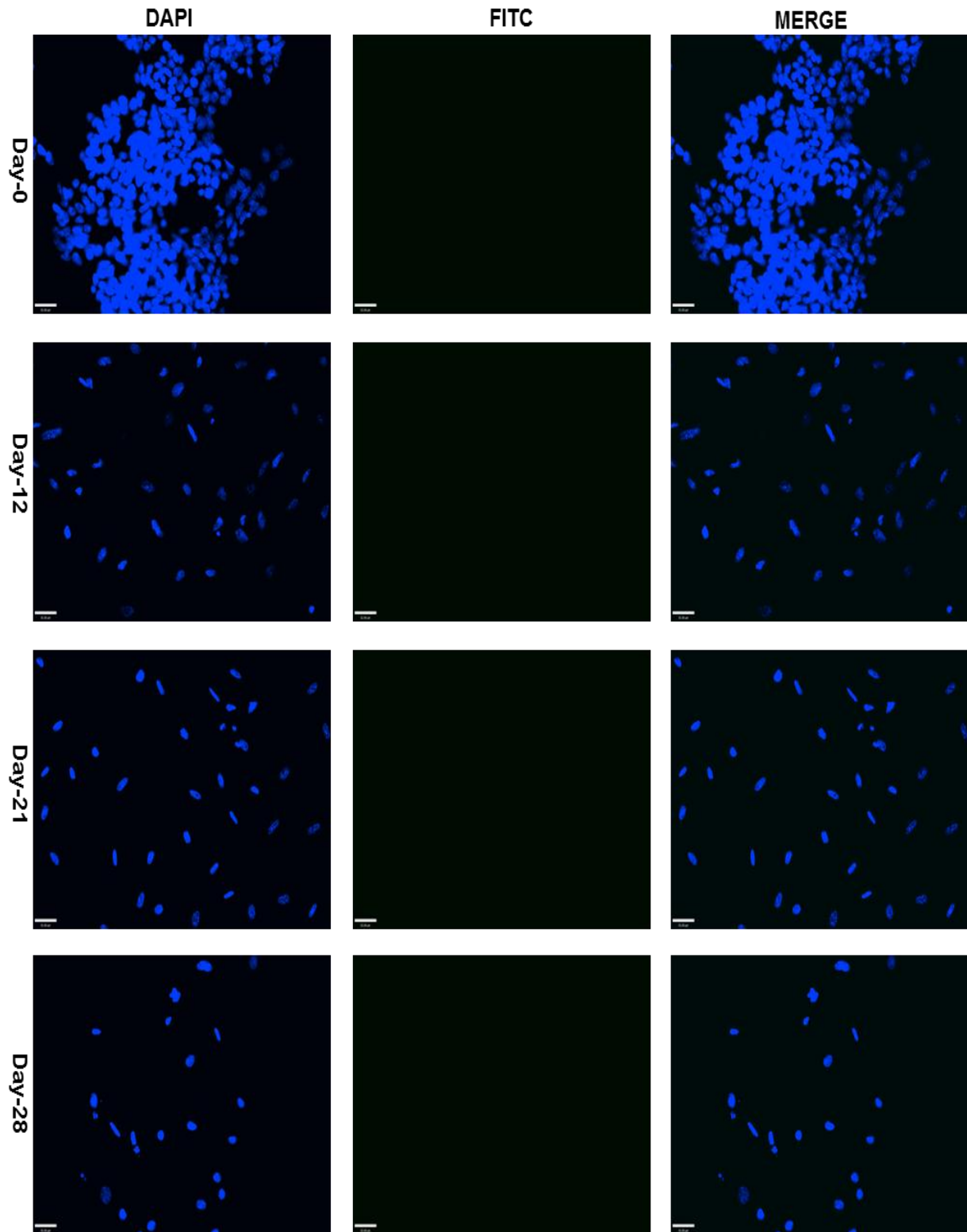


Figure 4.4: Mouse ES (E14tg2a) cells grown in neural medium were stained without primary and secondary antibodies to account for auto fluorescence and/or background staining (Dapi was used to stain the nucleus). No fluorescence was observed (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

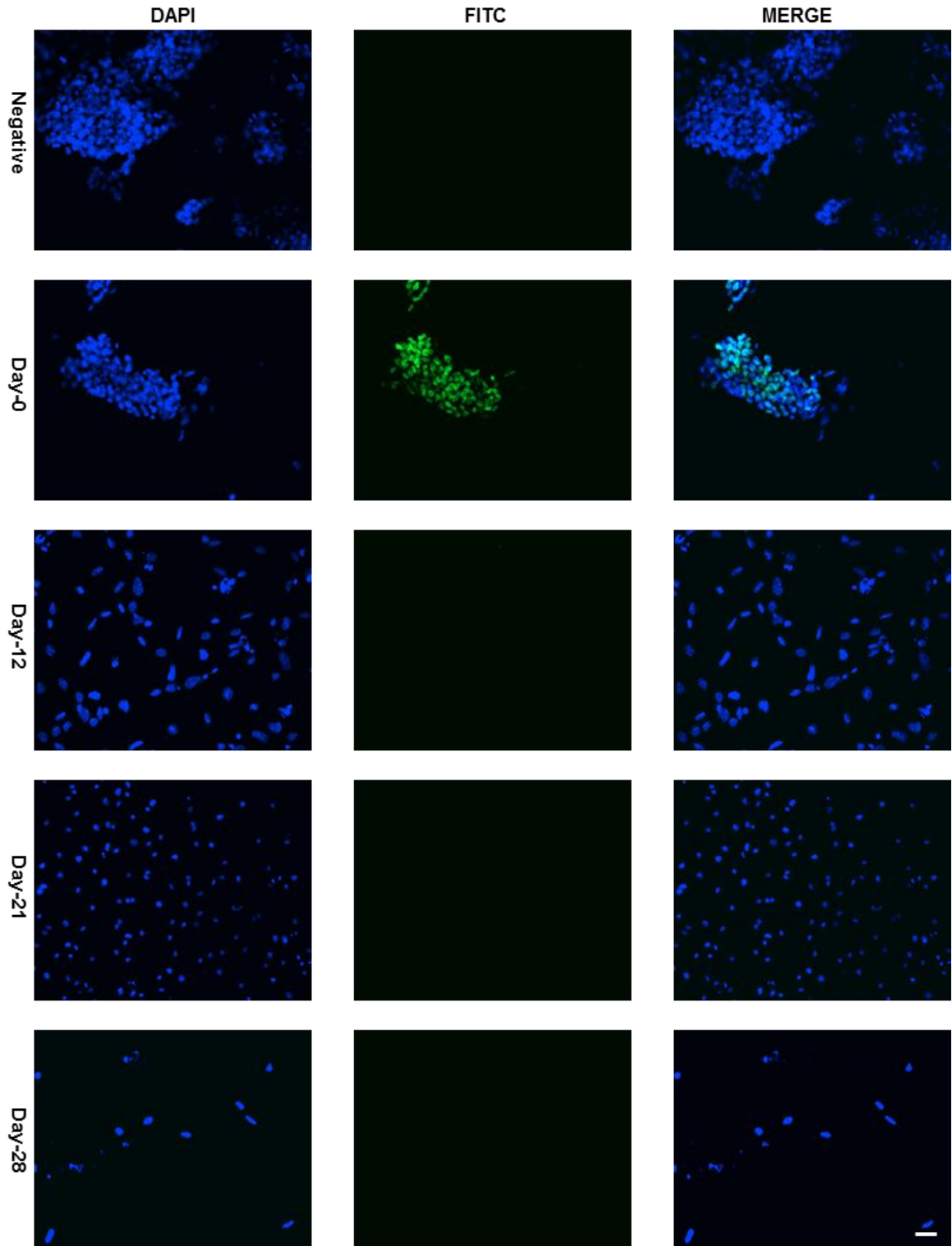


Figure 4.5: Images of E14Tg2a cells undergoing differentiation in neurogenic medium at day-0, 12, 21 and 28 after performing immunofluorescence. Antibody against Sox2 was used to stain the cells and almost cells of undifferentiated ES cell colony were found positive and no expression was detected at day-12, 21 and 28 (Dapi was used to stain nucleus and FITC conjugated secondary antibody to stain Sox2; all pictures were taken at 20X magnification and scale bar is 32 $\mu$ m)

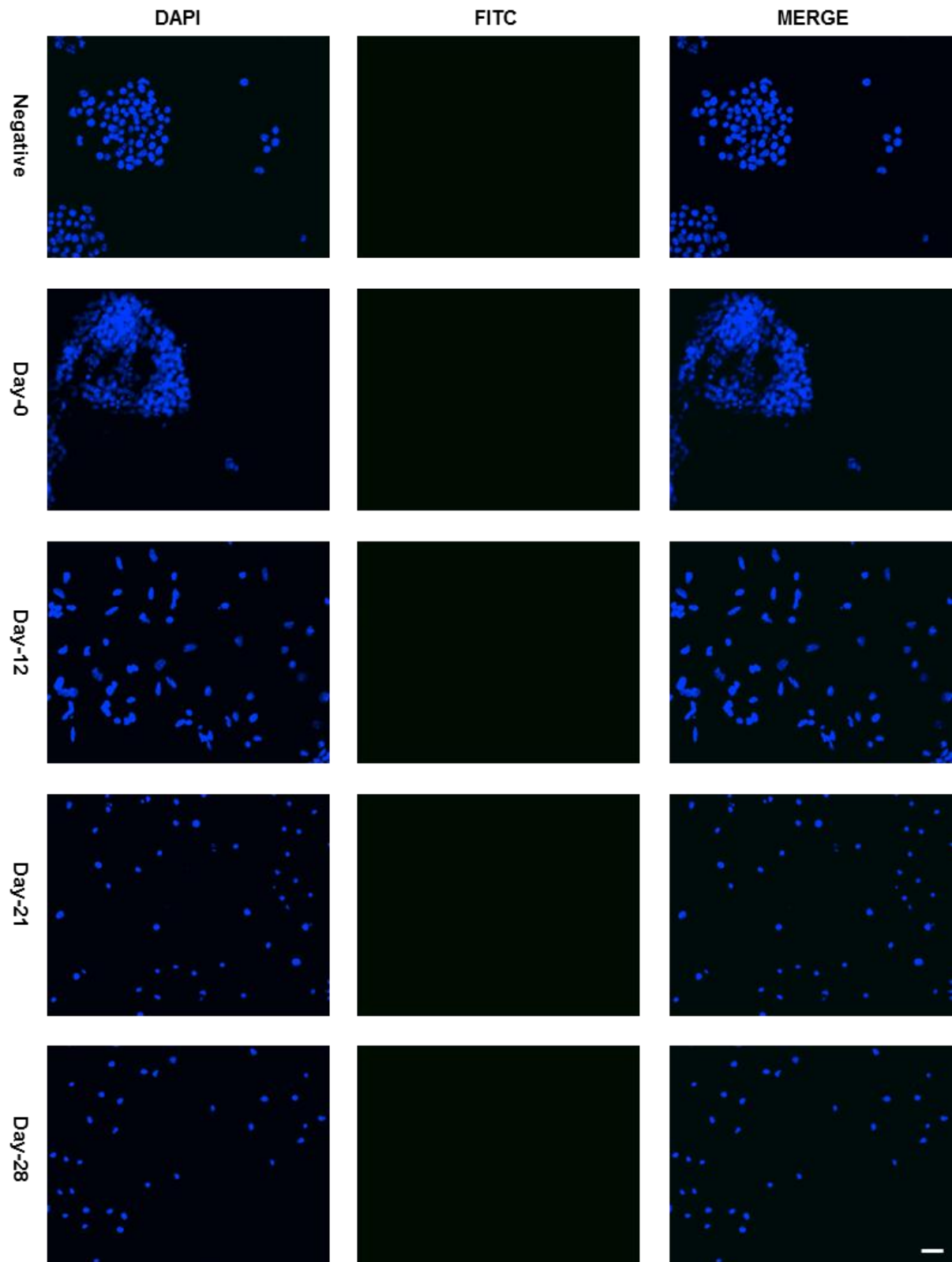


Figure 4.6: Images of E14Tg2a cells undergoing differentiation in neurogenic medium at day-0, 12, 21 and 28 after performing immunofluorescence. Antibody against Sox9 was used to stain the cells and no expression was detected at day-0, 12, 21 and 28 (Dapi was used to stain nucleus and FITC conjugated secondary antibody to stain Sox2; all pictures were taken at 20X magnification and scale bar is 32 $\mu$ m)

### **4.3.3. Molecular characterization of differentiated cells**

#### **4.3.3.1. Gene expression analysis by RT-PCR**

Gene expression profiling of embryonic stem cells pluripotency markers Oct4, Nanog, Sox2 and some of neural-markers i.e. Sox9, Nestin, Pax6 and beta-tubulin III were carried out employing random primed cDNAs prepared from RNA extracted from cells undergoing differentiation at different time-points. A house keeping gene GAPDH was used as endogenous control (section 3.3.4.1 for using GAPDH as a normalization control). Products of PCR were visualized by ethidium bromide stained agarose gels using AIDA image analysis software (Figure 4.7) and semi-quantification of gene products was carried out by taking ratio of gel bands to that of GAPDH (Figure 4.8-4.9).

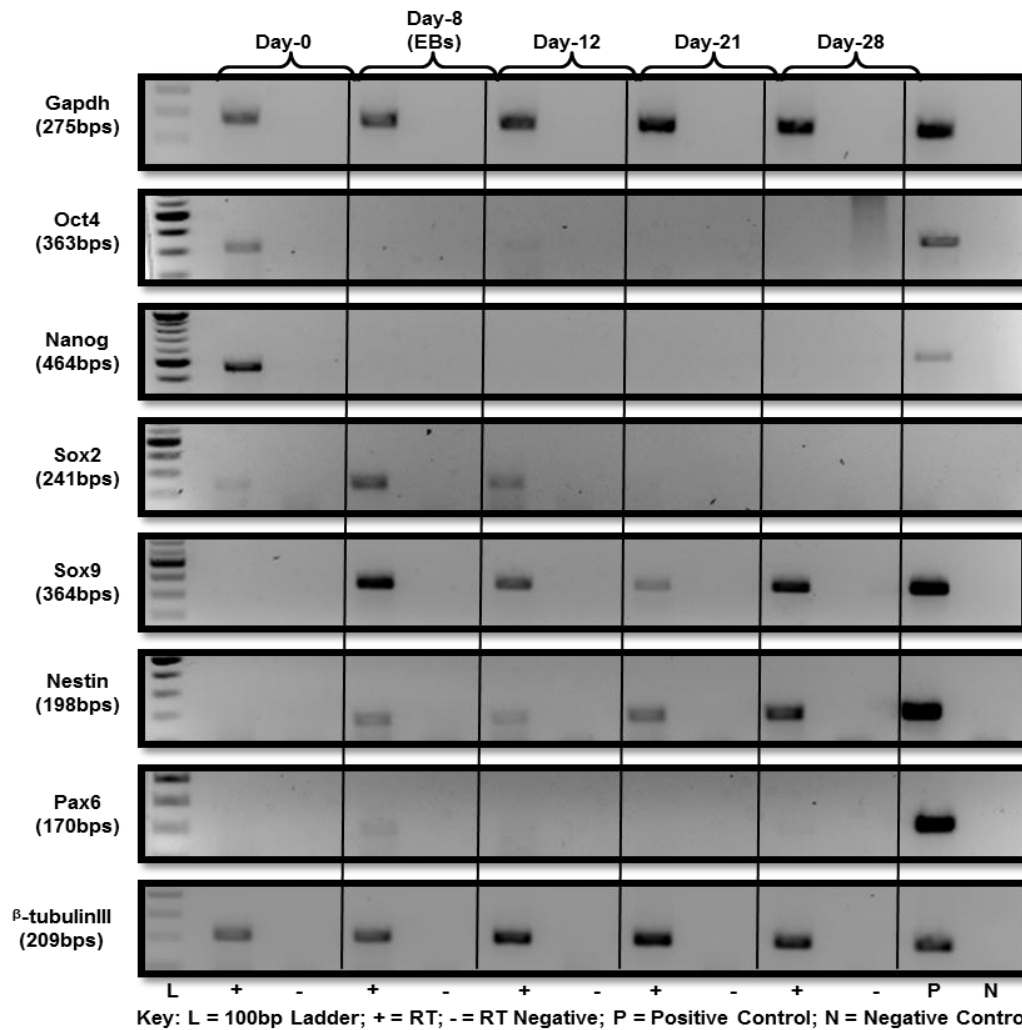


Figure 4.7: Gene expression profiling of mouse ES cells grown in neural differentiation medium using random primed cDNA prepared from cells harvested at day-0, 12, 21 and 28 following differentiation.

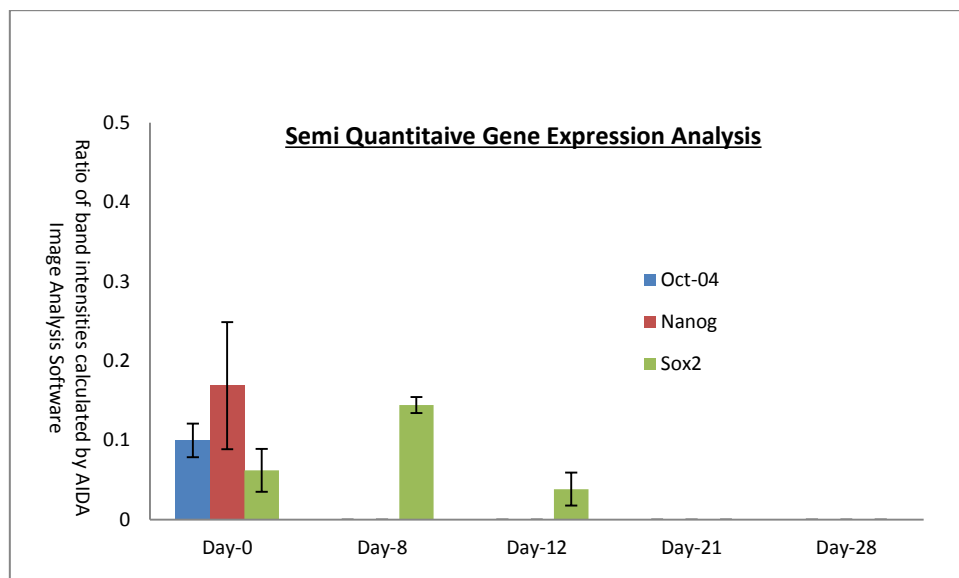


Figure 4.8: Semi-quantitative gene expression analysis showing expression trends of Oct-4, Nanog and Sox9 genes in mouse embryonic stem cells grown in neural differentiation medium. Results are presented as mean $\pm$ SEM.

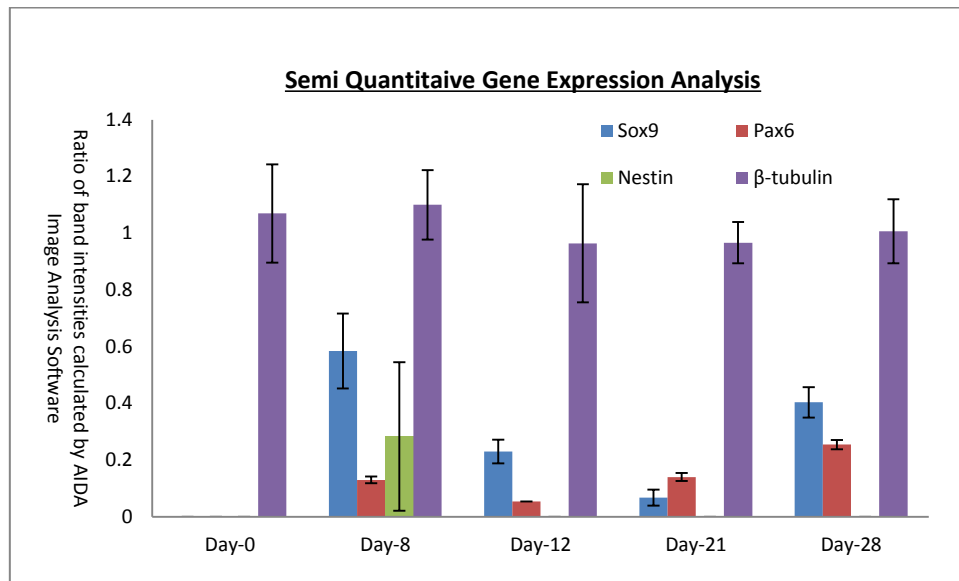


Figure 4.9: Semi-quantitative gene expression analysis showing expression trends of Sox9, Pax6, Nestin and beta-tubulin III genes in mouse embryonic stem cells grown in neural differentiation medium. Results are presented as mean±SEM.

Oct4, Nanog and Sox2 were used as markers of embryonic stem cells pluripotency and all were found to be expressed at day-0. Both Oct4 and Nanog were observed to be down regulated after day-0 as cells differentiate with a very small expression of Oct4 at day12. Sox2 was found to be expressed until day-12 and then beyond that no expression was found at RNA level (Figure 4.7 and 4.8).

Sox9 was observed to be up regulated at day-8 EB stage and then down regulated and seemed to be again up regulated at day-28 stage following plating of dissociated EBs (Figure 4.7 and 4.9).

Nestin was found to be upregulated at day-8 EBs and then found to be expressed until day-28. Pax6 expression was observed at day-8 and then appeared to be down regulated at day-12

post plating of dissociated EBs and no expression was detected at RNA level (Figure 4.7 and 4.9).

Beta tubulin III was included as neural specific marker and found to be expressed at all time-points and apparently peaking in expression at day-12 and lasting until day-28 (Figure 4.7 and 4.9).

#### 4.3.3.2. DNA methylation analysis of SRR2 by methylation sensitive PCR (MS-PCR)

MS-PCR was carried out in the similar way as already detailed in chapter 2 (section 2.3.2) and chapter 3 (section 3.3.4.2). SRR2 region of Sox2 was observed to be methylated at all time-points analysed i.e. day-8, 12, 21 and 28 (Figure 4.10). Controls shown in Figure 4.10 are already detailed in section 3.3.4.2. Upper panel of Figure 4.10 shows the multiplex PCR reaction products for controls where top-band is methylated Igf2R (so band is seen in both Uncut and *HpaII* lanes), middle-band is DNA sequence lacking any *MspI/HpaII* sites (band in all three lanes) and lower band is unmethylated sequence of Aprt promoter (only present in Uncut lane).

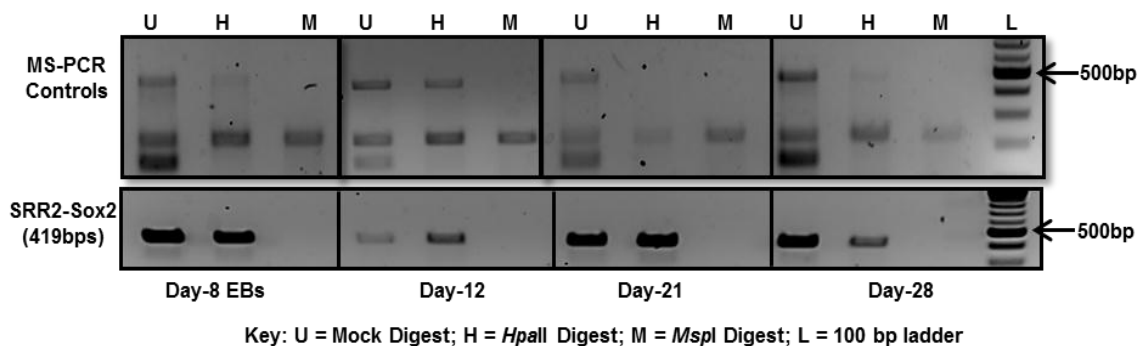


Figure 4.10: MS-PCR gels showing methylated product for Sox2-SRR2 at all time-points chosen for analysis. First the genomic DNA was extracted from ES cells at day-0, 12, 21 and 28, digested using *MspI* and *HpaII* restriction enzymes and then analysed by PCR.

#### 4.3.3.3. DNA methylation analysis of SRR2 by Bisulphite Sequencing

In order to further confirm the MS-PCR data and analyse the methylation status at each individual CpG dinucleotide in the SRR2 region of Sox2, DNA collected at each differentiation time point was treated with bisulphite, then PCR amplified and sequenced. The region analysed by direct sequencing contained three CpG dinucleotides that were found to be unmethylated at time-points selected for investigation (Figure 4.11).

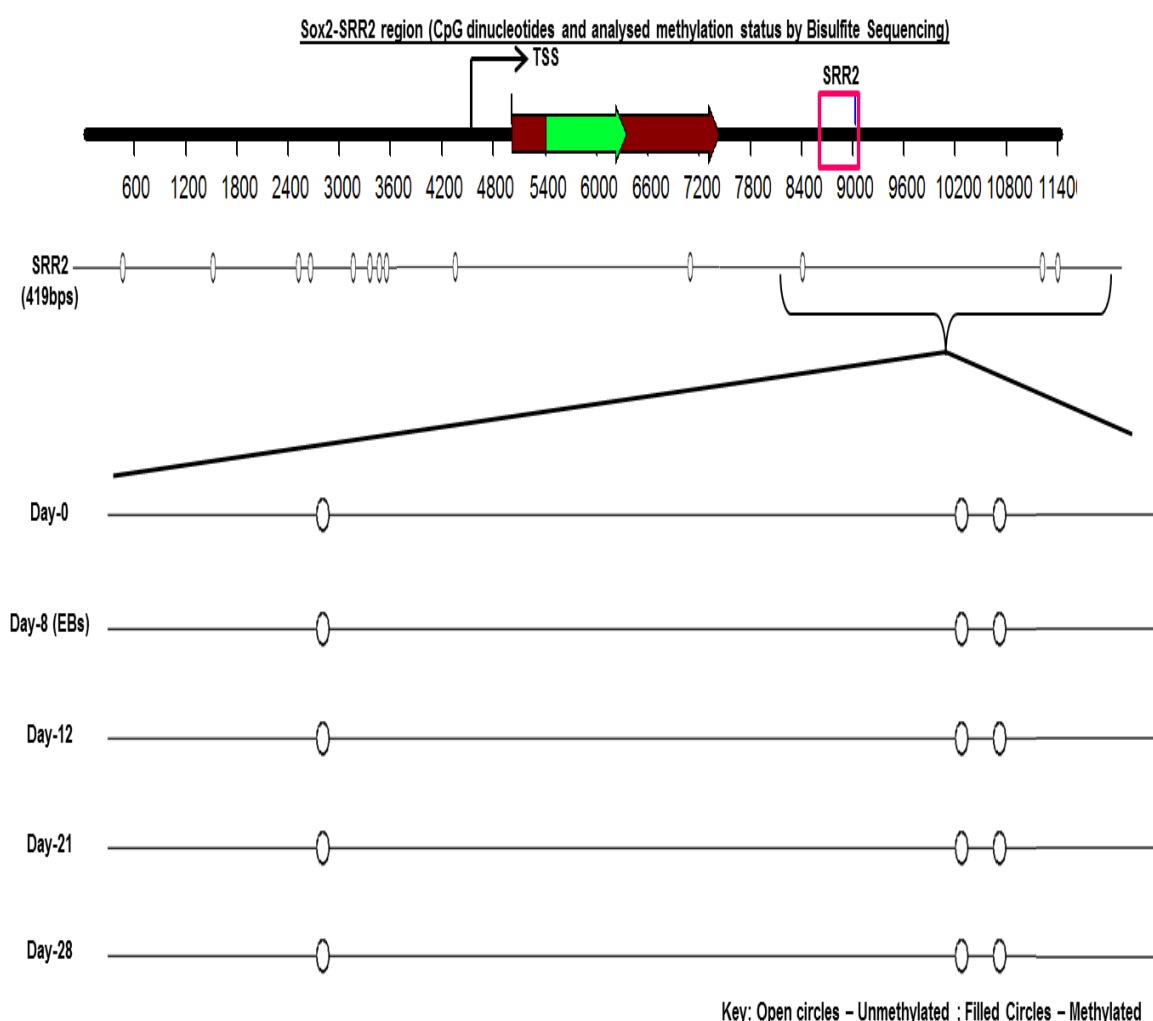


Figure 4.11: Bisulphite sequencing results of DNA methylation status of CpG sites in SRR2 region of Sox2. These three sites were found to be unmethylated in ES cells before differentiation and after culturing for 28 days in differentiation media at all time-points analysed.

#### **4.4. Discussion**

Mouse ES cells (E14Tg2a) were first grown in non-adherent bacterial dishes in EB medium to form EBs for 4 days and then retinoic acid (RA) was added to the culture medium and EBs grown for further 4 days. It has been experimentally shown by numerous research studies that a homogenous population of ES cells when treated with RA becomes enriched in neural precursors which subsequently can be differentiated to different neuronal subtypes using various inductive signals and selective media (Bible *et al.*, 2004; Bible *et al.*, 2007; Bain *et al.*, 1995; Strubing *et al.*, 1995; Fraichard *et al.*, 1995; Finlay *et al.*, 1996; Okabe *et al.*, 1996; Li *et al.*, 1998; Stavridis and Smith, 2003; Cai and Geabel, 2007). EBs obtained after 8 days were disaggregated by trypsinization and plated in neural differentiation medium (DMEM/F12 supplemented with neural specific supplement N2) in gelatine coated plates. Cells obtained from the same EB pool were also plated in medium lacking N2 with the objective to use this cell population as control since this was the only additional component to basal medium that could be omitted in parallel set of experiments if they were to use as control in spirit. These EB derived cells when grown in defined medium suffers from a significant reduction in cell number at first since they are transferred from serum-containing medium to serum-free medium, but those that survive are mostly neural progenitors which can be expanded in culture and induced to differentiate into neurons (Bible *et al.*, 2004; Bible *et al.*, 2007; Okabe *et al.*, 1996; Li *et al.*, 1998; Stavridis and Smith, 2003; Ying and Smith, 2003).

Neural differentiation of E14tg2a was first analysed by examining the specific neural morphology under microscope and this was evident after 24 hours of plating dissociated EBs. By day-4 after plating neurites can be seen forming dense and tangled networks with typical

neuronal morphology in N2 media as shown in Figure 4.3 which became more denser with time in culture (by second and third week). This was consistent with the findings reported by studies from which this protocol was derived (Bible *et al.*, 2004; Bible *et al.*, 2007). In the control medium, as expected, very few cells were found to attach 4-days after plating with a morphology resembling that of cells undergoing senescence and by day-6 almost all of the cells had died (Figure 4.3 presents some representative pictures). Cell death could be attributed to the fact that DMEM/F12 is a basal medium (Dulbecco and Freeman, 1959; Ham, 1965; complete composition Invitrogen Gibco 21331-020) and is unable to support growth of cells at its own unless supplemented with further additional supplements that aid in cell attachment, survival, proliferation and/or differentiation (Butler and Jenkins, 1989). And further augmenting the cell death was transfer of cells from serum-based medium to serum-free medium which on top of that also lacked any growth factors to support growth of cells (Ying and Smith, 2003). And this could possibly be more exasperated by pre-treatment of cells to become neural progenitors which also needed specialized environment to grow and expand which was only provided in parallel culture medium supplemented with N2. Since cells seeded in control media all died, this merits explanation what is considered control in such differentiation studies.

As detailed earlier in section 4.1.2 that for differentiation, ES cells are allowed to aggregate in the absence of LIF in suspension culture to form EBs for a defined time periods (in most cases 3-8 days), these EBs are then returned to serum-containing medium or serum-free medium either as aggregates or disaggregated to single cells, and allowed to further differentiate to produce terminally differentiated cell types. Most of the ES cell derived differentiations have been optimized using serum-containing media. Serum is added in cell culture medium as an essential growth supplement where it serves a source of broad range of

macromolecules, hormones, growth factors and provides attachment and spreading factors. But undefined composition of serum, batch to batch variations which often lead to phenotypic differences in cell cultures and risk of spreading contamination are major drawbacks and hence studies are now focusing more towards the use of serum-free media (Bruner *et al.*, 2010; Keller, 2005). In the absence of serum, a large number of molecules need to be added in media that could maintain cell adhesion, proliferation, growth and survival. Defined media are optimized through empirical approaches such that growth of particular cell types is favoured over others. When cells are transferred to serum-free media, it takes some time for cells to adapt to this new environment particularly if they were originally maintained in serum-containing media (van der Valk *et al.*, 2010). One way to overcome this is to seed cells in higher number, so here in this study (described in this chapter) cell seeding density used was adjusted according to original protocol keeping the same surface area to volume ratio as was optimized by them (Bible *et al.*, 2007; Ying and Smith, 2003)

Those differentiation studies that make use of serum-containing medium post EB stage use additional supplements to promote lineage induction/enrichment towards desired cell type. Because additional supplements are added in normal serum-containing basal medium, this necessitates setting up a parallel set of experiments using same EB derived cells under similar conditions but not receiving additional supplements which are then allowed to grow alongside the cells receiving additional supplements. Differentiation endpoint or enrichment of desired cell type is always studied using lineage specific markers (RNA, protein) and functional assaying of cells phenotype. Ultimate test of differentiated phenotype is achieved by transplanting these cells to appropriate animals models where they should retain their functionality (Keller, 2005; Wobus and Boheler, 2005). The idea behind setting up of these parallel set of experiments is to compare the enrichment of particular cell type in response to

added supplements compared to differentiation observed in serum only since serum itself contains inducing factors (undefined composition) as discussed above and is prone to batch to batch variations. In fact the pioneering study that demonstrated the differentiation potential of ES cells into derivative of all three germ layers when they removed the feeder layer maintaining undifferentiated state of cells in standard serum containing media did not use any additional factors (Doetschman *et al.*, 1985). These control culture does not imply that they will have cell types of interest only or will not have cell types of interest at all. These are mixed cell populations derived from same EB pool from where the differentiated cell types of interest are generated.

Traditionally cell populations grown in serum only medium where no additional supplements are added are called “controls” presumably because this is the only constant factor in such experiments while the additional supplements (dose, duration, combination, time of application) added is experimental variable which can be altered according to needs/research question. It should be noted here that this additional control group only serves to compare enrichment of desired/required cell type in response to added growth factors in serum compared to differentiation seen in serum only medium without addition of exogenous factors and is not a control *per se*. Differentiated cells are always analysed and characterized using features inherent to that particular cell type e.g. morphology, lineage specific markers, functional assessment of phenotype etc. Numerous studies that attempted directed differentiation of ES cells to other lineages using routine serum based media supplemented with some exogenous factors documented their findings in both serum only media and media supplemented with additional factors (an examples is chapter 3 of this study; few other examples are Buttery *et al.*, 2001; Hamazaki *et al.*, 2002).

In contrast studies that use serum-free defined media for directed differentiation towards cell type of interest make use of selective nature of medium to specifically allow growth of desired cell types and hence do not need a de facto control group. As detailed above that such a control group is technically needed in studies that are attempting to achieve differentiation in response to added supplements to routine serum based media so as to compare the differentiation seen with and without applied experimental treatment. But here in these studies using defined media, medium composition is optimized such that only cells able to respond to those signals can survive hence acting as filter in its own against unwanted cell types and is the only experimental variable that can be altered where by definition, control is something that is fixed/same in all experimental conditions tested.

For neural differentiation such protocols have become available which make use of defined media and hence not needing an inclusion of parallel set of experiments in contrast to differentiation studies making use of supplements in routine serum based media. As noted above that this control group only serves to compare the differentiation achieved under experimental conditions tested to what seen in serum only. Differentiated phenotype is always characterized by analysing the cell types generated. With use of defined media compositions such comparison is essentially not needed. Hence those preliminary studies that are aiming to screen for such defined milieu to get terminally differentiated cell types of interest use empirical combinations of different factors known from previous studies to determine the optimal media compositions. For example a study by Okabe *et al.*, in 1996 reported use of serum-free defined media for *in vitro* neural differentiation of mouse embryonic stem cells. This study aggregated ES cells as EBs for 4 days and then plated those aggregates in DMEM/F12 supplemented with insulin, transferrin, selenium and fibronectin (ITSFn) which was previously reported to be used for neural induction of embryonal

carcinoma cell line (Rizzino and Growley, 1990). They documented significant loss of cell viability at first when cultures were transferred from serum containing media to serum free formulation but ultimately they were able to get neural progenitors where more than 85% were nestin positive. Nestin has been a traditional marker associated with neural precursors that can subsequently give rise to neurons, glia and astrocytes (Landhal *et al.*, 1990). These nestin positive cells were then subsequently differentiated to neuronal cell types with high efficiency using defined medium and differentiated cells were analysed by expression of lineage specific markers and electrophysiology. This study did not include an explicit control rather compared data obtained across different experimental conditions tested to conclude which was optimal.

The principal objective of directed differentiation experiments is to identify conditions that would ideally favour the induction of particular lineage precursors (in EBs or monolayers of ES cells) and then use optimal selective media conditions to allow those precursors to proliferate and generate the pure cultures of terminally differentiated cell types. So eventually desired cell types can be produced *in vitro* under controlled conditions using defined media supplemented with inductive growth factors (Smith, 2001). For neural differentiation, which is one of the extensive studied *in vitro* differentiation, such two-step protocols are available. The study presented in this thesis used similar two step protocol of neural differentiation i.e. enrichment of neural progenitors in EBs using RA treatment and then selectively allowing neural precursors to proliferate in defined medium (DMEM/F12/N2) to differentiate towards neural cell types (Bible *et al.*, 2007; Bible *et al.*, 2004). Different studies have shown experimentally that a homogenous population of ES cells when treated with RA becomes enriched in neural precursors which subsequently can be differentiated to different neuronal subtypes using various inductive signals and selective media (Bible *et al.*, 2004; Bible *et al.*,

2007; Li *et al.*, 1998; Stavridis and Smith, 2003; Cai and Gabel, 2007). And one study has experimentally demonstrated that neural precursors generated by RA treatment (similar to one used in this protocol) can form cells of motoneurons and interneurons in anterior spinal cord when transplanted to embryonic chick neural tube showing that such a treatment could lead to cells which retain functionality when transplanted (Plachta *et al.*, 2004). As detailed above that such a study design which is biased towards enrichment of particular desired cell type using selective treatment does not have a bona fide control. And those studies from which this protocol was derived also did not include such an explicit control in their studies (Bible *et al.*, 2004; Bible *et al.*, 2007). Yet study documented in this chapter attempted to set up a parallel set of experiments as explicit control with the objective to compare differentiation seen with N2 and without N2 since N2 was the only additional component added to basal media. EBs grown for 8 days in suspension culture were disintegrated (section 2.2.4) and seeded in differentiation media (DMEM/F12/N2) and control media (DMEM/F12 alone). The idea to seed cells in DMEM/F12 was to use this group of cells as control but cells did not survive and reasons are discussed above. This step experimentally demonstrated that DMEM/F12 alone did not support growth of cells unless supplemented with additional growth factors/serum.

The experimental set up described in this chapter is different to one described in Chapter 3 (osteoblast differentiation) where differentiation was attempted by adding exogenous factors to serum based medium so control cultures were set up using supplements minus medium. This control contained the same pool of EB derived cells but not received additional supplements. While in neural differentiation experiment only additional supplement added in medium was N2 and so the only way to set up control cultures was to grow cells without N2. Such a step was redundant given that cells do not survive in DMEM/F12 alone as discussed

above. The other way to set up parallel control culture could possibly be using cells grown in absence of RA. But there are already studies available that attempted successful *in vitro* neural differentiation of mouse ES cells without RA treatment (Okabe *et al.*, 1996; Lee *et al.*, 2000; Rolletschek *et al.*, 2001). They all used specific growth factors in combination with serum-free defined medium and achieved differentiation to different types of neurons. Hence if such a step was included, it would at best served as an alternate strategy to achieve differentiation and not a control culture. The reason why probably neural cell types can be achieved in culture with and without RA is that neural induction is considered a default differentiation pathway during vertebrate embryogenesis under repressive control of BMP signalling (Munoz-Sunjuan and Brivanlou, 2002). Studies have very early on reported that if ES cells are allowed to differentiate under chemically defined serum-free conditions they preferentially express markers of neuroectoderm without addition of any additional growth factors (Wiles and Johansson, 1999). The problem however is that differentiated culture almost always contain different types of neural cells i.e. neurons, astrocytes, oligodendrocytes and glia and that's why variety of protocols are available to achieve enrichment of a particular cell type by addition of specific growth factors at different stage of differentiation subsequent to neural progenitors formation which is a common step in majority of differentiation protocols (Stavridis and Smith, 2003).

Whether there is an explicit control group or not, cells grown in differentiated media are always analysed and characterized by using established markers and functional assays for such cell types. In order to further characterize the differentiated cells beside morphology, a set of markers was selected for expression studies at RNA and protein level in accordance with the data reported for earlier studies reporting *in vitro* differentiation of embryonic stem cells in to neural lineage (Bain *et al.*, 1995; Bible *et al.*, 2004; Bible *et al.*, 2007; Stavridis

and Smith, 2003). This included Sox2, Sox9, Nestin, Pax6 and beta-tubulin III. Sox2 along with other members of SoxB family has been reported to regulate and co-ordinate neuroectodermal specification and commitment (Pevny and Placzek, 2005). More specifically, Sox2 has been reported to maintain neural progenitor identity (Graham *et al.*, 2003) with subsequent down regulation as these cells become committed. Here in this experiment Sox2 was found to be down regulated at day-8 EBs stage and then further reduction in expression level at day-12 and beyond that time point no expression was detected (Figure 4.7).

In order to confirm the molecular expression data, protein expression of Sox2 was also analysed by immunocytochemistry and only undifferentiated ES cells were found to be positive (Figure 4.5). No Sox2 protein expression was observed at day-12 which could be due to cells still containing RNA but not transcribing it to functional protein product. Post transcriptional gene silencing involving long non coding RNAs and small interfering RNAs is now considered important regulatory phenomenon particularly with reference to their role in embryonic stem cell differentiation (Tay *et al.*, 2008; Dinger *et al.*, 2008). Indeed, Sox2 gene has been shown to be embedded within an intron of long non-coding RNA which transcribes in the same orientation and has been documented to have regulatory roles during vertebrate development (Amaral *et al.*, 2009).

Sox9 showed marked up regulation at day-8 EB stage at RNA level (Figure 4.7) and it has been reported to be up regulated prior to gliogenesis shifting potential of neural progenitors from neurogenic to gliogenic and disappears from oligodendrocyte lineage once cells differentiate terminally (Stolt *et al.*, 2003). It has been shown that retinoic acid treated ES cells form precursor cells with the characteristics of radial glial cells which eventually

differentiate into glutamatergic neurons (Bibel *et al.*, 2007). Sox9 was down regulated after day-8 EB stage and seemed to be up regulated at day-28 after plating of cells in neural differentiation medium. However, no Sox9 protein expression was detected at any time point along the whole differentiation time course starting from day-0 to day-28 (Figure 4.6). As described earlier, microRNAs are known to play an important role in gene regulation and an earlier study has reported the role of miR-124 in Sox9 protein suppression but mRNA expression is unaffected in adult neurogenesis (Cheng *et al.*, 2009).

Nestin is a neuroepithelial stem cell intermediate filament protein and, a well-established marker of neural stem cells and has been widely employed as marker in studies exploring neural differentiation of ES cells (Landhal *et al.*, 1990; Yaworsky and Kappan, 1999). It has been considered as a multipotent neural lineage marker expressed both in neurons and glia (Lenka *et al.*, 2002). It has been shown to be expressed in developing nervous system, neural stem cells from sub ventricular zone and to become down regulated when the cells differentiate (Wei *et al.*, 2002). Here in these studies nestin was observed to be up regulated at day-8 (EBs) stage and found to be expressed until day-28. As protein expression studies were not carried out, mRNA expression at this late stage could possibly mean that RNA not transcribed into functional protein through mechanisms similar to those described above. Alternatively, this expression may be accounted for by a subpopulation of progenitor cells still present in culture.

Pax6 has been reported to play an important role in neural precursor's proliferation, cortical lamination, and development of basal ganglia, neuronal survival and neurogenic fate determination (Kallur *et al.*, 2008). Pax6 has been shown to undergo dynamic changes in expression as ES cells become committed to neuronal differentiation (Gao *et al.*, 2011).

mRNA expression analysis in these experiments showed that Pax6 is up regulated in EBs after 8 days of differentiation which should be rich in neural precursors after RA treatment. Beyond day-8 no Pax6 expression was detected at RNA level (Figure 4.6). Earlier studies have shown that Pax6 mRNA was not detectable after day-12 in ES cells differentiated to neuronal cell types under similar conditions (Bible *et al.*, 2007; Bible *et al.*, 2004).

Beta-tubulin III belongs to the tubulin class of proteins and shows expression specifically in post mitotic neurons (Lee *et al.*, 1990), it is a classic marker to assess neural differentiation of embryonic stem cells (Bain *et al.*, 1995). In this study beta tubulin III was found to be expressed starting from day-0. It has been shown that undifferentiated mouse ES cells express beta III tubulin (Ginis *et al.*, 2004) though their role in undifferentiated cells has not been studied so far. Beta III tubulin was found be markedly upregulated after day-8 of differentiation and expression lasted until day-28.

Taken together these morphological and molecular data shows that ES are differentiating in this neurogenic media and results obtained are comparable to studies reported earlier. All of the markers associated with neural progenitor's identity tested in this study i.e. Sox2, Sox9, Pax6 and Nestin were found to be upregulated in day-8 EBs compared to no expression at day-0 undifferentiated cells. Then there appeared to be visible upregulation of beta-tubulin III, a marker extensively used to characterize differentiated neural cells. But it must be emphasized that no attempt to ascertain the purity of differentiated culture was made, hence it cannot be said with certainty that how much of differentiated population were neurons. Future work would be targeted to purify the differentiated cells and electrophysiology measurements would be carried out to determine whether neurons are actually forming active synaptic connections. It should also be noted here that the primary objective of the study

presented in this thesis was to study DNA methylation changes occurring in regulatory regions of Sox2 during differentiation of mouse ES cells when they are differentiated under different culture conditions. Hence differentiation was attempted using previously reported optimized protocols and no attempt was made to modify/refine them at this stage of analysis. But in future efforts would be made to compare and contrast different differentiation strategies to select the optimal one when repeating this experiment.

Epigenetic mechanisms of gene regulation involving DNA methylation, histone modifications and regulatory RNAs have now started to emerge as important mechanisms governing the neural specification of neural stem cells (Sanosaka *et al.*, 2010). The information about different regulatory regions of Sox2 and rationale for doing DNA methylation analysis has already been detailed in Chapter 1 section 1.5.3 and 1.6. Different regulatory regions of Sox2 have been implicated in directing region specific expression in cells of differing origin e.g. expression can be seen in telencephalon but not spinal cord stem cells (Zappone *et al.*, 2000).

SRR2, a regulatory region of Sox2 was found to be constitutively methylated at all time-points in the differentiated population studied in this chapter when analysed by MS-PCR. Given the limitation of the technique to resolve methylation status at each individual CpG, bisulphite sequencing was then carried out (section 3.4 for discussion about various techniques to study DNA methylation) . Unfortunately the whole region of SRR2 could not be sequenced as direct sequencing attempts were unsuccessful and time limitation did not allow attempting cloning of the fragment and subsequent clonal analysis. But fortunately analysed region contains the sequence which is core SRR2 sequence and contains an enhancer site which is under the control of Sox2/Oct4 complex (Tomioka *et al.*, 2002). This

enhancer has been reported as neural stem cell specific in telencephalon region (Miyagi *et al.*, 2006).

Three CpGs located in this enhancer region in SRR2 were analysed by direct DNA sequencing after bisulphite conversion of genomic DNA and they all were found to be unmethylated in undifferentiated and differentiated cells as shown in Figure 4.8. It has been shown by another study that this region exerts its enhancer activity in embryonic stem cells and neural stem cells but do not function when cells differentiate (Miyagi *et al.*, 2004). Furthermore an earlier study has shown the differential methylation at SRR2 in neurons v/s astrocytes generated from human neural precursors suggesting that terminally differentiated neurons use methylation of this region to silence the gene (Sikorska *et al.*, 2008). Here no methylation was observed at analysed three CpG sites found in the enhancer region suggesting possible involvement of other gene silencing mechanisms independent of DNA methylation such as histone modifications. Also there is possibility that heterogeneous nature of culture did mask the low level of methylation. So in future experiments effort would be focused towards establishing a pure population of cell types of interest. It could also be possible that other CpG sites not analysed in this study could have become methylated during differentiation. Hence further work extending the analysis to all CpG sites and also investigating chromatin structure surrounding the region would help in understanding the role of this region in gene silencing once cells become committed and differentiate.

**Chapter 5 - DNA methylation analysis of Sox2 regulatory regions SRR1  
and SRRR2 in mouse mesenchymal stem cells (MSCs) cultured in  
osteoblast differentiation medium**

## **5.1.Introduction**

### **5.1.1. Mesenchymal stem cells (MSCs) as a model to study osteogenesis**

Mesenchymal stem cells are mainly derived from bone marrow though have been shown to exist in many other tissues such as hair follicles, muscles, liver etc. (Bianco *et al.*, 2008). Bone marrow derived mesenchymal stem cells or also known as stromal stem cells; they are considered to be self-renewing and having multilineage differentiation potential forming different mesoderm derivatives including bones, cartilage and adipose tissues (Pittenger *et al.*, 1999). Recently bone marrow derived MSCs been also shown to transdifferentiate into other lineages derived from both ectoderm and endoderm such as neurons and hepatocytes (Charbord, 2010). The most salient features of MSCs are their readiness to grow in culture; as they have displayed and suppression of immune response *in vivo*; MSCs have already been in use clinically to assess the safety and efficiency of bone marrow transplants in human subjects (Ghannam *et al.*, 2010).

Bone tissue engineering has become an active focus of research due to increased number of people being affected with osteodegenerative disorders including but not limited to osteoporosis, osteoarthritis, skeletal deficiencies (Heng *et al.*, 2004). Thus *in vitro* differentiation of MSCs into bone tissues in a variety of experimental systems have made them a promising system to be used in cell based therapies for bone tissue engineering and regenerative medicine (Meijer *et al.*, 2007). Besides being used for therapies, they offer a model system to study osteogenesis which is under complex control of myriads of molecular mechanisms and signalling networks (Lian *et al.*, 2006).

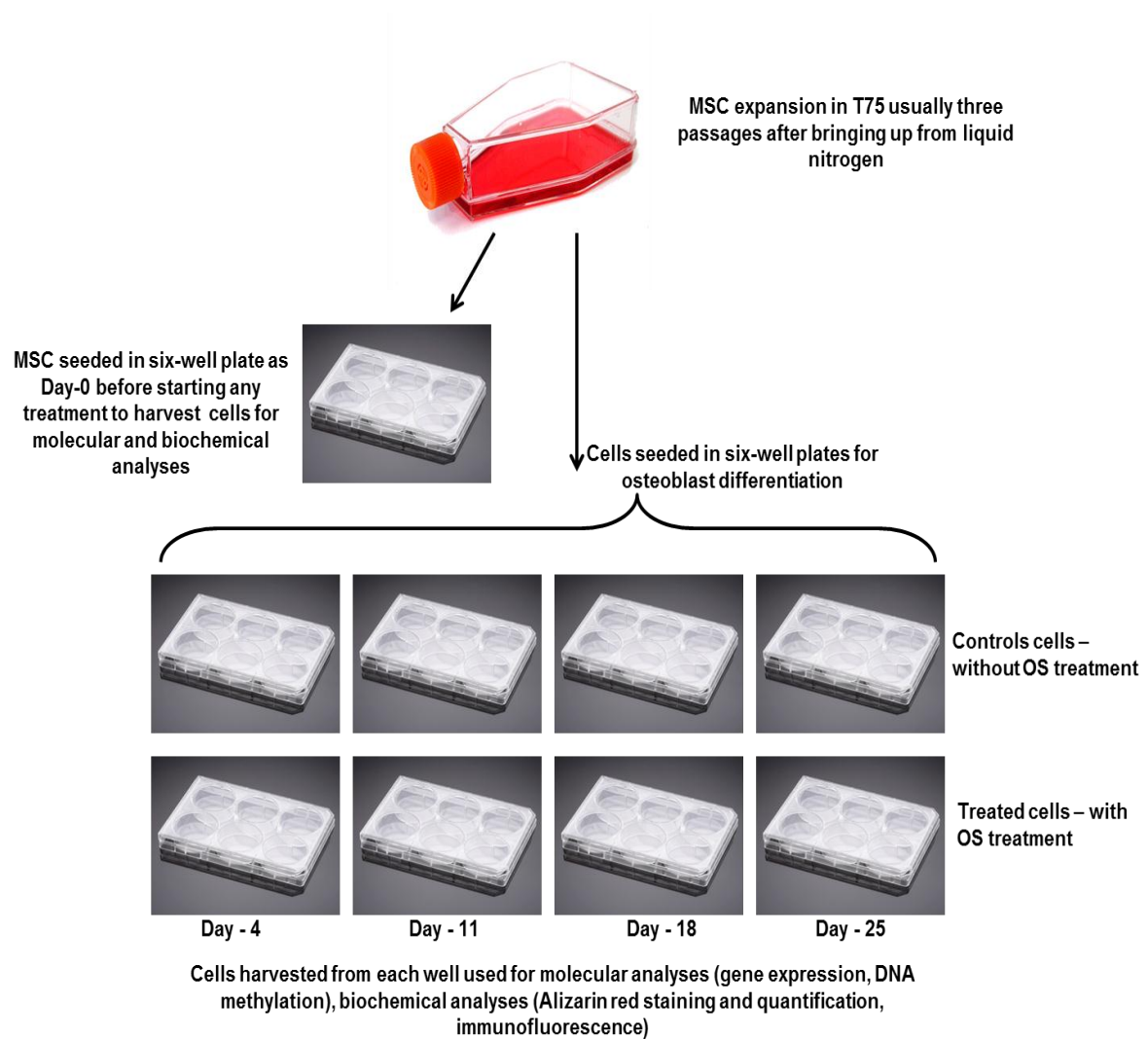
However, the low frequency of MSCs in bone marrow and limited self-renewal capacity has created a need to explore and exploit differentiation potential of pluripotent ES cells into bones which have been shown to regenerate themselves indefinitely under proper culture conditions (D'Ippolito *et al.*, 1999; Duplomb *et al.*, 2007). Furthermore bone formation has been thought to involve a hierarchy of precursor populations existing at various stages of bone development starting mainly from mesenchymal stem cells and going through osteoprogenitors, preosteoblasts and osteoblasts though their well-defined boundaries and compartmentalization are still under investigation (Long, 2001). It has been proposed that MSCs are osteoblastic precursors or pre-engaged cells originating from a primitive progenitor and hence might not allow investigating the early stages of bone development where ES could potentially overcome this issue (Duplomb *et al.*, 2007; Long, 2001). Nevertheless, MSCs have been extensively investigated *in vitro* for bone formation (section 3.1.1 in chapter 3 for detailed description of osteogenesis), so can also serve as positive control for ES derived osteoblast differentiation.

### **5.1.2. Sox2 in MSCs**

There are only limited studies so far investigating the role of Sox2 in MSCs in general and in osteoblasts in particular. Sox2 has been shown to maintain expansion and differentiation potential of MSCs (Go *et al.*, 2008). Though MSCs can renew themselves and have been used to repair bone disorders (Horwitz *et al.*, 1999), but with time they tend to lose their differentiation potential (D'Ippolito *et al.*, 1999). Hence understating the regulation of Sox2 in MSCs and during their differentiation would aid in refining methodologies attempting to use these cells for therapeutic purposes.

The specific role of Sox2 in osteoblasts is already described in chapter 3 section 3.1.3. In short, data available to date has shown Sox2 to be present in osteoprogenitors favouring their proliferation over terminal differentiation to osteoblasts. Given that MSCs are physiological precursors of osteoblasts, they were differentiated to osteoblasts in parallel with mouse ES cells using the same protocol and other experimental factors. The aim of these experiments was to study the molecular and biochemical changes occurring in both cell types when they are differentiated using identical experimental conditions. It was hoped that data obtained by this study could potentially help in understanding the regulatory role played by Sox2 in bone development, and conserved/shared gene regulatory pathways between these cell types.

## 5.2. Experimental work plan



## **5.3. Results**

### **5.3.1. Differentiation of mMSCs (D1 cell line) into osteoblasts**

#### **5.3.1.1. Cell Culture**

D1 – a mouse mesenchymal stem cell line (Diduch *et al.*, 1993) was retrieved from liquid nitrogen and cultured on tissue culture treated flasks with routine passaging on confluence to generate enough number of cells to set up differentiation and for cryopreservation. Some representative images of D1 stem cell line are shown in Figure 5.1.

MSCs cells were first seeded in six-well tissue culture plates in an undifferentiated state and allowed to reach confluence before changing to medium containing OS supplements as described in chapter 2 section 2.1.5. Cells grown in medium lacking OS supplements were taken as control. It has been reported that MSCs grown in presence of OS supplements form colonies with mineral deposition which can be further confirmed by microscopy and Alizarin Red staining. As shown in Figure 5.2, the D1 cell line when cultured under similar conditions, displays a gradual differentiation towards the osteogenic lineage with increasing density of dark granules that stained red with alizarin red dye.

#### **5.3.1.2. Alizarin red extraction and quantification**

Alizarin red dye was extracted after staining and quantified as described in detail in chapter 2 section 2.3.1. The amount of alizarin red dye extracted was normalized to DNA content of cells (section 2.3.2 and 3.3.1.2). Figure 5.3 and 5.4 shows digital images of the cells in a well

of six-well plate after staining with alizarin red dye. Cells grown in medium containing osteogenic supplements showed a significant increase in amount of alizarin red dye extracted in comparison to cells grown in medium devoid of them as shown in figure 5.5. Figure 5.6 is the DNA calibration curve used to normalize the amount of alizarin red dye. It needs to be highlighted here from data statistics point of view that all the measurements of alizarin red staining and quantification assays were made from two independent series of experiments done in triplicates due to same reasons discussed in Chapter 3 section 3.3.2.

### **MSCs grown in medium with and without Osteogenic Supplements**

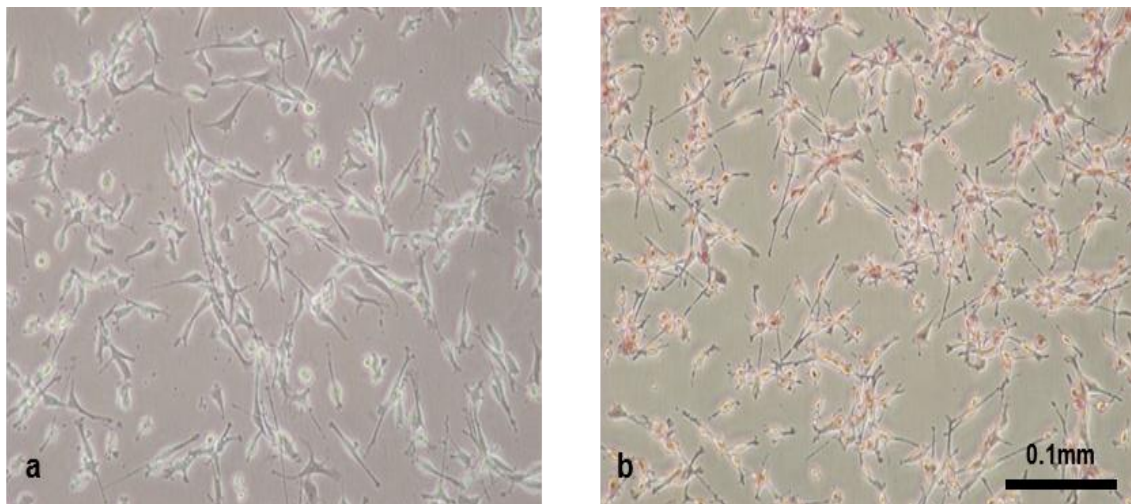


Figure 5.1: MSCs at day-0 prior to starting differentiation treatment (a) and stained with alizarin red dye (b). (All pictures were taken at 10X magnification).

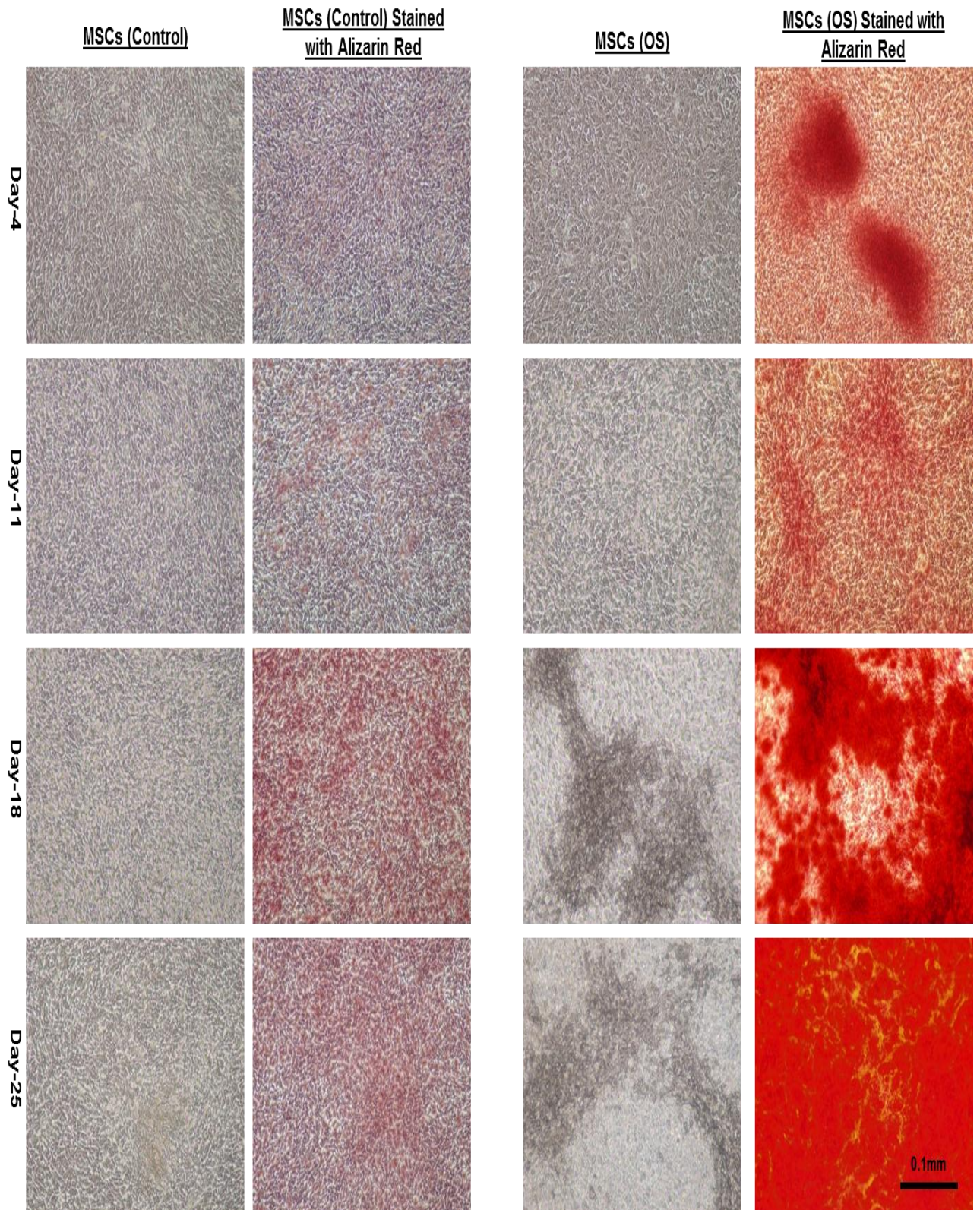


Figure 5.2: MSCs grown in medium containing and lacking osteogenic supplements for 4 weeks and then stained with Alizarin red dye. Dark areas surrounding cells are visible and stained red with dye (All pictures were taken at 10X magnification). It has been already detailed in section 3.3.1.2 in Chapter 3 that alizarin red stain in its own could not be taken as a measure of mineral deposition and so further data supplementing these observation is presented in section 5.3.1.3 and 5.3.2.

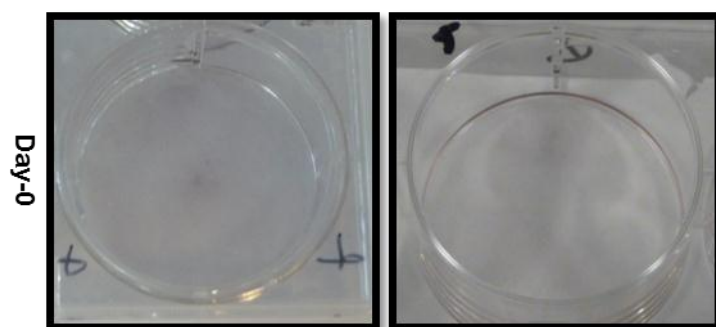


Figure 5.3: MSCs at day-0 prior to start of differentiation treatment and stained with alizarin red dye in six-well plates. No nonspecific staining was observed.

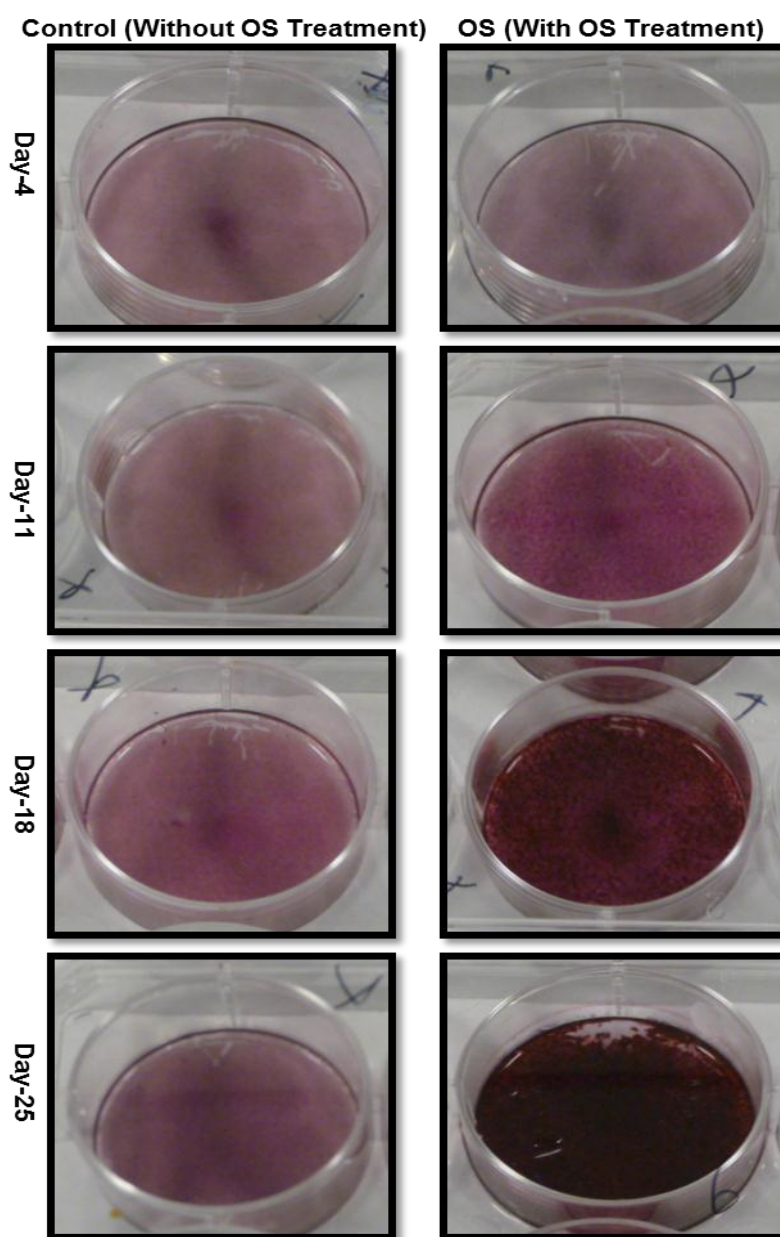


Figure 5.4: MSCs subjected to osteogenic differentiation for four weeks and stained with alizarin red dye for mineral deposition in six-well plates at various time points. Significantly intense staining is apparent in treated cells after two weeks compared to non-treated cell population.

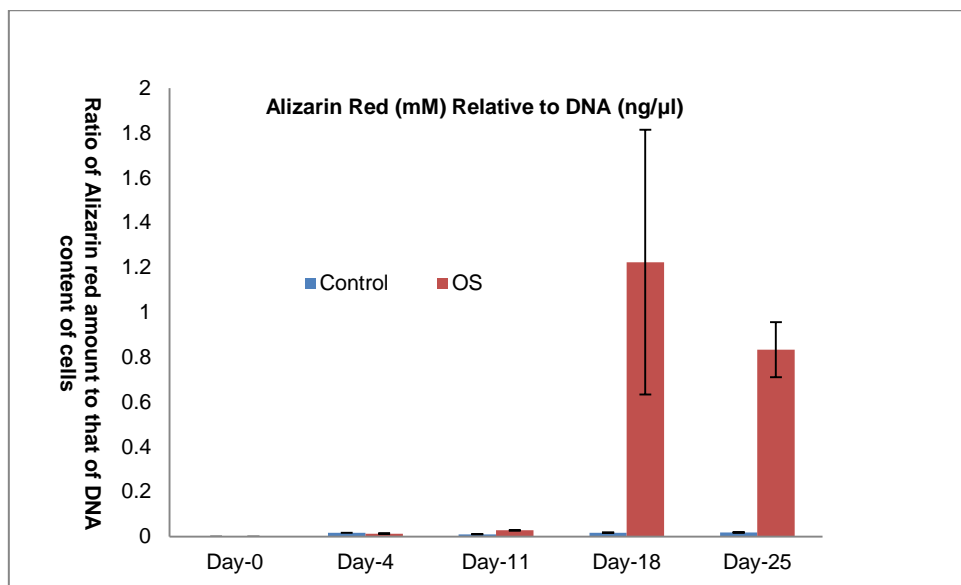


Figure 5.5: Mineral deposition was detected by alizarin red dye and then dye was acid extracted and quantified using a colourimetric assay. Data are collected from two plates, each analysed in triplicates and expressed as mean $\pm$ SEM (n=2). There appeared to be a significant difference in amount of mineral deposition in treated and untreated cells.

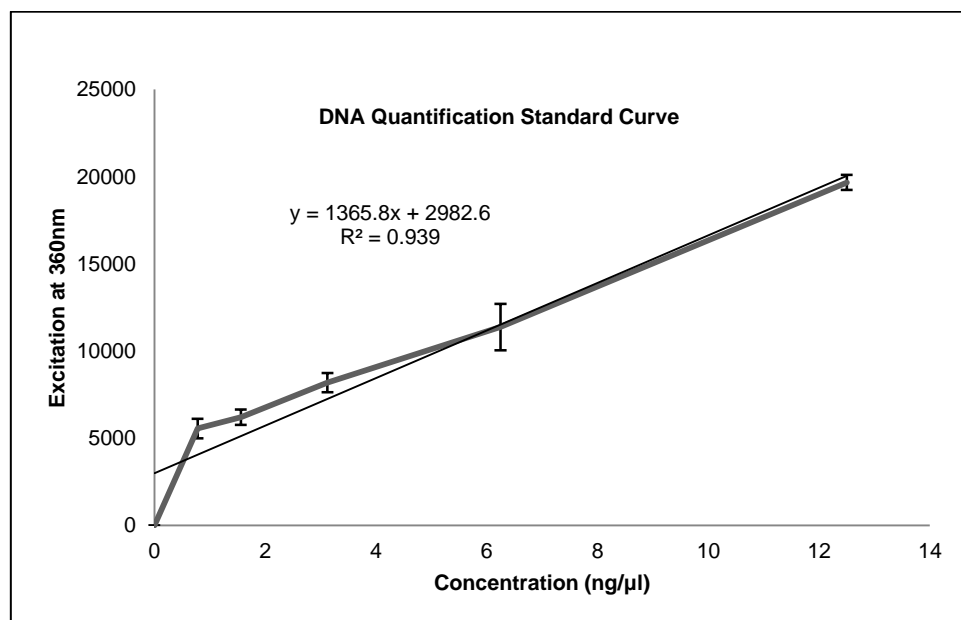


Figure 5.6: DNA calibration curve generated to normalize the amount of mineral deposition relative to cell contents of plates (data is collected from two plates and each analysed in triplicates so mean $\pm$ SEM with n=2)

### **5.3.1.3.Immunofluorescence**

Immunofluorescence was performed using antibodies against Sox2, Sox9, Osterix and Osteocalcin to detect the localization and expression of these marker proteins. Almost all of the cells in undifferentiated MSC culture were found to express Sox2 protein (Figure 5.10). Then only some of the cells were found to express Sox2 protein at day-4 in control cells and after wards no signal was detected (Figure 5.10). Cells grown in medium containing OS supplements did not show expression at any time point analysed beyond day-0 as shown in Figure 5.11.

Sox9 was detected in MSCs at day-0 but not all of the cells were observed to be expressive. Cells grown in medium lacking OS supplements showed a low level of protein expression in nuclei at day-4, day-11 and very few cells at day-18. No signal was observed at day-25 (Figure 5.12). While cells grown in medium containing OS supplements appeared to show gradual increase in positive signal with less positive cells at day-4 and day-11 and more at day-18 and day-25 (Figure 5.13).

Osterix protein was not found to be expressed at any time points in cells grown with and without OS promoting factors (Figure 5.14 and 5.15).

A strong cytosolic signal was observed for Osteocalcin starting from day-0 in undifferentiated MSCs and cells at later points i.e. day-18 and day-25 showed a high level of expression in both control (Figure 5.16) and OS populations (Figure 5.17). There was marked difference in level of expression in cells grown in OS medium.

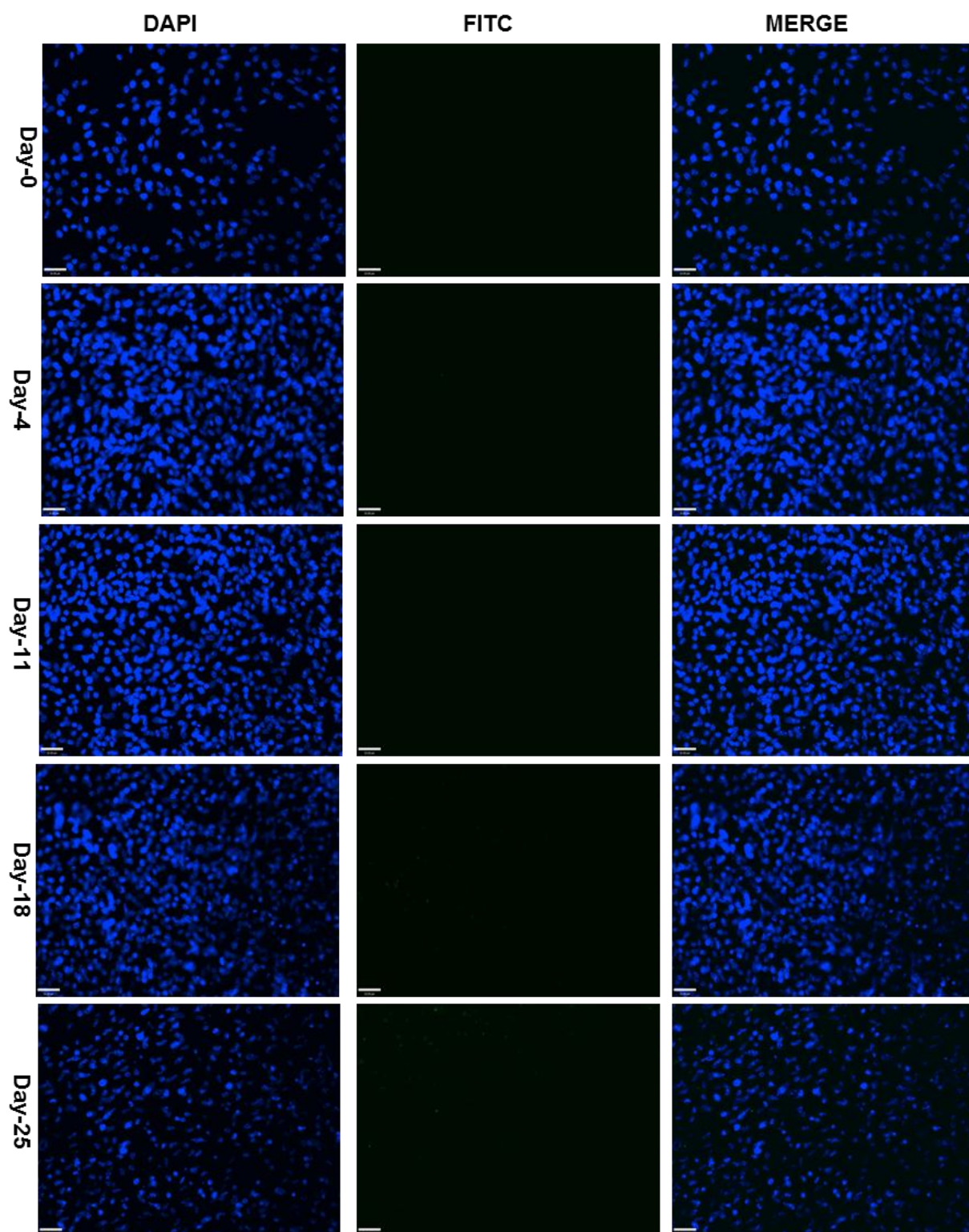


Figure 5.7: Mouse MSC (D1) cells grown in control medium were stained without primary and secondary antibodies to account for auto fluorescence and/or background staining (Dapi was used to stain the nucleus). No fluorescence was observed (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

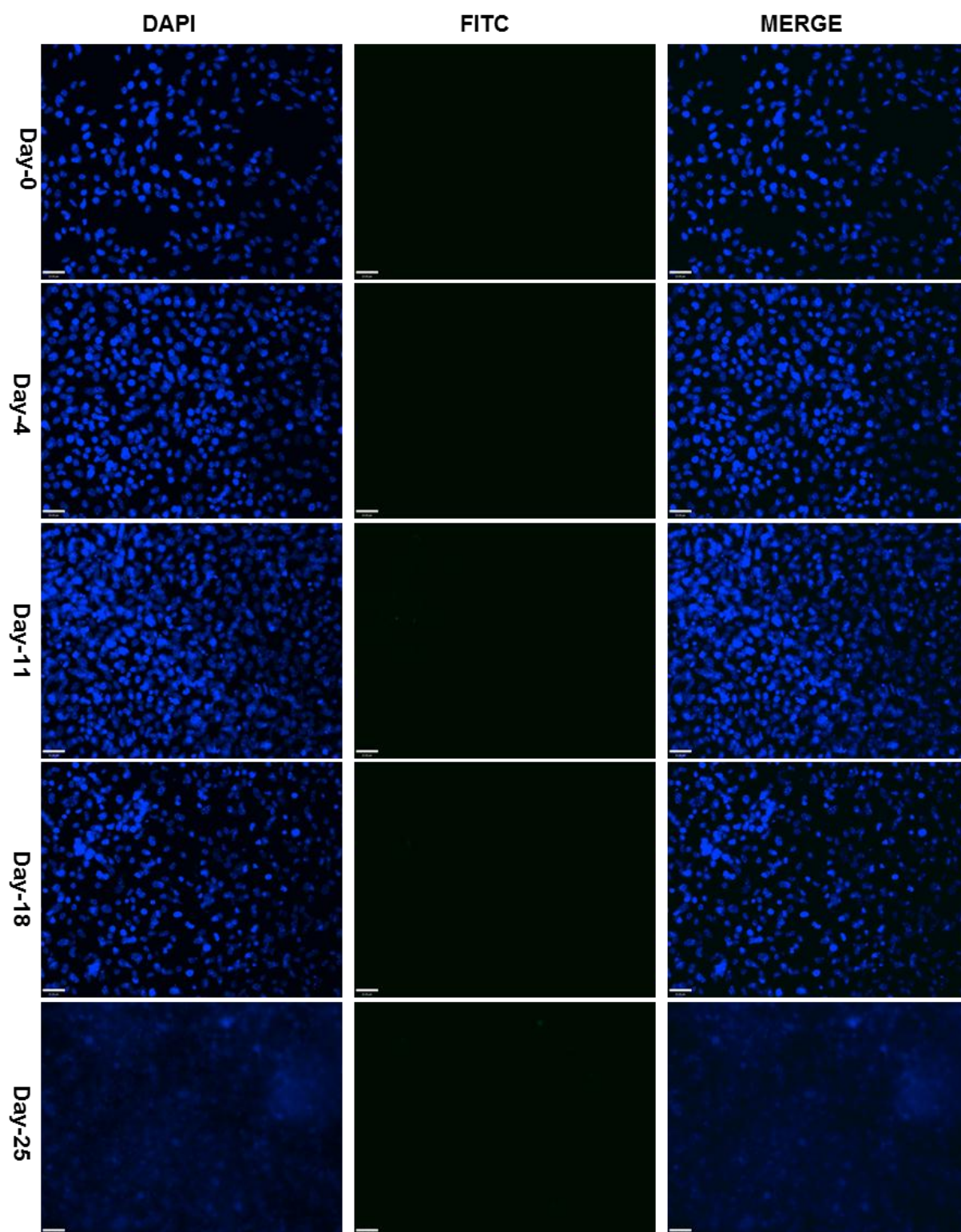


Figure 5.8: Mouse MSC (D1) cells grown in OS medium were stained without primary and secondary antibodies to account for auto fluorescence and/or background staining (Dapi was used to stain the nucleus). No fluorescence was observed (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

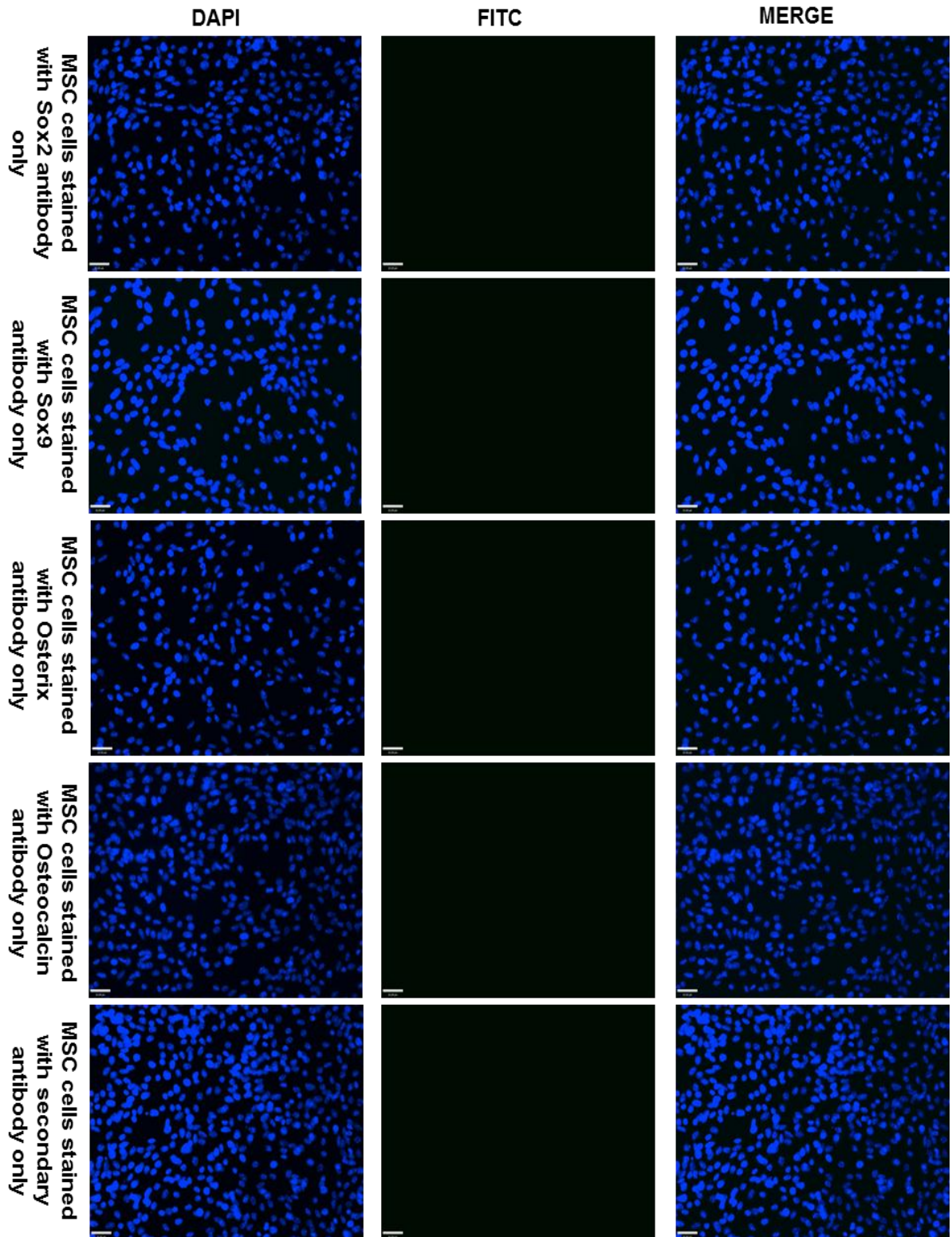


Figure 5.9: Mouse MSC (D1) cells stained without primary antibodies only and secondary antibody only to account for nonspecific fluorescence and/or background staining (Dapi was used to stain the nucleus). No fluorescence was observed (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

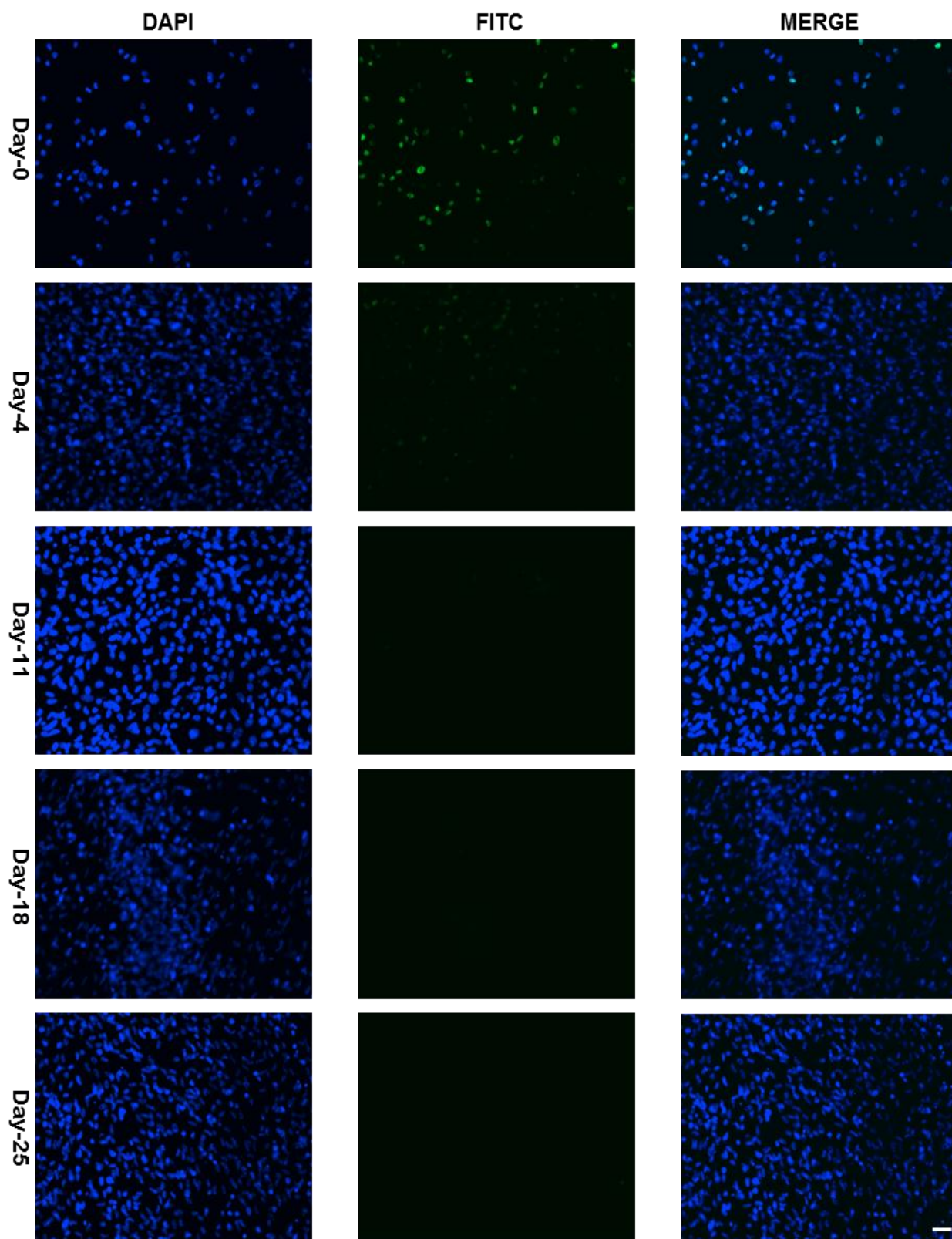


Figure 5.10: MSCs grown in medium lacking osteogenic supplements stained using antibody against Sox2 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox2) and found to be expressive at day-0 with some expression at day-4. No expression was detected after that. (All pictures were taken at 20X magnification and scale bar is 32µm).

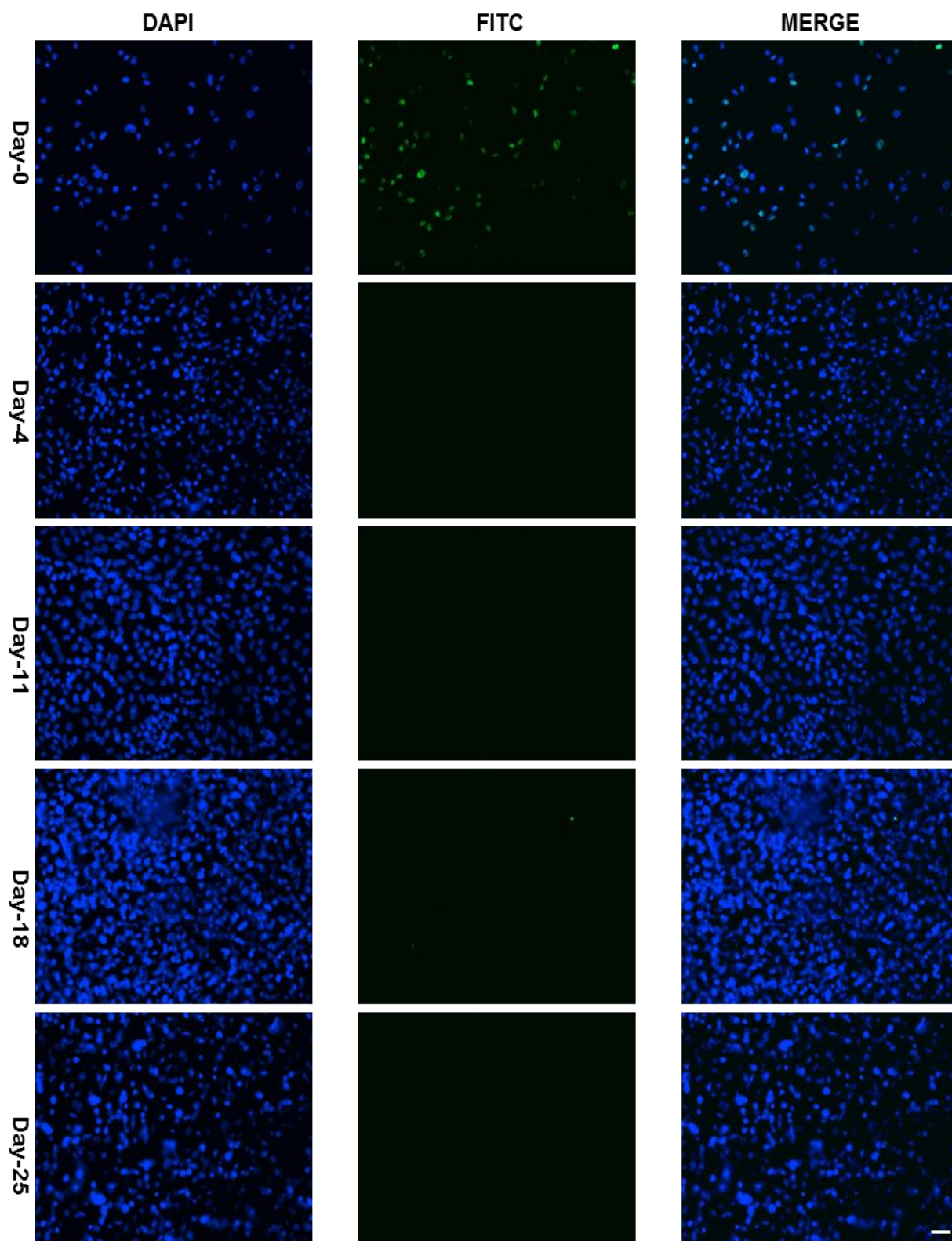


Figure 5.11: MSCs grown in medium containing osteogenic supplements stained using antibody against Sox2 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox2). Sox2 was found to be expressed by cells at day-0. No expression was detected beyond day-0 (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

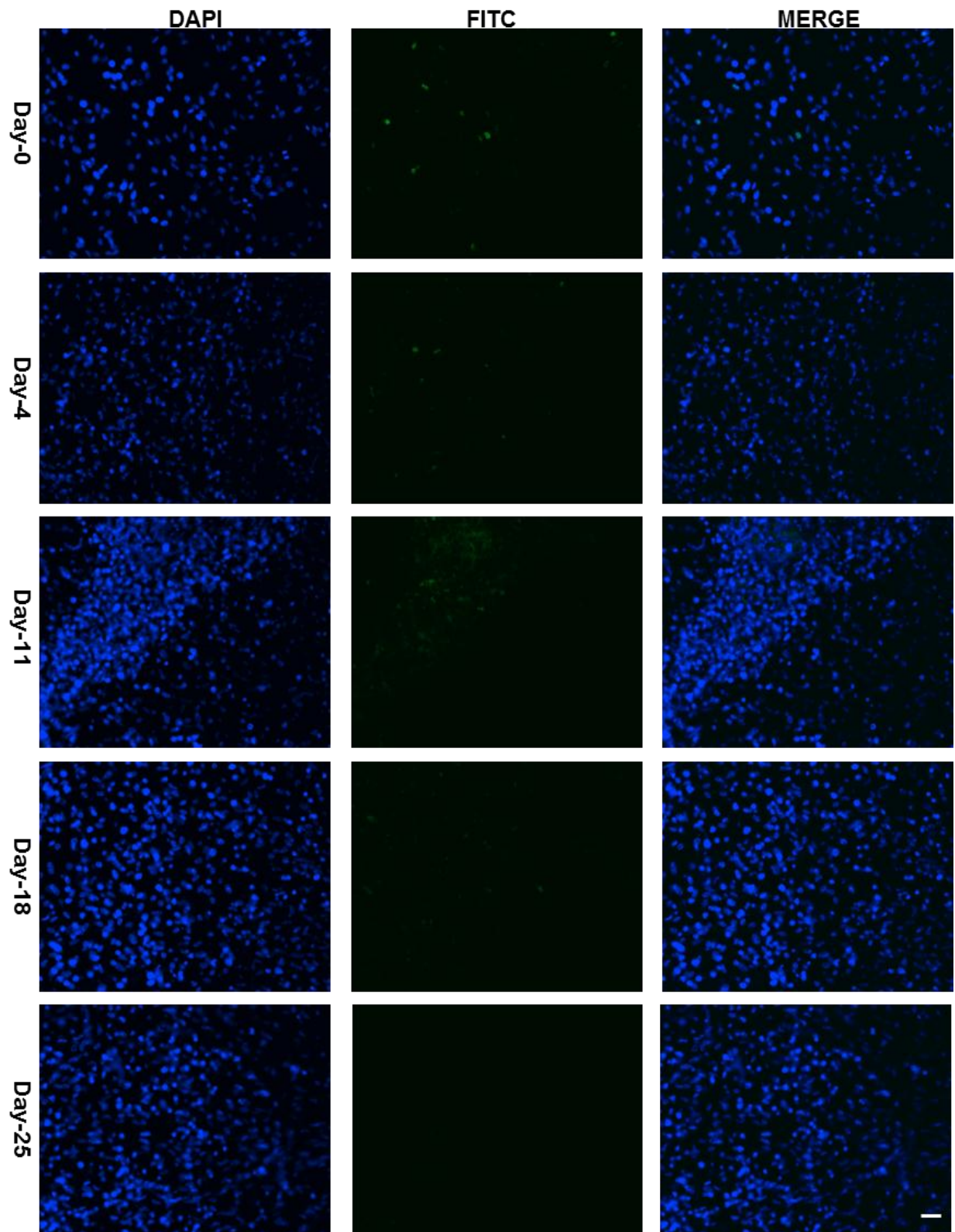


Figure 5.12: MSCs grown in medium lacking osteogenic supplements stained using antibody against Sox9 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox9). Sox9 was found to be expressed by cells at day-0. Few cells were found to be positive for Sox9 at day-4, more at day-11 than day-18 and no expression at day-25 (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

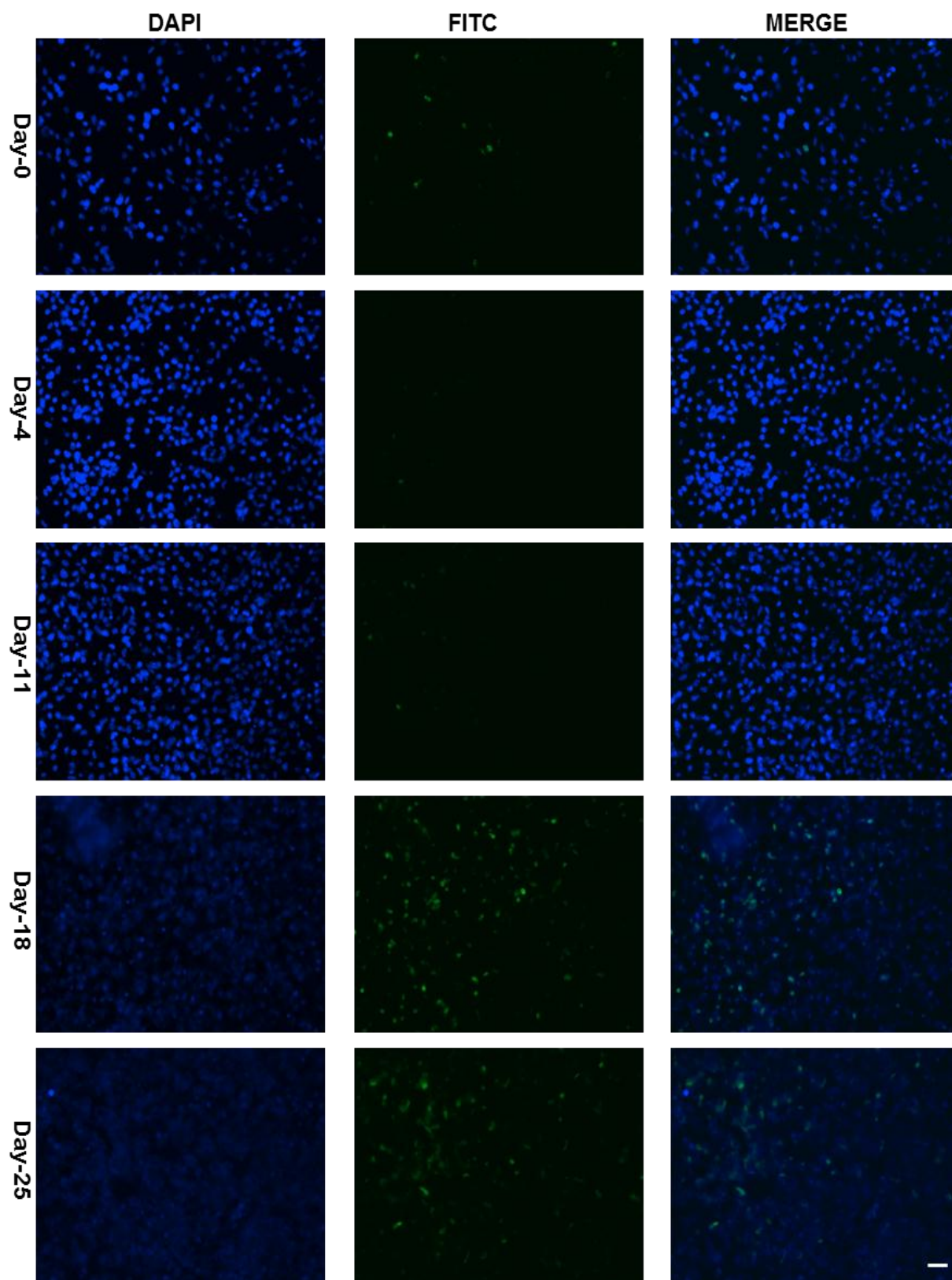


Figure 5.13: MSCs grown in medium containing osteogenic supplements stained using antibody against Sox9 protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Sox9). Sox9 was found to be expressed by cells at day-0. Few cells were found to be positive for Sox9 at day-4 and then gradual increase at day-11, day-18 and day-25 (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

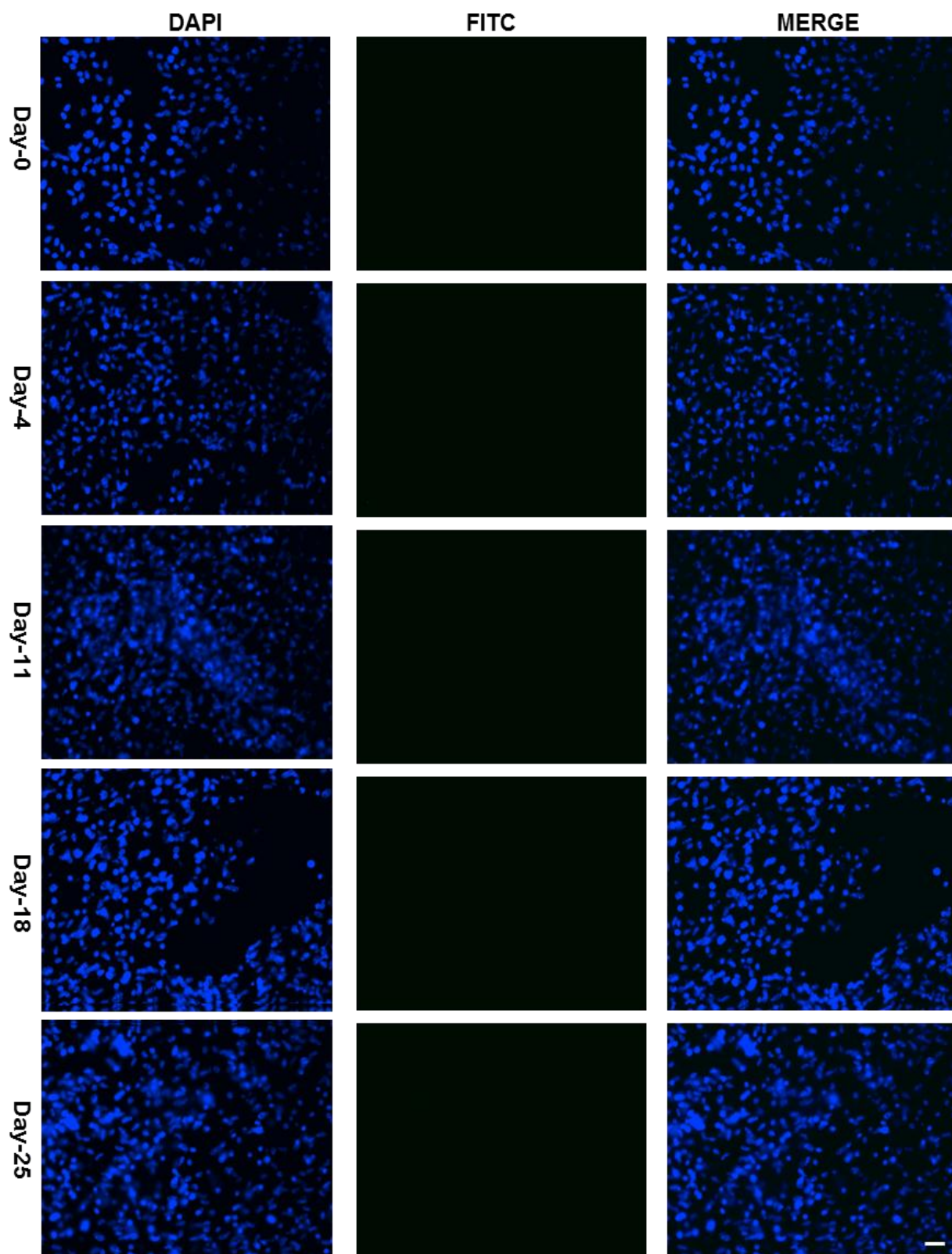


Figure 5.14: MSCs grown in medium lacking osteogenic supplements stained using antibody against Osterix protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osterix). No signal was detected at any time point analysed (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

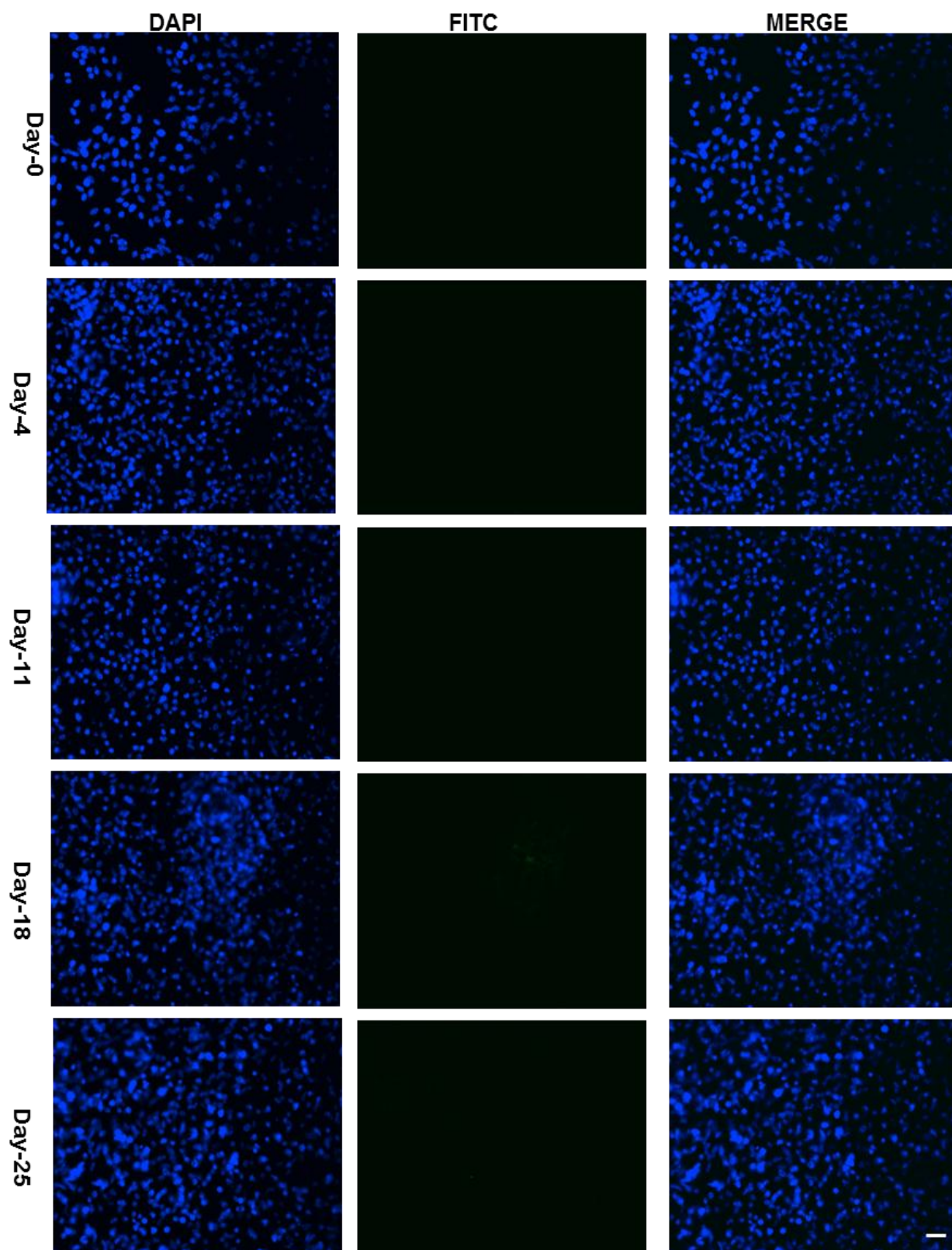


Figure 5.15: MSCs grown in medium containing osteogenic supplements stained using antibody against Osterix protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osterix). No positive signal was observed at day-0, 4, 11, 18 and 25 (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

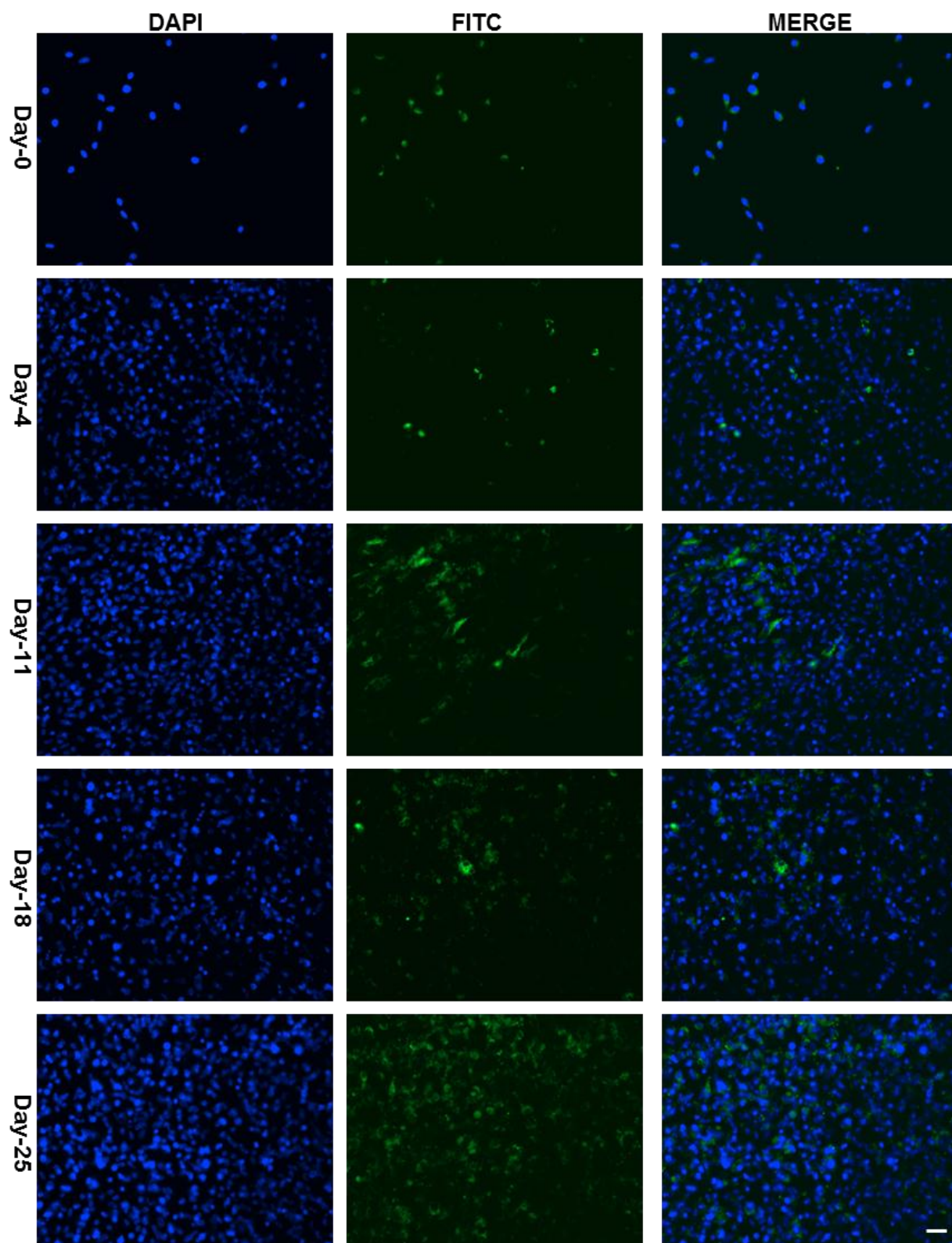


Figure 5.16: MSCs grown in medium lacking osteogenic supplements stained using antibody against Osteocalcin protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osteocalcin). Cell found to be positive starting at day-0 with gradual increase in intensity of signal at day-4, 11, 18 and 25 (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

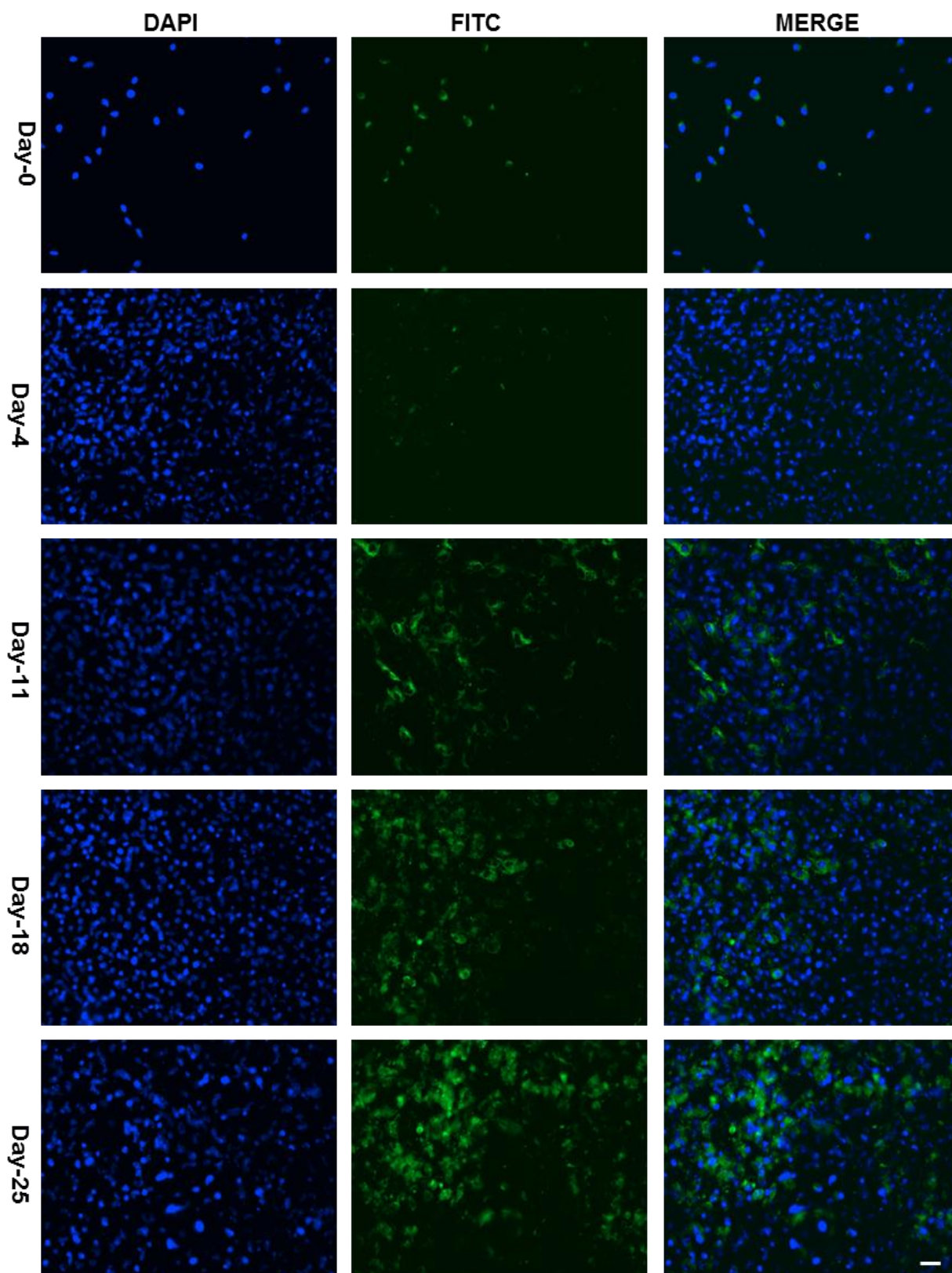


Figure 5.17: MSCs grown in medium containing osteogenic supplements stained using antibody against Osteocalcin protein (Dapi was used to stain the nucleus and FITC conjugated secondary antibody to localize Osteocalcin). A positive signal was first seen at day-0 with increased with time along the differentiation time course (All pictures were taken at 20X magnification and scale bar is 32 $\mu$ m).

### 5.3.2. Molecular Analysis

#### 5.3.2.1. Gene Expression Analysis by reverse transcription PCR (RT-PCR)

Gene expression studies with GAPDH as internal control and Sox9, Alkaline phosphatase, Runx2, Osteirx, Osteopontin, and Osteocalcin as osteo-specific markers were carried out on random primed cDNA synthesized from RNA, extracted from cells at different time-points during differentiation (section 2.2.3). Figure 5.18 displays representative results obtained from these analyses.

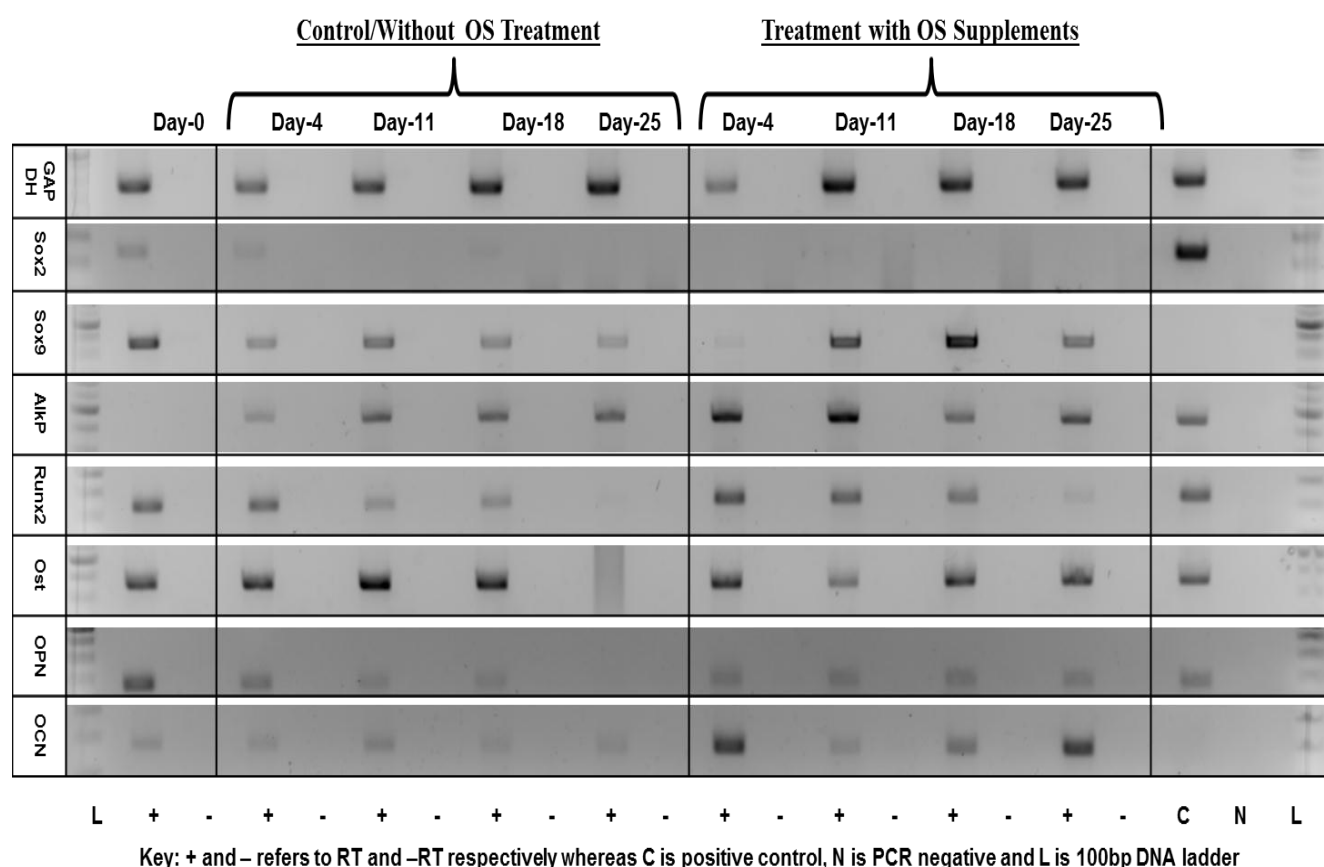


Figure 5.18: Gene expression analysis using random primed cDNAs of MSC osteogenic time course. GAPDH was used as internal control. These are the results from experiments done in triplicates from three independent biological replicates (n=3).

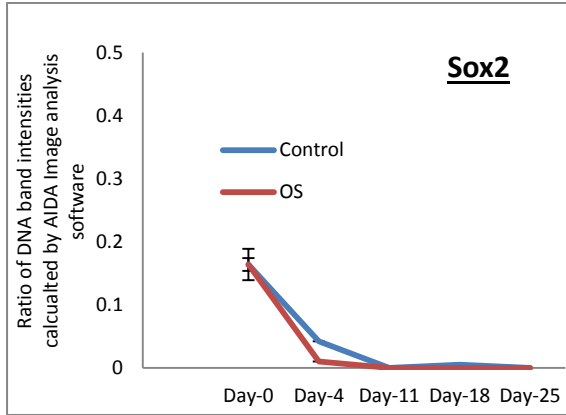


Figure 5.19: Semi-quantitative gene expression analysis showing expression of Sox2 in mouse mesenchymal stem cells grown in Control and OS medium for 25 days. Results are presented as mean $\pm$ SEM (n=3).

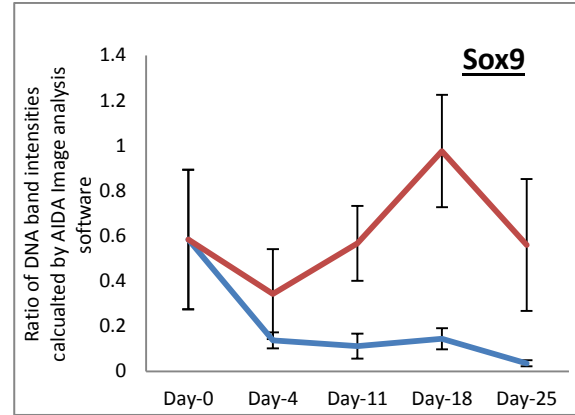


Figure 5.20: Semi-quantitative gene expression analysis showing expression of Sox9 in mouse mesenchymal stem cells grown in Control and OS medium for 25 days. Results are presented as mean $\pm$ SEM (n=3).

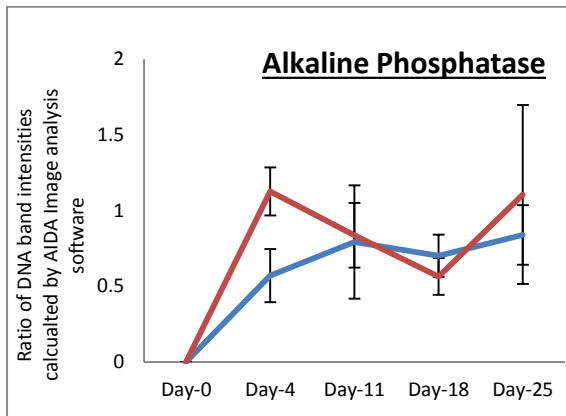


Figure 5.21: Semi-quantitative gene expression analysis showing expression of Alkaline Phosphatase in mouse mesenchymal stem cells grown in Control and OS medium for 25 days. Results are presented as mean $\pm$ SEM (n=3).

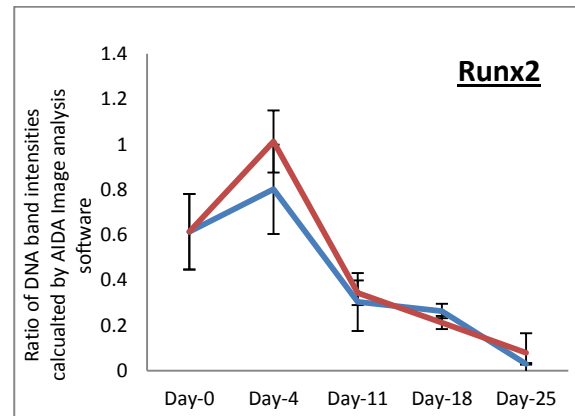


Figure 5.22: Semi-quantitative gene expression analysis showing expression of Runx2 in mouse mesenchymal stem cells grown in Control and OS medium for 25 days. Results are presented as mean $\pm$ SEM (n=3).

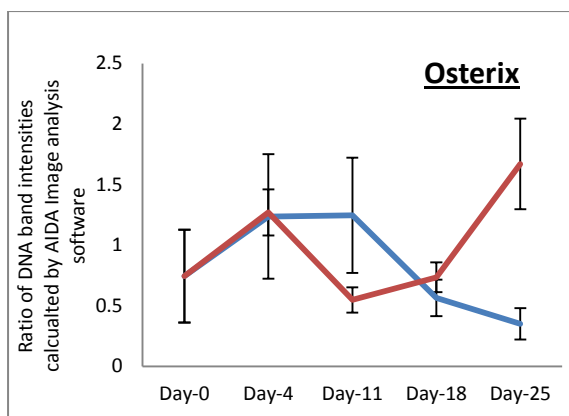


Figure 5.23: Semi-quantitative gene expression analysis showing expression of Osterix in mouse mesenchymal stem cells grown in Control and OS medium for 25 days. Results are presented as mean $\pm$ SEM (n=3).

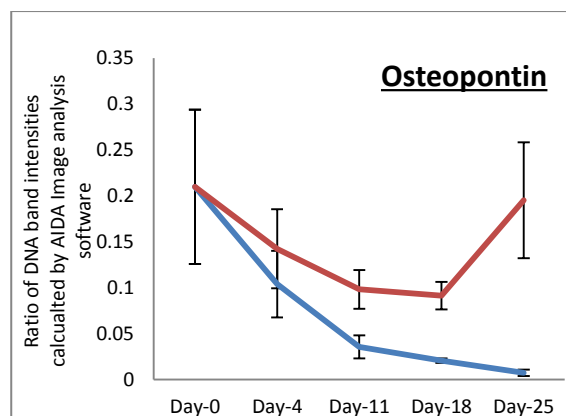


Figure 5.24: Semi-quantitative gene expression analysis showing expression of Osteopontin in mouse mesenchymal stem cells grown in Control and OS medium for 25 days. Results are presented as mean $\pm$ SEM (n=3).

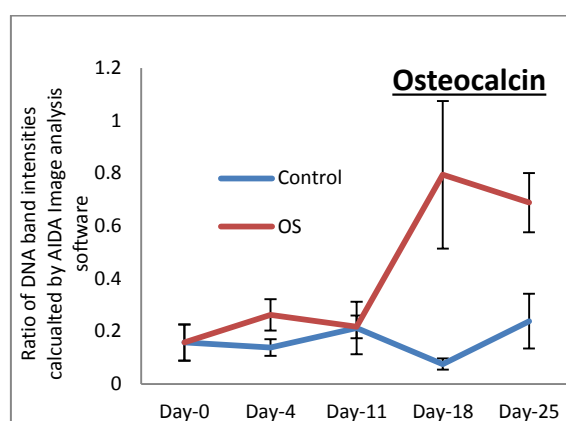


Figure 5.25: Semi-quantitative gene expression analysis showing expression of Osteocalcin in mouse mesenchymal stem cells grown in Control and OS medium for 25 days. Results are presented as mean $\pm$ SEM (n=3).

Sox2 was found to be expressed at day-0 and then a very low level of expression was observed at day-4 in control cells which correlates well with the protein expression as observed by immunofluorescence (Figure 5.10). While cells grown in medium containing OS supplements did not show Sox2 expression at RNA level (Figure 5.18 and 5.19) and protein level (Figure 5.11) after day-0.

Sox9 seemed to be showing differential gene expression where expression appears to be stronger at day-18 and day-25 in OS cells in comparison to cells grown in control medium (Figure 5.18 and 5.20). The same was observed with Sox9 expression at protein level by immunofluorescence (Figure 5.12 and 5.13).

Alkaline phosphatase appeared to be upregulated at day-4 in OS cells and then going down at day-18. Cells grown in control medium showed similar pattern of expression but less than OS treated cells (Figure 5.18 and 5.21). Similarly Runx2 found to be down regulating after day-4 along differentiation time course in both treated and non-treated cell populations (Figure 5.18 and 5.22).

Osterix, Osteopontin and Osteocalcin were found to be expressed at all time-points examined where Osteoclain seems to be expressed in higher levels at later points of differentiation i.e. day-25 (Figure 5.18, 5.23-5.25). Osteocacilin protein was also found to be expressed at all time-points with stronger signal at day-18 and day-25 (Figure 5.16 and 5.17).

#### **5.3.2.2. Analysis of DNA methylation at SRR1 and SRR2 by MS-PCR**

In order to look at DNA methylation changes occurring at SRR1 and SRR2, MS-PCR was carried out in the similar way as already detailed in chapter 2 section 2.3.2.4 and chapter 3 section 3.2.3.2. Both SRR1 and SRR2 regions of Sox2 were observed to be methylated at all time-points i.e. day-0, 4, 11, 18 and 25 in both undifferentiated and differentiated cells grown in osteogenic differentiation medium (Figure 5.28-5.29).

Figure 5.26 shows the result of PCR carried out on DNA prepared from MSCs grown in control and OS medium at different time points and then digested with *MspI* and *HpaII* enzymes. A region of DNA containing *Msp* sites but reported to be unmethylated was used as control (section 3.2.3)

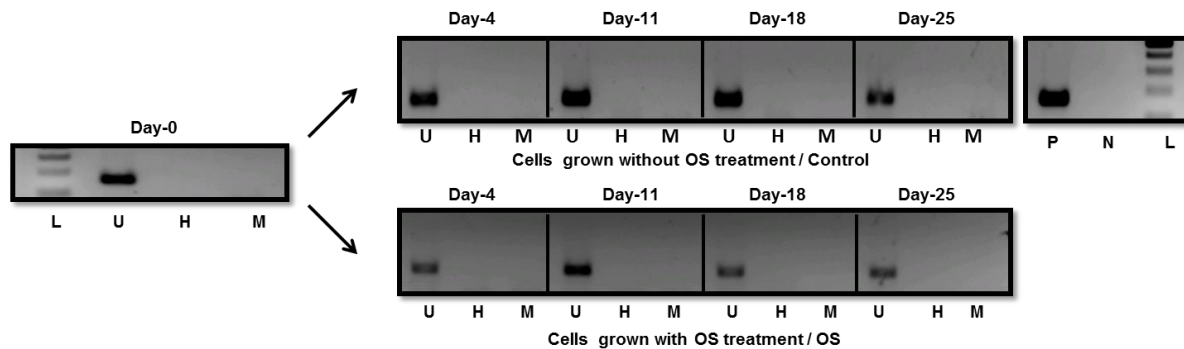


Figure 5.26: mAprt promoter region used as control and showing that no amplified product was observed in *MspI* and *HpaII* digested samples so digestion is not partial.

Figure 5.27 is the result of PCR carried out on same set of samples as described above. This is the genomic region known to be methylated and contains *Msp* sites.

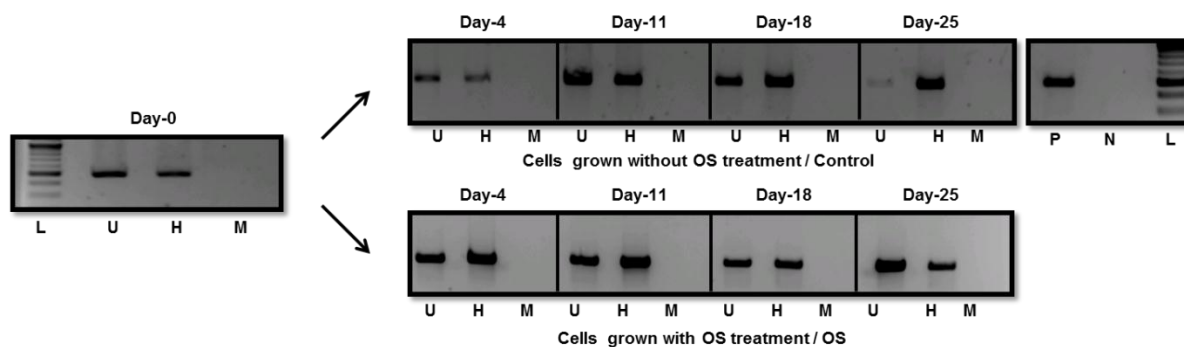


Figure 5.27: mIgf2R-DMR2 region used as control and showing that no amplified product was observed in *MspI* digested samples and only *HpaII* digested fragments amplified.

SRR1 and SRR2 regions of Sox2 were found to be methylated at time points selected for analysis in both control and OS treated cell populations (Figure 5.28 and 5.29). This technique only allows to study DNA methylation at any genomic region that contains *Msp* sites and when all of them are methylated. And if any of these in unmethylated or partially methylated then due to digestion of DNA amplified product won't be seen.

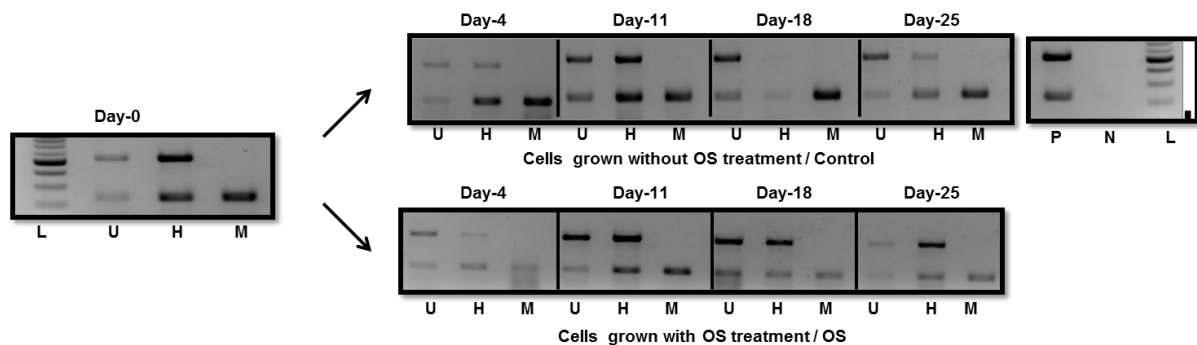


Figure 5.28: SRR1 region of Sox2 was found to be methylated at every time point examined by MS-PCR

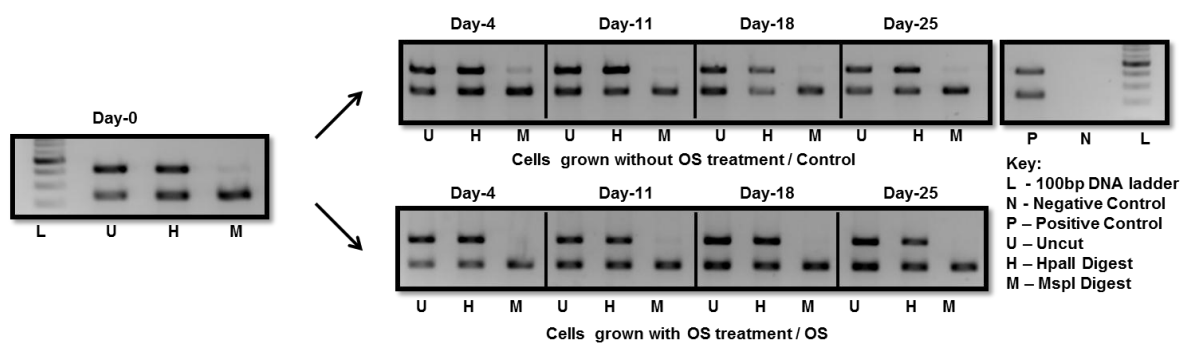


Figure 5.29: SRR2 region of Sox2 was found to be methylated at day-0, 4, 11, 18 and 25 in both control and OS cells by MS-PCR

## 5.4. Discussion

Bone development *in vitro* has been traditionally analysed by the mineralized bone nodules formation (Heng *et al.*, 2008). As is apparent in Figure 5.2, mineralization as black layer around the cells under the microscope can be clearly seen at day-11 of differentiation showing a gradual increase by day-25. Further staining with alizarin red dye showed the darkening of deposited granular matrix while control cells did not show such a strong staining. This dye was subsequently extracted and quantified using an already described assay to assess the extent of mineralization (Gregory *et al.*, 2004). Figure 5.5 shows very clearly that treated population of MSCs have produced more stain than non-treated cells and the difference is really apparent after two weeks of culture. As detailed in the discussion section of the chapter 3 (section 3.4), medium used for osteogenic fate induction could lead to non-specific mineralization. Therefore, further characterization of differentiating MSCs cells was carried out using cell surface and other molecular markers by immunocytochemistry and gene expression profiling.

As described previously osteogenesis is a complex process and the expression pattern of various markers associated with osteoblasts is organized temporally and sequentially (Zur Nieden *et al.*, 2003). Type I collagen, Alk-P and osteonectin have been reported to appear at the end of the proliferation phase; osteopontin followed by bone sialoprotein and Runx2 at the start of mineralization phase; and finally osteocalcin at high level during mineralization. All these markers can have varying times of expression onset in culture dependent on cell line and methods of differentiation (Duplomb *et al.*, 2007).

Sox2 has been reported to maintain self-renewing osteoprogenitor cell population and favours their maintenance over differentiation into mature osteoblasts (Roy *et al.*, 2010). It has also been reported that cells impaired in osteogenic differentiation show high level of Sox2 expression (Muraglia *et al.*, 2008). Moreover, Sox2 has been reported to inhibit *in vitro* murine osteoblastic differentiation by inhibiting Wnt signalling (Mansukhain *et al.*, 2005). Here Sox2 was observed both at RNA and protein level at day-0 in undifferentiated cells and then very small expression was detected at day-4 in control cells. Cells grown in OS medium did not show Sox2 expression neither at RNA level nor at protein level hinting at the possibility that cells could have become committed and are no longer in proliferative phase.

Sox9 is known to be expressed in mesenchymal condensation during the deposition of cartilage and plays a central role in skeletogenesis (Wright *et al.*, 1995). Based on the location of MSCs, they differentiate to form chondrocytes or osteoblasts and Sox9 is one of the important determining factor (Fujii *et al.*, 2005). Here in these experiments Sox9 was found to be down regulated both at RNA and protein level after two weeks of MSC culture in differentiation medium compared to control cells. Cells maintained a lower level of RNA and protein expression at all time-points analysed except day-25 in control where no protein was detected.

Runx2 and Osterix are two central transcription factors in determining the commitment of osteoprogenitors cells to osteoblasts where Osterix is thought to act downstream of Runx2 (Komori *et al.*, 2006). Runx2 was found to be upregulated at day-4 in OS cells and then down regulated by day-25. A significant expression of Osterix was detected starting from day-0 and at all time-points during differentiation while no protein expression was detected at any time point. A previous study has shown that Osterix expression stimulates mesenchymal stem cell

proliferation in a partially committed stage of osteoblast precursor (Kim *et al.*, 2005). Similarly alkaline phosphatase, osteopontin and osteocalcin which are reported to be expressed in higher amounts in osteoblasts as detailed in chapter 3 (section 3.4), were observed to be present at all time-points analysed in both control and OS treated population.

Together these observations suggest that this MSC population seems to be already enriched in expression of markers associated with late stage of osteoblasts and at the same time expressing marker associated with early stages of osteoblast commitment at later stages i.e. in third and fourth week of culture. This could be due to the heterogeneity in starting population that some of the cells have already become committed to osteoprogenitors and hence are preosteogenic. There is also the possibility of asynchronous differentiation i.e. some cells have yet to start mineralizing so expressing early stage markers while others have mineralized.

DNA methylation has been reported as a major mechanism of gene silencing (discussed in chapter 3 section 3.4). Two regulatory regions of Sox2 namely SRR1 and SRR2 were chosen for analysis (section 1.5 and 1.6) and found to be constitutively methylated at all time-points when analysed with MS-PCR. Given the limitation of the technique to resolve DNA methylation status at each CpG site, bisulphite sequencing was attempted. But unfortunately none of the region could be PCR amplified after bisulphite treatment which could then be analysed by DNA sequencing. Hence further work is needed to optimize the reaction conditions for PCR amplification.

Adult stem cells have been now given focus regarding neoplastic transformation so that they could be exploited for their differentiation potential while keeping the self-renewing capacity

(Serakinci *et al.*, 2004). Further work investigating the mechanisms regulating differentiation in this transformed cell line would be of interest to gain a molecular insight into induction of proosteogenic potential as is apparent by these observations.

## **Chapter 6 – Discussion**

## 6. Discussion

Development is a process resulting in sequential reduction of differentiation potential of pluripotent progenitors involving activation or repression of various gene networks as cells acquire more specialized characteristics. This differentiation into specialized tissues and organs proceeds without any changes in DNA sequences of genomes of the cells so can be regarded as under epigenetic control (Reik, 2007). Indeed it has been experimentally shown that genomes from differentiated cells of animals can be used to clone animals proving that cells do not lose their genetic material during differentiation; rather regulated and controlled gene expression determine and maintains cell fate (Gurdon *et al.*, 1975; Wilmut *et al.*, 1997).

Regulation at the transcriptional level is central to the process because both spatial and temporal expression of transcribed proteins determines the identity of a cell, tissue or organ (Prior and Walter, 1996). An important component of transcriptional regulation is transcription factors which bind to DNA and co-ordinate the cellular decision making process, making it irreversible and free of errors which could potentially lead to developmental abnormalities and disorders. Lately, such transcription regulators have been identified that maintain the differentiation potential of embryonic stem cells, and forced expression of these factors can even reprogram somatic genomes to an undifferentiated state (Takahashi *et al.*, 2006; 2007; Park *et al.*, 2008).

Epigenetic modifications such as DNA methylation of CpG dinucleotides, modification of histone tails mainly by acetylation and methylation, other non-histone proteins and regulatory RNAs affect and change gene expression patterns during cellular differentiation along with transcription factors (Jaenisch and Young, 2008). All of these factors acting together lead to

the generation of cell and tissue specific gene expression patterns reflected in their genotype under given circumstances and govern their phenotype (Morgan *et al.*, 2005). So interplay between these factors at different stages of development regulates pluripotency of progenitors and their differentiation at the right time for example by maintaining reversible repressive expression of genes required at later stage of development at earlier stages and/or irreversible silencing of pluripotency associated genes after cellular commitment and differentiation (Reik, 2007).

Embryonic stem cells have been shown to be able to differentiate into all lineages derived from embryos *in vitro* and hence can be used as a model system not only to study developmental programming and reprogramming events but also, once these events are known can be used to create differentiated cell types for transplantation therapies in clinical applications (Solter and Gearhart, 1999). Sox2 has been reported to be indispensable for mouse embryonic stem cells self-renewal and also, is one of the transcription factors used to reprogram somatic cells.

This study specifically looked at DNA methylation status of two evolutionary conserved regulatory regions of Sox2 namely SRR1 and SRR2 in undifferentiated mouse embryonic stem cells and after subjecting them to differentiation treatment leading to osteogenic and neural lineages that are derived from germinal ectoderm and mesoderm respectively. Mouse mesenchymal stem cells, the physiological precursor of osteoblasts were subjected to a similar differentiation treatment in parallel, so as to compare and contrast the changes in DNA methylation at these regions when same cell types are obtained from embryonic and adult origin stem cells. The objective was to examine the role of DNA methylation of regulatory regions of Sox2 in cell type specific expression pattern.

DNA sequences of short length often known as regulatory elements are basic components in gene regulation and can be found in promoter, enhancer, near the transcription start site (TSS) or far away from TSS and can be either *cis* or *trans* acting (Sikorski and Buratowski, 2009). The combinatorial action of a given set of regulatory elements for a particular gene would determine its expression in tissue specific and environmental specific manner (Latchman, 2004; Levine and Tjian, 2003). The Sox2 gene has been reported to contain two evolutionary conserved upstream and downstream (~4kb from transcription start sites) enhancers designated as Sox2 Regulatory Region 1 and 2 (SRR1 and SRR2) in mouse and humans (Tomioka *et al.*, 2002; Sikorska *et al.*, 2008).

SRR1 contains binding site for POU transcription factors while SRR2 binds Oct4-Sox2 and are essential for Sox2 expression both in ES cells and neural stem cells (Catena *et al.*, 2004; Tomioka *et al.*, 2002). It has been previously shown that these regulatory sequences control region specific expression of Sox2 in neural stem cells such that expression is seen in telencephalon but not spinal cord stem cells (Miyagi *et al.*, 2006; Zappone *et al.*, 2000). Another study has found that these enhancers exhibit differential DNA methylation and histone H3 acetylation during human neural progenitor's differentiation into astrocytes and neurons affecting expression level of Sox2 in these different cell types (Sikorska *et al.*, 2008). Their study has found that methylation of SRR2 enhancer particularly at highly conserved site +4250, correlated with silencing of Sox2 expression in neurons while SRR1 methylation in both astrocyte and neurons linked to gene silencing and cell cycle exit. They concluded that differential DNA methylation of these two enhancers of Sox2 in neural precursors and terminally differentiated cells mediate neurodevelopment and regulate gene expression transiently and permanently.

Given that these sequences are highly conserved in mouse, this study looked at differentiating neuronal cells from embryonic stem cells and three individual CpGs analysed in core enhancer region of SRR2 were found to be unmethylated in both undifferentiated ES cells and differentiated cells. At the level of this analysis, it could not be concluded that all of the CpG sites remained unmethylated during differentiation. It is also possible that differentiated cells contained a smaller proportion of terminally differentiated neurons and presence of other cell types as cells were not sorted prior to analysis might, have potentially masked the low level of methylation (chapter 4).

Both SRR1 and SRR2 appeared to be methylated in ES cells grown in medium promoting differentiation towards osteoblasts when analysed by MS-PCR except at day-0 (undifferentiated state). As described above, these two enhancers contribute towards ES specific expression of Sox2 so could be unmethylated in undifferentiated cells. This could also be due to the fact that major epigenetic reprogramming events take place upon cell differentiation and commitment (Reik *et al.*, 2001). So these regions might be unmethylated at all of CpG sites in undifferentiated ES cells and upon differentiation at the day-3 embryo bodies stage start setting up some methylation marks.

MS-PCR analysis described in Chapter 2 section 2.3.3 showed that both SRR1 and SRR2 became methylated at day-3 and then appeared to be methylated at all time-points analysed thereafter. No Sox2 expression was detected at the RNA level after seven days of treatment and after 14 days at both protein and RNA levels. The three CpGs in the SRR2 region that were analysed in detail were found unmethylated at every time point tested during differentiation suggesting that no changes in methylation status are taking place at these sites. However, it cannot be excluded that other CpG sites in SRR1 and SRR2 not tested in this

study might have become methylated upon differentiation. Hence further studies extending the analysis of DNA methylation to all CpG sites within this region are required to determine whether DNA methylation within these regions plays any role in silencing of Sox2 under the differentiation conditions used.

MSCs are physiological precursors of bone cells and they were subjected to differentiate towards osteoblast lineage under similar conditions to that of ES cells with the objective to profile and then compare DNA methylation changes in cells derived from both adult and embryonic origin (chapter 5). Both SRR1 and SRR2 were found to be methylated at all time points in undifferentiated and differentiated MSCs when analysed by MS-PCR. Unfortunately it was not possible within the time frame of this study to analyse individual CpGs using bisulphite sequencing due to technical limitations (Chapter 5 section 5.3). As detailed earlier (chapter 3.4 and 4.4) that these regions have been studied and implicated in Sox2 expression in ESCs and neural stem cells so far, so could be possible that they are methylated in MSCs. Sox2 expression was detected only in undifferentiated MSCs and not in differentiated cells both at RNA and protein level so there is a possibility of DNA methylation leading to gene silencing. This seems quite interesting on one hand given that no data regarding DNA methylation is available about these regions in MSCs so far. But since this MSC cell line is immortalized hence, it cannot be excluded that methylation of these regions might be a consequence of cell line establishment and maintenance rather than actual physiological phenomena and hence need to be supplemented with data from primary cell types. Indeed some of the earlier studies have looked at and reported differences in gene expression patterns and DNA methylation in immortalized cell lines (Caliskan *et al.*, 2011; Wild *et al.*, 2010)

In summary, this is the first study looking at DNA methylation status of two regulatory regions of Sox2 i.e. SRR1 and SRR2 in undifferentiated mouse embryonic stem cells and upon their differentiation when grown in osteoblast and neural medium in a time course dependent manner. Similarly no study has so far described the DNA methylation profile of these regulatory regions of Sox2 in undifferentiated and differentiated mouse mesenchymal stem cells. Data obtained in this study hints towards the possible involvement of differential DNA methylation in regulating Sox2 expression in different cell types. There seems to be promising differences in methylation status of these two regulatory regions of Sox2 not only between cell types i.e. embryonic v/s adult but also lineages i.e. osteoblasts v/s neural though still preliminary and incomplete. Given the importance of epigenetic regulation in cell commitment and differentiation, analysis of these regulatory regions will provide a valuable insight into molecular mechanisms of gene regulation and how they contribute towards cell fate decisions and switch between different cell types. As these regions are highly conserved in mouse and humans, it could be proposed that regulatory mechanisms might also be shared hence making mouse embryonic stem cells a good model system to study regulation of Sox2 during *in vitro* differentiation.

## 7. Appendices

### **7.1. Chemicals, Medium and Reagents**

<b>Chemical Name</b>	<b>Catalogue No.</b>	<b>Supplier</b>
Gelatin	G1393	Sigma
Bacterial petri dishes	633102	Greiner
Tri Reagent	T9424	Sigma
DNase	04536282001	Roche
RNasin	Promega N2511	Promega
Reverse Transcriptase	11785826001	Roche
Thermopol Buffer	B9004S	BioLabs
dNTPs	U1240	Promega
Taq polymerase	M0267L	BioLabs (NEB)
Orange G	03756	Fischer Scientific
Glycerol	G8773	Sigma
Phase lock tubes	129025	Qiagen
<i>MspI</i>	R0106S	BioLabs
<i>HpaII</i>	R0171S	BioLabs
EZ DNA methylation kit	D5006	Zymo Research
DMEM + glutamax	61965-026	Invitrogen Gibco
Fetal Calf Serum	EU-000-FE	SLI
MEM Non essential amino acids	11140-035	Invitrogen Gibco
Sodium pyruvate	11360-039	Invitrogen Gibco

Penicillin/Streptomycin	15070-063	Invitrogen Gibco
2-Betamercaptoethanol	M6250	Sigma
LIF	ESG1107	Millipore Chemicon
DMSO	C6295	Sigma
DMEM	21885-025	Invitrogen Gibco
Fetal Bovine Serum	16170-07	Invitrogen Gibco
Beta glycerol phosphate	410990250	Acros Organics
Ascorbic Acid Phosphate	A8690-54	Sigma
Dexamethasone	D4902	Sigma
Retinoic Acid	R2625	Sigma
DMEM:F12	21331-020	Invitrogen Gibco
N2 Supplement	17502-048	Invitrogen Gibco
Glutamax	35050-038	Invitrogen Gibco
Neurobasal Medium	21103-049	Invitrogen Gibco
B27 Supplement	17504-044	Invitrogen Gibco
Alizarin Red S	A5533	Sigma
Phenol	P4557	Sigma
Chloroform	C2432	Sigma
Sodium Acetate	9740	Sigma
Proteinase K	P2308	Sigma
Sox2 antibody	ab97959	abcam
Sox9 antibody	AB5535	Millipore

Osterix antibody	ab22522	abcam
Osteocalcin antibody	sc-30045	Sant Cruz Biotechnology
FITC conjugated anti rabbit anitbody	DI-1488-1.5	Vector Laboratoreis
Vectashield	H-1200	Vector Laboratoreis

## **7.2. Medium and Buffer Compositions**

### **Complete ES medium**

DMEM + glutamax (4.5g/l D-glucose + GlutaMax)

18% Fetal Calf Serum (v/v) (ES Grade)

1X MEM Non essential amino acids (100X)

1X Sodium pyruvate (100X)

1X Penicillin/Streptomycin (100X)

0.1mM 2-Betamercaptoethanol (100mM liquid)

1000U/ml LIF (10000000 U/ml)

### **Freezing Medium**

90% Fetal Calf Serum (v/v)

10% DMSO (v/v)

### **EB Medium**

DMEM (1g/l D-glucose + Pyruvate + GlutaMax)

15% Serum (not ES Grade)

1X Non essential amino acids (100X)

1X Penicillin/Streptomycin (100X)

0.1mM 2-Betamercaptoethanol (100mM liquid)

### **Osteogenic Differentiation Medium**

DMEM + glutamax (1g/l D-glucose + GlutaMax)

15% Fetal Calf Serum (v/v) (not ES Grade)

10mM Beta glycerol phosphate (1M stock in DMEM, filter sterilise at 4°C for 1 month)

50µM Ascorbic Acid Phosphate (50mM stock in DMEM, filter sterilise at 4°C for 1 month)

10 uM Dexamethasone (1mM stock in EtOH, keep at -20°C)

1X Penicillin/Streptomycin (100X)

### **Retinoic Acid**

5mM stock in DMSO (light sensitive)

### **N2 Medium**

DMEM:F12

1X N2 Supplement (100X)

0.5X Glutamax (100X)

1X Penicillin/Streptomycin (100X)

### **Neurobasal Medium**

Neurobasal Medium

1X B27 Supplement (50X)

1X Penicillin/Streptomycin (100X)

### **Cell Lysis Buffer**

[Note: All chemicals were of analytical grade from Sigma, unless specified separately]

10mM Tris-HCl pH 8.0

10mM NaCl

10mM EDTA pH 8.0

0.5% SDS (w/v)

50µg/ml Protienase K (10mg/ml)

**2X DMSO Buffer**

20% DMSO (v/v)

32mM Ammonium Sulphate

20mM Betamercaptoethanol

134mM Tris-HCl pH 8.8

### 7.3. Primers Sequences for RT-PCR and MS-PCR

Gene	Primer ID	Primer Sequence	Product (bps)	Annealing Temperature
<b>mAprt</b>	mAprt1 (RT)	GGTAGCTCACAAAGGTCCT		
	mAprt2 (F) mAprt3 (R)	GGAAATCCAGAAAGATGCCT TCTAGCCAGCTCCTCAGTCA	227	60°C
<b>Gapdh</b>	mGapdh(F)	CCCACTAACATCAAATGGGG	275	60°C (Ying <i>et al.</i> , 2008)
	mGapdh(R)	CCTTCCACAATGCCAAAGTT		
<b>Sox2 (cDNA)</b>	J11Sx2 (cDF)	CTGCGGAAAAAAACCACCAATC	241	62°C
	J12Sx2 (cDR)	TTTGCGAACTCCCTGCGAAG		
<b>Sox9 (cDNA)</b>	J13 Sx9cD F	GAAGTCGGTGAAGAACGGACAAG	364 (973 on genomic DNA)	62°C
	J14 Sx9cD R	GGGTGGCAAGTATTGGTCAAACTC		
<b>Igf2 – DMR2 (MS-PCR)</b>	M115 (F)	CGTGGCACTTTTGAGTTCATCTCTC	489	65°C
	M114 (R)	ATTCTGGTTGTGCCGAGTTGCGAG		
<b>mAprt (MS-PCR)</b>	M116 (F)	TCTTCCCCGACTTCCCAATC	164	60°C
	M117 (R)	GAACAAGGACAGGAGAGTGACCC		
<b>CSa (MS-PCR)</b>	CSa_s (F)	TGGTTGGCATTATCCCTAGAAC	133	59°C (Weber <i>et al.</i> , 2007)
	CSa_as (R)	GCAACATGGCAACTGGAAACA		
<b>Runx2 (cDNA)</b>	mRunx2_F	CCTGAACTCTGCACCAAGTC	234	64°C (Kawaguchi <i>et al.</i> , 2005)
	mRunx2_R	GAGGTGGCAGTGTCATCATC		
<b>Osteopontin (cDNA)</b>	mOsteopontin_F	TGCACCCAGATCCTATAGCC	186	64°C
	mOsteopontin_R	CTCCATCGTCATCATCATCG		
<b>Osteocalcin (cDNA)</b>	mOsteocalcin_F	CCCTGCTTGTGACGAGCTAT	170	52°C

	mOsteocalcin_R	ACTTGCAGGGCAGAGAGAGA		
<b>Alk-P (cDNA)</b>	mAlp_F	GAAGACGTGGCGGTCTTTGC	457	64°C (Chen <i>et al.</i> , 2006)
	mAlp_R	GGAATCTGTGCAGTCTGTG		
<b>Osterix (cDNA)</b>	mOsterx_F	CCTCTGCGGGACTCAACAAC	355	64°C (Kawaguchi <i>et al.</i> , 2005)
	mOsterix_R	TGCCTGGACCTGGTGAGATG		
<b>Nestin (cDNA)</b>	mNestin_F	TCCCTTTCTCCCCCTTAAAA	198	52°C
	mNestin_R	ACTGAGCAGCTGGTTCTGC		
<b>Pax6 (cDNA)</b>	mPax6_F	GGCTTTGTGCAAGATTCTCC	170	60°C
	mPax6_R	CTCGGCCTAGTGGCTTCTTT		
<b>Beta III tubulin</b>	mβIII tubulin_F	AAGGTAGCCGTGTGTGACATC	209	60°C (Ahn <i>et al.</i> , 2004)
	mβIII tubulin_R	ACCAGGTCATTCATGTTGCTC		
<b>Sox2 – SRR1</b>	Sox2 – SRR1 – F	AAGTAACCCTGATGGTTTAAGC TC	536	62°C
	Sox2 – SRR1 – R	ACGCAGCGTTCTCAGGAT		
<b>Sox2 – SRR2</b>	Sox2 – SRR2 – F	AAGGCACCAAGAACCAGAAA	419	62°C
	Sox2 – SRR2 – R	GCCCGGGAAATTCTTTTAGA		

#### 7.4. Primer Optimizations

All primer pairs were first optimized using temperature gradient, varied PCR buffers and  $Mg^{2+}$  concentrations to find optimal annealing and amplification parameters for PCRs. Below are some gel pictures showing various optimization experiments results.

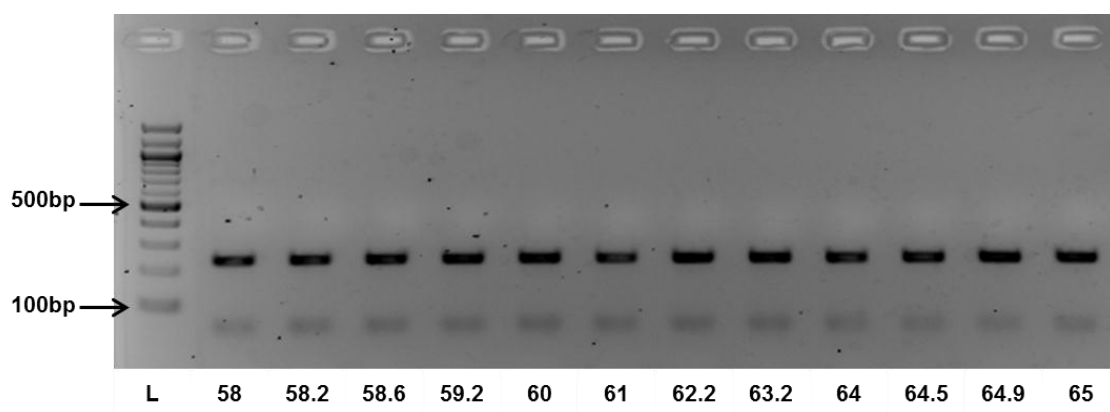


Figure 7.1: Temperature gradient (58-65°C) for Sox2 (241bps) primer pairs

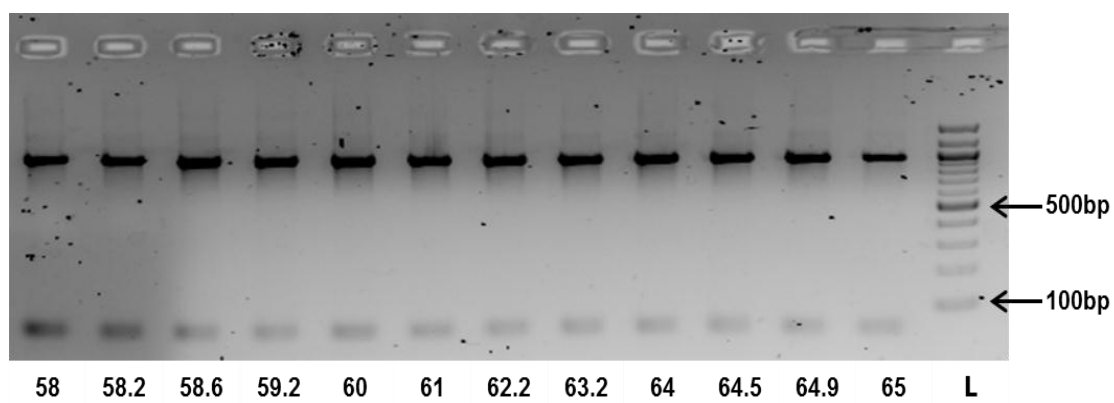


Figure 7.2: Temperature gradient (58-65°C) for Sox9 (963bps) primer pairs

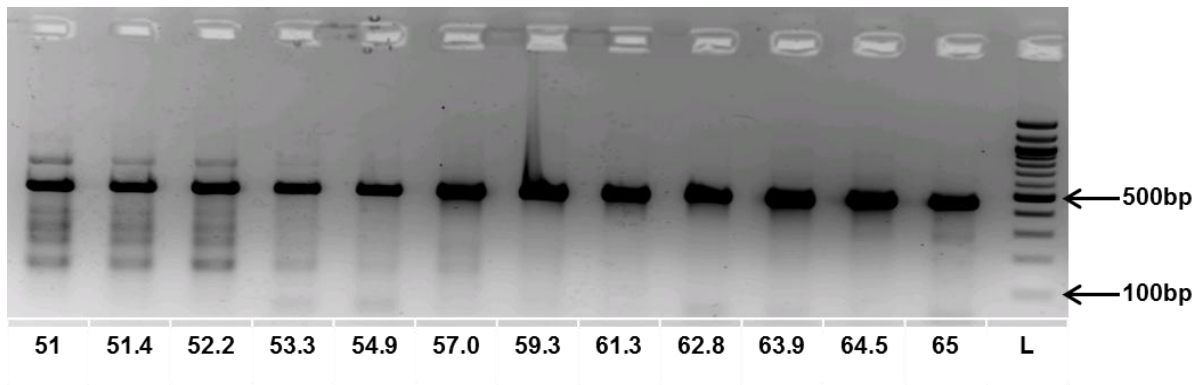


Figure 7.3: Temperature gradient (58-65°C) for Alkaline phosphatase (457bps) primer pairs

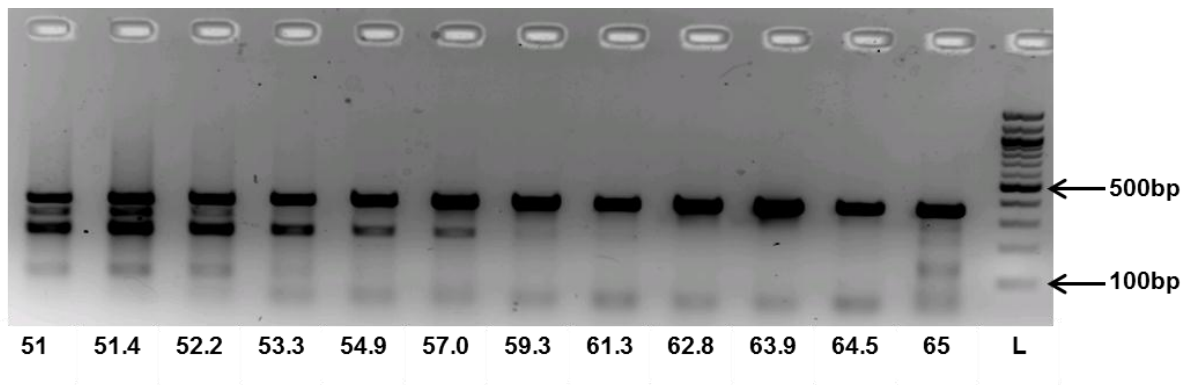


Figure 7.4: Temperature gradient (58-65°C) for Ostetix (355bps) primer pairs

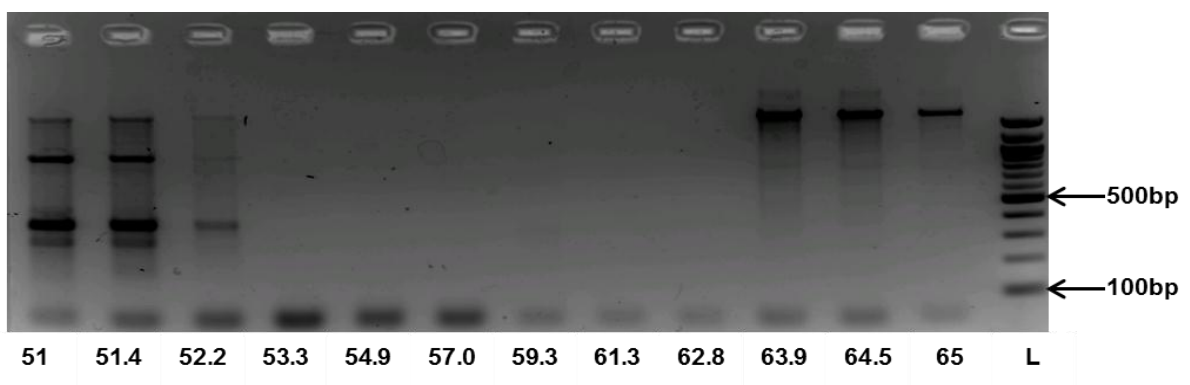


Figure 7.5: Temperature gradient (58-65°C) for Osteopontin (1.4Kb) primer pairs

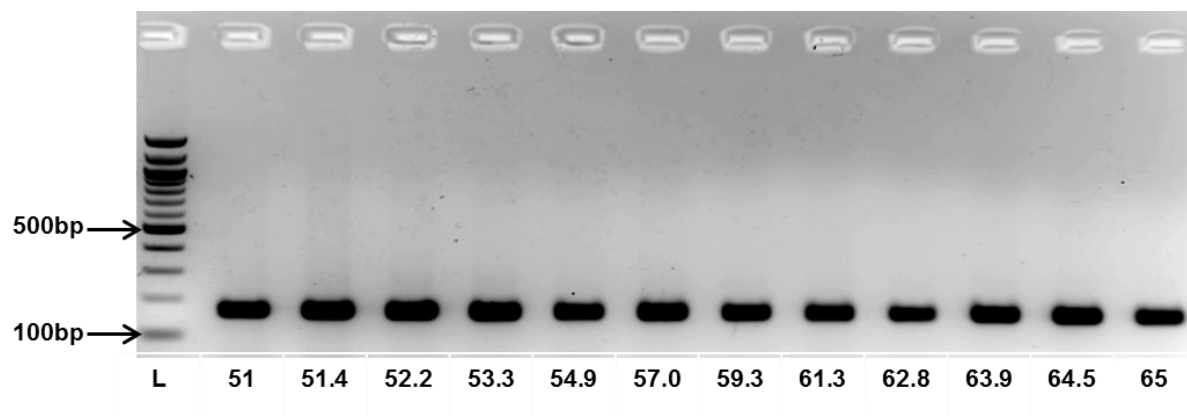


Figure 7.6: Temperature gradient (58-65°C) for Osteocalcin (170bps) primer pairs

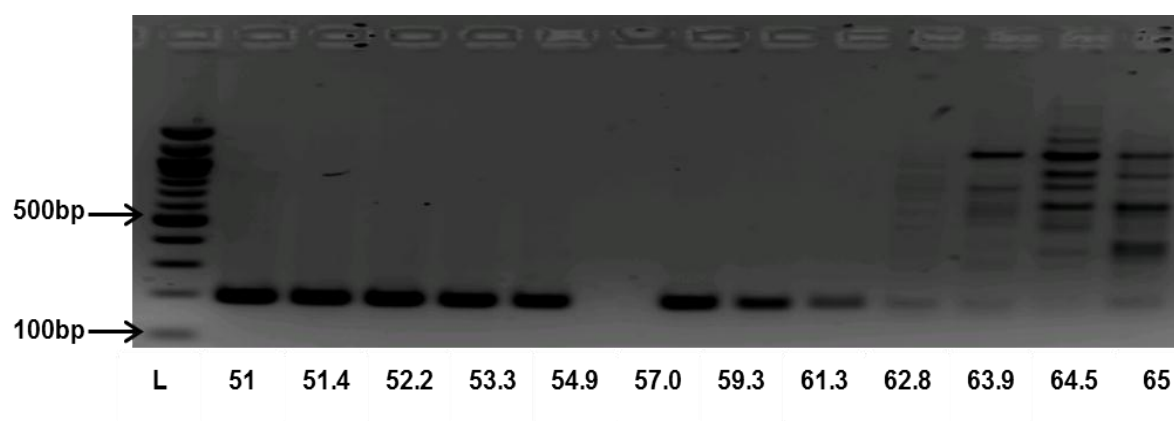


Figure 7.7: Temperature gradient (58-65°C) for Nestin (198bps) primer pairs

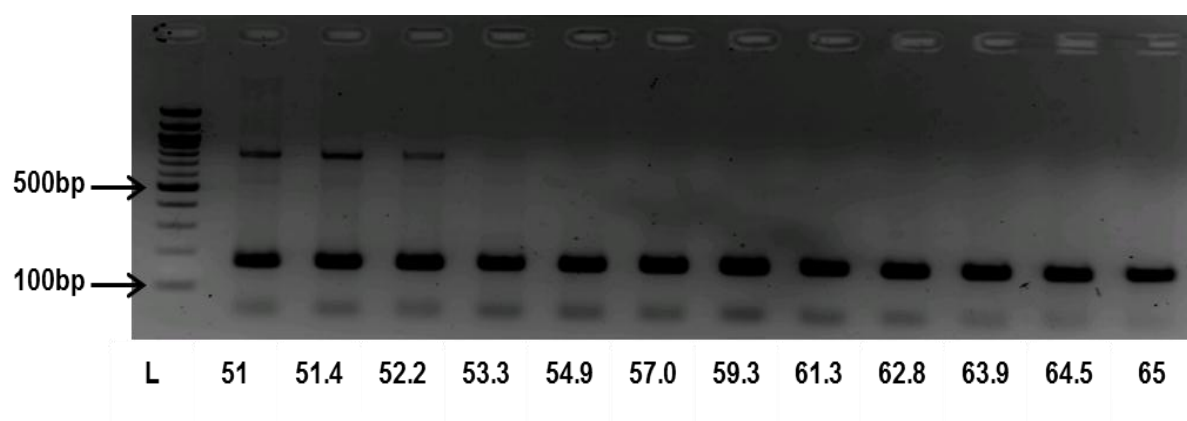


Figure 7.8: Temperature gradient (58-65°C) for Pax6 (170bps) primer pairs

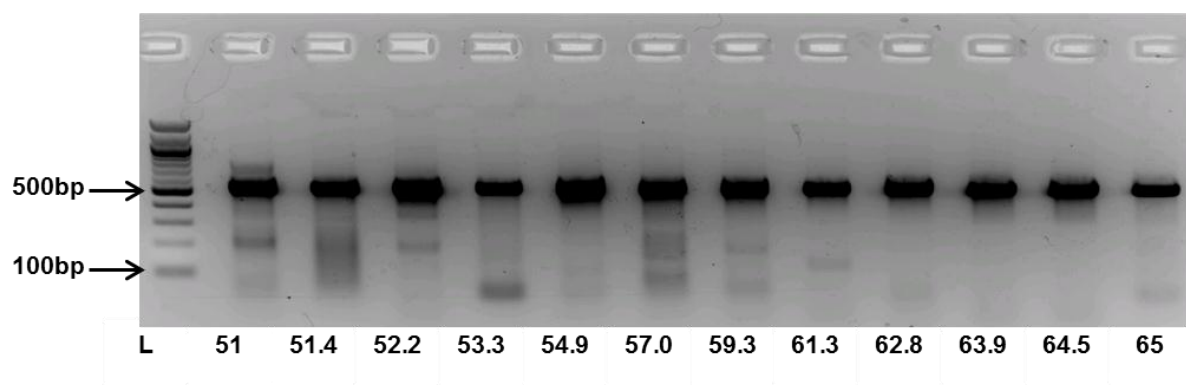


Figure 7.9: Temperature gradient (58-65°C) for SRR1 (536bps) primer pairs

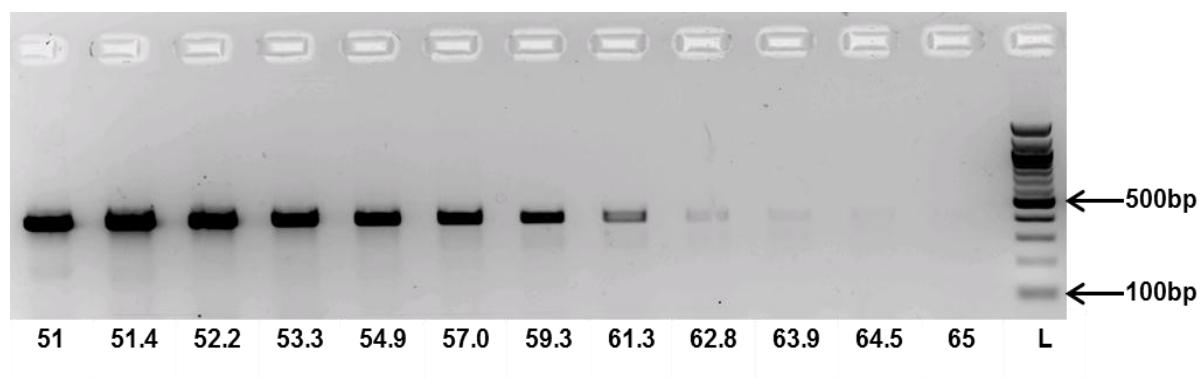


Figure 7.10: Temperature gradient (58-65°C) for SRR2 (419bps) primer pairs

## **7.5. Sequencing Chromatograms**

## **8. Bibliography**

## **References**

- Abranches, E., M. Silva, et al. (2009). "Neural differentiation of embryonic stem cells in vitro: a road map to neurogenesis in the embryo." PLoS One 4(7): e6286.
- Ahn, J. I., K. H. Lee, et al. (2004). "Temporal expression changes during differentiation of neural stem cells derived from mouse embryonic stem cell." J Cell Biochem 93(3): 563-578.
- Alberts B, Johnson A, Lewis J, et al. (2002). Molecular Biology of the Cell. 4th edition. New York: Garland Science.
- Alfred, R., T. Gareau, et al. (2010). "Serum-free scaled up expansion and differentiation of murine embryonic stem cells to osteoblasts in suspension bioreactors." Biotechnol Bioeng 106(5): 829-40.
- Amaral, P. P., C. Neyt, et al. (2009). "Complex architecture and regulated expression of the Sox2ot locus during vertebrate development." RNA 15(11): 2013-2027.
- Ambrosetti, D., G. Holmes, et al. (2008). "Fibroblast growth factor signaling uses multiple mechanisms to inhibit Wnt-induced transcription in osteoblasts." Mol Cell Biol 28(15): 4759-4771.
- Ambrosetti, D. C., C. Basilico, et al. (1997). "Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites." Mol Cell Biol 17(11): 6321-9.
- Anderson, D. J., F. H. Gage, et al. (2001). "Can stem cells cross lineage boundaries?" Nature Medicine 7: 393-395.
- Antequera, F. and A. Bird (1993). "Number of CpG islands and genes in human and mouse." Proc Natl Acad Sci U S A 90(24): 11995-9.

- Aubin, J. E., & Triffitt, J. (2002). Mesenchymal stem cells and the osteoblast lineage.
- Aubin, J. E. (1998). "Advances in the osteoblast lineage." *Biochem Cell Biol* 76(6): 899-910.
- Avilion, A. A., S. K. Nicolis, et al. (2003). "Multipotent cell lineages in early mouse development depend on SOX2 function." *Genes Dev* 17(1): 126-40.
- Bain, G., D. Kitchens, et al. (1995). "Embryonic stem cells express neuronal properties in vitro." *Dev Biol* 168(2): 342-357.
- Bain, G., W. J. Ray, et al. (1996). "Retinoic acid promotes neural and represses mesodermal gene expression in mouse embryonic stem cells in culture." *Biochem Biophys Res Commun* 223(3): 691-694.
- Bannister, A. J. and T. Kouzarides (2005). "Reversing histone methylation." *Nature* 436(7054): 1103-6.
- Bartel, D. P. (2004). "MicroRNAs: genomics, biogenesis, mechanism, and function." *Cell* 116(2): 281-97.
- Basu-Roy, U., D. Ambrosetti, et al. (2010). "The transcription factor Sox2 is required for osteoblast self-renewal". *Cell Death Diff.* 17: 1345-1353.
- Benayahu, D., Y. Kletter, et al. (1989). "Bone marrow-derived stromal cell line expressing osteoblastic phenotype in vitro and osteogenic capacity in vivo." *J Cell Physiol* 140(1): 1-7.
- Ben-Tabou de-Leon, S. and E. H. Davidson (2007). "Gene regulation: gene control network in development." *Annu Rev Biophys Biomol Struct* 36: 191.
- Bernstein, B. E., M. Kamal, et al. (2005). "Genomic maps and comparative analysis of histone modifications in human and mouse." *Cell* 120(2): 169-81.
- Bernstein, B. E., T. S. Mikkelsen, et al. (2006). "A bivalent chromatin structure marks key developmental genes in embryonic stem cells." *Cell* 125(2): 315-26.

- Bernstein, E., E. M. Duncan, et al. (2006). "Mouse polycomb proteins bind differentially to methylated histone H3 and RNA and are enriched in facultative heterochromatin." *Mol Cell Biol* 26(7): 2560-9.
- Bernstein, E. and C. D. Allis (2005). "RNA meets chromatin." *Genes Dev* 19(14): 1635-55.
- Bestor, T. H. and D. Bourc'his (2004). "Transposon silencing and imprint establishment in mammalian germ cells." *Cold Spring Harb Symp Quant Biol* 69: 381-7.
- Bianco, P., P. G. Robey, et al. (2008). "Mesenchymal stem cells: revisiting history, concepts, and assays." *Cell Stem Cell* 2(4): 313-319.
- Bibel, M., J. Richter, et al. (2007). "Generation of a defined and uniform population of CNS progenitors and neurons from mouse embryonic stem cells." *Nat Protoc* 2(5): 1034-1043.
- Bibel, M., J. Richter, K. Schrenk, K. L. Tucker, V. Staiger, M. Korte, M. Goetz and Y. A. Barde (2004). "Differentiation of mouse embryonic stem cells into a defined neuronal lineage." *Nat Neurosci* 7(9): 1003-1009.
- Binderman, I., R. M. Greene, et al. (1979). "Calcification of differentiating skeletal mesenchyme in vitro." *Science* 206(4415): 222-5.
- Bird, A., M. Taggart, et al. (1985). "A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA." *Cell* 40(1): 91-9.
- Bird, A. (2007). "Perceptions of epigenetics." *Nature* 447(7143): 396-8.
- Bird, A. P. (1980). "DNA methylation and the frequency of CpG in animal DNA." *Nucleic Acids Res* 8(7): 1499-504.
- Bird, A. P. (1986). "CpG-rich islands and the function of DNA methylation." *Nature* 321(6067): 209-13.

- Blau, H. M., T. R. Brazelton, et al. (2001). "The Evolving Concept of a Stem Cell: Entity or Function? ." *Cell* 105(7): 829-841.
- Bock, C., S. Reither, et al. (2005). "BiQ Analyzer: visualization and quality control for DNA methylation data from bisulphite sequencing." *Bioinformatics* 21(21): 4067-4068.
- Bortvin, A., K. Eggan, et al. (2003). "Incomplete reactivation of Oct4-related genes in mouse embryos cloned from somatic nuclei." *Development* 130(8): 1673-80.
- Botquin, V., H. Hess, et al. (1998). "New POU dimer configuration mediates antagonistic control of an osteopontin preimplantation enhancer by Oct-4 and Sox-2." *Genes Dev* 12(13): 2073-90.
- Bourne, S., J. M. Polak, S. P. Hughes and L. D. Buttery (2004). "Osteogenic differentiation of mouse embryonic stem cells: differential gene expression analysis by cDNA microarray and purification of osteoblasts by cadherin-11 magnetically activated cell sorting." *Tissue Eng* 10(5-6): 796-806.
- Bouwman, P. and S. Philipsen (2002). "Regulation of the activity of Sp1-related transcription factors." *Mol Cell Endocrinol* 195(1-2): 27-38.
- Bowles, J., G. Schepers, et al. (2000). "Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators." *Dev Biol* 227(2): 239-55.
- Boyer, L. A., K. Plath, et al. (2006). "Polycomb complexes repress developmental regulators in murine embryonic stem cells." *Nature* 441(7091): 349-53.
- Boyer, L. A., T. I. Lee, et al. (2005). "Core transcriptional regulatory circuitry in human embryonic stem cells." *Cell* 122(6): 947-56.
- Boyes, J. and A. Bird (1991). "DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein." *Cell* 64(6): 1123-34.

- Bradbury, E. M. (1992). "Reversible histone modifications and the chromosome cell cycle." *Bioessays* 14(1): 9-16.
- Brandeis, M., D. Frank, I. Keshet, Z. Siegfried, M. Mendelsohn, A. Nemes, V. Temper, A. Razin and H. Cedar (1994). "Sp1 elements protect a CpG island from de novo methylation." *Nature* 371(6496): 435-438.
- Brunner, D., J. Frank, H. Appl, H. Schoffl, W. Pfaller and G. Gstraunthaler (2010). "Serum-free cell culture: the serum-free media interactive online database." *ALTEX* 27(1): 53-62.
- Burdon, T., C. Stracey, et al. (1999b). "Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells." *Developmental Biology* 210(1): 30-43.
- Burdon, T., I. Chambers, et al. (1999a). "Signalling mechanisms regulating self-renewal and differentiation of pluripotent embryonic stem cells." *Cells Tissues Organs* 165(3-4): 131-43.
- Butler, M., and Jenkins, H (1989). "Nutritional aspects of the growth of animals cells in culture". *Journal of Biotechnology*. 12(2): 97-110
- Buttery, L. D., S. Bourne, et al. (2001). "Differentiation of osteoblasts and in vitro bone formation from murine embryonic stem cells." *Tissue Eng* 7(1): 89-99.
- Cai, C. and L. Grabel (2007). "Directing the differentiation of embryonic stem cells to neural stem cells." *Dev Dyn* 236(12): 3255-3266.
- Caley, D. P., R. C. Pink, et al. (2010). "Long noncoding RNAs, chromatin, and development." *ScientificWorldJournal* 10: 90-102.
- Caliskan, M., D. A. Cusanovich, et al. (2011). "The effects of EBV transformation on gene expression levels and methylation profiles." *Hum Mol Genet* 20(8): 1643-1652.

- Catena, R., C. Tiveron, et al. (2004). "Conserved POU binding DNA sites in the Sox2 upstream enhancer regulate gene expression in embryonic and neural stem cells." *J Biol Chem* 279(40): 41846-41857.
- Cerutti, H. and J. A. Casas-Mollano (2006). "On the origin and functions of RNA-mediated silencing: from protists to man." *Curr Genet* 50(2): 81-99.
- Chadee, D. N., M. J. Hendzel, et al. (1999). "Increased Ser-10 phosphorylation of histone H3 in mitogen-stimulated and oncogene-transformed mouse fibroblasts." *J Biol Chem* 274(35): 24914-20.
- Chambers, I., D. Colby, et al. (2003). "Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells." *Cell* 113(5): 643-55.
- Chambers, I., J. Silva, et al. (2007). "Nanog safeguards pluripotency and mediates germline development." *Nature* 450(7173): 1230-4.
- Chambers, I. and A. Smith (2004). "Self-renewal of teratocarcinoma and embryonic stem cells." *Oncogene* 23(43): 7150-60.
- Chan, H. M. and N. B. La Thangue (2001). "p300/CBP proteins: HATs for transcriptional bridges and scaffolds." *J Cell Sci* 114(Pt 13): 2363-2373.
- Chapman, E. J. and J. C. Carrington (2007). "Specialization and evolution of endogenous small RNA pathways." *Nat Rev Genet* 8(11): 884-96.
- Charbord, P. (2010). "Bone marrow mesenchymal stem cells: historical overview and concepts." *Hum Gene Ther* 21(9): 1045-1056.
- Chavez, L., J. Jozefczuk, C. Grimm, J. Dietrich, B. Timmermann, H. Lehrach, R. Herwig and J. Adjaye (2010). "Computational analysis of genome-wide DNA methylation during the differentiation of human embryonic stem cells along the endodermal lineage." *Genome Res* 20(10): 1441-1450.

- Chen, T., Y. Ueda, et al. (2003). "Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b." *Mol Cell Biol* 23(16): 5594-605.
- Cheng, L. C., E. Pastrana, et al. (2009). "miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche." *Nat Neurosci* 12(4): 399-408.
- Chentoufi, J., M. Hott, et al. (1993). "Kinetics of in vitro mineralization by an osteogenic clonal cell line (C1) derived from mouse teratocarcinoma." *Differentiation* 53(3): 181-9.
- Clarke, D. L., C. B. Johansson, et al. (2000). "Generalized potential of adult neural stem cells." *Science* 288(5471): 1660-1663.
- Collignon, J., S. Sockanathan, et al. (1996). "A comparison of the properties of Sox-3 with Sry and two related genes, Sox-1 and Sox-2." *Development* 122(2): 509-20.
- Coucouvanis, E. and G. R. Martin (1995). "Signals for death and survival: a two-step mechanism for cavitation in the vertebrate embryo." *Cell* 83(2): 279-287.
- Davidson, E. H., D. R. McClay, et al. (2003). "Regulatory gene networks and the properties of the developmental process." *Proc Natl Acad Sci U S A* 100(4): 1475-1480.
- Davidson, E. H., J. P. Rast, et al. (2002). "A genomic regulatory network for development." *Science* 295(5560): 1669-1678.
- Delaval, K., J. Govin, et al. (2007). "Differential histone modifications mark mouse imprinting control regions during spermatogenesis." *EMBO J* 26(3): 720-9.
- Delgado, S., M. Gomez, et al. (1998). "Initiation of DNA replication at CpG islands in mammalian chromosomes." *EMBO J* 17(8): 2426-35.

- Diduch, D. R., M. R. Coe, et al. (1993). "Two cell lines from bone marrow that differ in terms of collagen synthesis, osteogenic characteristics, and matrix mineralization." *J Bone Joint Surg Am* 75(1): 92-105.
- Dinger, M. E., K. C. Pang, et al. (2008). "Differentiating protein-coding and noncoding RNA: challenges and ambiguities." *PLoS Comput Biol* 4(11): e1000176.
- Dinger, M. E., P. P. Amaral, T. R. Mercer, K. C. Pang, S. J. Bruce, B. B. Gardiner, M. E. Askarian-Amiri, K. Ru, G. Solda, C. Simons, S. M. Sunkin, M. L. Crowe, S. M. Grimmond, A. C. Perkins and J. S. Mattick (2008). "Long noncoding RNAs in mouse embryonic stem cell pluripotency and differentiation." *Genome Res* 18(9): 1433-1445.
- D'Ippolito, G., P. C. Schiller, et al. (1999). "Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow." *J Bone Miner Res* 14(7): 1115-1122.
- Doench, J. G., C. P. Petersen, et al. (2003). "siRNAs can function as miRNAs." *Genes Dev* 17(4): 438-42.
- Doetschman, T. C., H. Eistetter, M. Katz, W. Schmidt and R. Kemler (1985). "The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium." *J Embryol Exp Morphol* 87: 27-45.
- Duan, X., E. Kang, et al. (2008). "Development of neural stem cell in the adult brain." *Curr Opin Neurobiol* 18(1): 108-115.
- Dulbecco, R. and G. Freeman (1959). "Plaque production by the polyoma virus." *Virology* 8(3): 396-397.
- Duplomb, L., M. Dagouassat, et al. (2007). "Concise review: embryonic stem cells: a new tool to study osteoblast and osteoclast differentiation." *Stem Cells* 25(3): 544-552.

- Duplomb, L., M. Dagouassat, et al. (2007). "Differentiation of osteoblasts from mouse embryonic stem cells without generation of embryoid body." *In Vitro Cell Dev Biol Anim* 43(1): 21-4.
- Eggan, K., H. Akutsu, et al. (2001). "Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation." *Proc Natl Acad Sci U S A* 98(11): 6209-14.
- Evans, M. J. and M. H. Kaufman (1981). "Establishment in culture of pluripotential cells from mouse embryos." *Nature* 292: 154-156.
- Evans, M. J. and M. H. Kaufman (1983). "Pluripotential cells grown directly from normal mouse embryos." *Cancer Surv.* 2: 185-208.
- Feil, R., Walter, J., Allen, N. D., and Reik, W. (1994). Developmental control of allelic methylation in the imprinted mouse *Igf2* and *H19* genes. *Development* 120, 2933-2943.
- Ferrari, S., V. R. Harley, et al. (1992). "SRY, like HMG1, recognizes sharp angles in DNA." *EMBO J* 11(12): 4497-506.
- Ferri, A. L., M. Cavallaro, et al. (2004). "Sox2 deficiency causes neurodegeneration and impaired neurogenesis in the adult mouse brain." *Development* 131(15): 3805-3819.
- Fields, S. and M. Johnston (2005). "Cell biology. Whither model organism research?" *Science* 307(5717): 1885-1886.
- Finley, M. F., S. Devata and J. E. Huettner (1999). "BMP-4 inhibits neural differentiation of murine embryonic stem cells." *J Neurobiol* 40(3): 271-287.
- Fouse, S. D., Y. Shen, et al. (2008). "Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation." *Cell Stem Cell* 2(2): 160-9.

- Fraichard, A., O. Chassande, G. Bilbaut, C. Dehay, P. Savatier and J. Samarut (1995). "In vitro differentiation of embryonic stem cells into glial cells and functional neurons." *J Cell Sci* 108 ( Pt 10): 3181-3188.
- Fujii, T., T. Ueno, et al. (2005). "Immunohistochemical analysis of Sox9 expression in periosteum of tibia and calvaria after surgical release of the periosteum." *Acta Histochem* 106(6): 427-437.
- Fuks, F. (2005). "DNA methylation and histone modifications: teaming up to silence genes." *Curr Opin Genet Dev* 15(5): 490-5.
- Gao, J., J. Wang, et al. (2011). "Regulation of Pax6 by CTCF during induction of mouse ES cell differentiation." *PLoS One* 6(6): e20954.
- Ghannam, S., C. Bouffi, et al. (2010). "Immunosuppression by mesenchymal stem cells: mechanisms and clinical applications." *Stem Cell Res Ther* 1(1): 2.
- Gilbert, S. F. (2000). *Developmental Biology*, Sunderland (MA): Sinauer Associates.
- Ginis, I., Y. Luo, T. Miura, S. Thies, R. Brandenberger, S. Gerecht-Nir, M. Amit, A. Hoke, M. K. Carpenter, J. Itskovitz-Eldor and M. S. Rao (2004). "Differences between human and mouse embryonic stem cells." *Dev Biol* 269(2): 360-380.
- Go, M. J., C. Takenaka, et al. (2008). "Forced expression of Sox2 or Nanog in human bone marrow derived mesenchymal stem cells maintains their expansion and differentiation capabilities." *Exp Cell Res* 314(5): 1147-1154.
- Goldberg, A. D., C. D. Allis, et al. (2007). "Epigenetics: a landscape takes shape." *Cell* 128(4): 635-8.
- Graham, V., J. Khudyakov, et al. (2003). "SOX2 functions to maintain neural progenitor identity." *Neuron* 39(5): 749-765.
- Graham, V., Khudyakov, J., Ellis, P. & Pevny, L. SOX2 functions to maintain neural progenitor identity. *Neuron* 39, 749-765 (2003).

- Gregory, C. A., W. G. Gunn, et al. (2004). "An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction." *Anal Biochem* 329(1): 77-84.
- Grunstein, M. (1997). "Histone acetylation in chromatin structure and transcription." *Nature* 389(6649): 349-52.
- Guan, K., J. Rohwedel and A. M. Wobus (1999). "Embryonic stem cell differentiation models: cardiogenesis, myogenesis, neurogenesis, epithelial and vascular smooth muscle cell differentiation in vitro." *Cytotechnology* 30(1-3): 211-226.
- Gubbay, J., J. Collignon, et al. (1990). "A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes." *Nature* 346(6281): 245-50.
- Gurdon, J. B., R. A. Laskey, et al. (1975). "The developmental capacity of nuclei transplanted from keratinized skin cells of adult frogs." *J Embryol Exp Morphol* 34(1): 93-112.
- Ham, R. G. (1965). "CLONAL GROWTH OF MAMMALIAN CELLS IN A CHEMICALLY DEFINED, SYNTHETIC MEDIUM." *Proc Natl Acad Sci U S A* 53: 288-293.
- Hamazaki, T., Y. Iiboshi, M. Oka, P. J. Papst, A. M. Meacham, L. I. Zon and N. Terada (2001). "Hepatic maturation in differentiating embryonic stem cells in vitro." *FEBS Lett* 497(1): 15-19.
- Hannenhalli, S. and S. Levy (2001). "Promoter prediction in the human genome." *Bioinformatics* 17 Suppl 1: S90-6.
- Harley, V. R., R. Lovell-Badge, et al. (1994). "Definition of a consensus DNA binding site for SRY." *Nucleic Acids Res* 22(8): 1500-1.

- Hay, R. T. (2001). "Protein modification by SUMO." *Trends Biochem Sci* 26(5): 332-333.
- He, L. and G. J. Hannon (2004). "MicroRNAs: small RNAs with a big role in gene regulation." *Nat Rev Genet* 5(7): 522-31.
- Hendrich, B. and A. Bird (1998). "Identification and characterization of a family of mammalian methyl-CpG binding proteins." *Mol Cell Biol* 18(11): 6538-47.
- Heng, B. C., T. Cao, et al. (2004). "Strategies for directing the differentiation of stem cells into the osteogenic lineage in vitro." *J Bone Miner Res* 19(9): 1379-1394.
- Hooper, M., K. Hardy, et al. (1987). "HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells." *Nature* 326(6110): 292-295.
- Horwitz, E. M., D. J. Prockop, et al. (1999). "Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta." *Nat Med* 5(3): 309-313.
- Hurd, P. J., A. J. Bannister, K. Halls, M. A. Dawson, M. Vermeulen, J. V. Olsen, H. Ismail, J. Somers, M. Mann, T. Owen-Hughes, I. Gout and T. Kouzarides (2009). "Phosphorylation of histone H3 Thr-45 is linked to apoptosis." *J Biol Chem* 284(24): 16575-16583.
- Hutvagner, G. and P. D. Zamore (2002). "A microRNA in a multiple-turnover RNAi enzyme complex." *Science* 297(5589): 2056-60.
- Hwang, Y. S., Polak, J. M., and Mantalaris, A. (2008). In vitro direct osteogenesis of murine embryonic stem cells without embryoid body formation. *Stem Cells Dev* 17, 963-970.
- Ioshikhes, I. P. and M. Q. Zhang (2000). "Large-scale human promoter mapping using CpG islands." *Nat Genet* 26(1): 61-3.

- Istrail, S. and E. H. Davidson (2005). "Logic functions of the genomic cis-regulatory code." *Proc Natl Acad Sci U S A* 102(14): 4954-4959.
- Itskovitz-Eldor, J., M. Schuldiner, et al. (2000). "Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers." *Mol Med* 6(2): 88-95.
- Jablonka, E. and M. J. Lamb (2002). "The changing concept of epigenetics." *Ann N Y Acad Sci* 981: 82-96.
- Jaenisch, R. and R. Young (2008). "Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming." *Cell* 132(4): 567-82.
- Jenuwein, T. and C. D. Allis (2001). "Translating the histone code." *Science* 293(5532): 1074-80.
- Johnson, E. S. (2004). "Protein modification by SUMO." *Annu Rev Biochem* 73: 355-382.
- Kallur, T., R. Gisler, et al. (2008). "Pax6 promotes neurogenesis in human neural stem cells." *Mol Cell Neurosci* 38(4): 616-628.
- Kamachi, Y., M. Iwafuchi, et al. (2009). "Evolution of non-coding regulatory sequences involved in the developmental process: reflection of differential employment of paralogous genes as highlighted by Sox2 and group B1 Sox genes." *Proc Jpn Acad Ser B Phys Biol Sci* 85(2): 55-68.
- Kamachi, Y., M. Uchikawa, et al. (1998). "Involvement of Sox1, 2 and 3 in the early and subsequent molecular events of lens induction." *Development* 125(13): 2521-32.
- Kamachi, Y., M. Uchikawa, et al. (2000). "Pairing SOX off: with partners in the regulation of embryonic development." *Trends Genet* 16(4): 182-7.
- Kamachi, Y., S. Sockanathan, et al. (1995). "Involvement of SOX proteins in lens-specific activation of crystallin genes." *EMBO J* 14(14): 3510-9.

- Kapranov, P., J. Cheng, et al. (2007). "RNA maps reveal new RNA classes and a possible function for pervasive transcription." *Science* 316(5830): 1484-1488.
- Karsenty, G., H. M. Kronenberg, et al. (2009). "Genetic control of bone formation." *Annu Rev Cell Dev Biol* 25: 629-648.
- Karsenty, G. (1999). "The genetic transformation of bone biology." *Genes Dev* 13(23): 3037-3051.
- Kawaguchi, J., P. J. Mee, et al. (2005). "Osteogenic and chondrogenic differentiation of embryonic stem cells in response to specific growth factors." *Bone* 36(5): 758-69.
- Keller, G. (2005). "Embryonic stem cell differentiation: emergence of a new era in biology and medicine." *Genes Dev* 19(10): 1129-1155.
- Keshet, I., J. Yisraeli, et al. (1985). "Effect of regional DNA methylation on gene expression." *Proc Natl Acad Sci U S A* 82(9): 2560-4.
- Kim, Y. J., H. N. Kim, et al. (2006). "The bone-related Zn finger transcription factor Osterix promotes proliferation of mesenchymal cells." *Gene* 366(1): 145-151.
- Klose, R. J. and A. P. Bird (2006). "Genomic DNA methylation: the mark and its mediumtors." *Trends Biochem Sci* 31(2): 89-97.
- Kolasinska-Zwierz, P., T. Down, et al. (2009). "Differential chromatin marking of introns and expressed exons by H3K36me3." *Nat Genet* 41(3): 376-81.
- Kolchanov, N. A., T. I. Merkulova, et al. (2007). "Combined experimental and computational approaches to study the regulatory elements in eukaryotic genes." *Brief Bioinform* 8(4): 266-74.
- Komori, T. (2006). "Regulation of osteoblast differentiation by transcription factors." *J Cell Biochem* 99(5): 1233-1239.
- Kornberg, R. D. and Y. Lorch (1999). "Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome." *Cell* 98(3): 285-94.

- Koshland, D. and A. Strunnikov (1996). "Mitotic chromosome condensation." *Annu Rev Cell Dev Biol* 12: 305-33.
- Kouzarides, T. (2007). "Chromatin modifications and their function." *Cell* 128(4): 693-705.
- Kozhevnikova, M. N., A. S. Mikaelian, et al. (2008). "[Molecular and genetic regulation of osteogenic differentiation of mesenchymal stromal cells]." *Izv Akad Nauk Ser Biol*(3): 261-271.
- Kuersten, S. and E. B. Goodwin (2003). "The power of the 3' UTR: translational control and development." *Nat Rev Genet* 4(8): 626-37.
- Laird, P. W. (2010). "Principles and Challenges of genome wide DNA methylation analysis." *Nat Rev Genet* 11(3): 191-203.
- Lan, F., P. E. Bayliss, et al. (2007). "A histone H3 lysine 27 demethylase regulates animal posterior development." *Nature* 449(7163): 689-94.
- Lande-Diner, L. and H. Cedar (2005). "Silence of the genes--mechanisms of long-term repression." *Nat Rev Genet* 6(8): 648-54.
- Latchman, D. S. (2004). *Transcription Factors and Human Disease*. Eukaryotic Transcription Factors (Fourth Edition). London, Academic Press: 293-340.
- Laudet, V., D. Stehelin, et al. (1993). "Ancestry and diversity of the HMG box superfamily." *Nucleic Acids Res* 21(10): 2493-501.
- Lee, D. Y., C. Teyssier, et al. (2005b). "Role of protein methylation in regulation of transcription." *Endocr Rev* 26(2): 147-70.
- Lee, J. T. (2010). "The X as model for RNA's niche in epigenomic regulation." *Cold Spring Harb Perspect Biol* 2(9): a003749.
- Lee, K. K. and J. L. Workman (2007). "Histone acetyltransferase complexes: one size doesn't fit all." *Nat Rev Mol Cell Biol* 8(4): 284-295.

- Lee, M. G., C. Wynder, et al. (2005a). "An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation." *Nature* 437(7057): 432-5.
- Lee, M. K., J. B. Tuttle, et al. (1990). "The expression and posttranslational modification of a neuron-specific beta-tubulin isotype during chick embryogenesis." *Cell Motil Cytoskeleton* 17(2): 118-132.
- Lee, M. K., Tuttle, J. B., Rebhun, L. I., Cleveland, D. W. & Frankfurter, A. The expression and posttranslational modification of a neuron-specific beta-tubulin isotype during chick embryogenesis. *Cell motility and the cytoskeleton* 17, 118-132, doi:10.1002/cm.970170207 (1990).
- Lee, T. I., R. G. Jenner, et al. (2006). "Control of developmental regulators by Polycomb in human embryonic stem cells." *Cell* 125(2): 301-13.
- Lee, T. I. and R. A. Young (2000). "Transcription of eukaryotic protein-coding genes." *Annu Rev Genet* 34: 77-137.
- Lefebvre, V., B. Dumitriu, et al. (2007). "Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors." *Int J Biochem Cell Biol* 39(12): 2195-214.
- Lendahl, U., L. B. Zimmerman and R. D. McKay (1990). "CNS stem cells express a new class of intermediate filament protein." *Cell* 60(4): 585-595.
- Lengner, C. J., M. Q. Hassan, et al. (2005). "Nkx3.2-mediated repression of Runx2 promotes chondrogenic differentiation." *J Biol Chem* 280(16): 15872-9.
- Lenka, N., Z. J. Lu, et al. (2002). "Quantitation and functional characterization of neural cells derived from ES cells using nestin enhancer-mediated targeting in vitro." *J Cell Sci* 115(Pt 7): 1471-1485.
- Levine, M. and E. H. Davidson (2005). "Gene regulatory networks for development." *Proc Natl Acad Sci U S A* 102(14): 4936-4942.

- Levine, M. and R. Tjian (2003). "Transcription regulation and animal diversity." *Nature* 424(6945): 147-151.
- Li, E. (2002). "Chromatin modification and epigenetic reprogramming in mammalian development." *Nat Rev Genet* 3(9): 662-73.
- Li, M., L. Pevny, R. Lovell-Badge and A. Smith (1998). "Generation of purified neural precursors from embryonic stem cells by lineage selection." *Curr Biol* 8(17): 971-974.
- Li, Q., K. R. Peterson, et al. (2002). "Locus control regions." *Blood* 100(9): 3077-3086.
- Lian, J., C. Stewart, et al. (1989). "Structure of the rat osteocalcin gene and regulation of vitamin D-dependent expression." *Proc Natl Acad Sci U S A* 86(4): 1143-7.
- Lian, J. B., G. S. Stein, et al. (2006). "Networks and hubs for the transcriptional control of osteoblastogenesis." *Rev Endocr Metab Disord* 7(1-2): 1-16.
- Lim, L. P., N. C. Lau, et al. (2003). "The microRNAs of *Caenorhabditis elegans*." *Genes Dev* 17(8): 991-1008.
- Lindahl, T. (1982). "DNA repair enzymes." *Annu Rev Biochem* 51: 61-87.
- Liu, C. L., T. Kaplan, et al. (2005). "Single-nucleosome mapping of histone modifications in *S. cerevisiae*." *PLoS Biol* 3(10): e328.
- Loh, Y. H., Q. Wu, et al. (2006). "The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells." *Nat Genet* 38(4): 431-40.
- Long, M. W. (2001). "Osteogenesis and bone-marrow-derived cells." *Blood Cells Mol Dis* 27(3): 677-690.
- Luger, K., A. W. Mader, et al. (1997). "Crystal structure of the nucleosome core particle at 2.8 Å resolution." *Nature* 389(6648): 251-60.

- Macleod, D., J. Charlton, et al. (1994). "Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island." *Genes Dev* 8(19): 2282-92.
- Maden, M. (2007). "Retinoic acid in the development, regeneration and maintenance of the nervous system." *Nat Rev Neurosci* 8(10): 755-765.
- Mansukhani, A., D. Ambrosetti, et al. (2005). "Sox2 induction by FGF and FGFR2 activating mutations inhibits Wnt signaling and osteoblast differentiation." *J Cell Biol* 168(7): 1065-76.
- Martin, C. and Y. Zhang (2005). "The diverse functions of histone lysine methylation." *Nat Rev Mol Cell Biol* 6(11): 838-49.
- Martin, C. and Y. Zhang (2007). "Mechanisms of epigenetic inheritance." *Curr Opin Cell Biol* 19(3): 266-72.
- Martin, G. R. (1981). "Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells." *Proc Natl Acad Sci USA* 78: 7634-7638.
- Maston, G. A., S. K. Evans, et al. (2006). "Transcriptional regulatory elements in the human genome." *Annu Rev Genomics Hum Genet* 7: 29-59.
- Masui, S., Y. Nakatake, et al. (2007). "Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells." *Nat Cell Biol* 9(6): 625-35.
- Masumi, A. (2011). "Histone acetyltransferases as regulators of nonhistone proteins: the role of interferon regulatory factor acetylation on gene transcription." *J Biomed Biotechnol* 2011: 640610.
- Masumi, A. (2011). "Histone acetyltransferases as regulators of nonhistone proteins: the role of interferon regulatory factor acetylation on gene transcription." *J Biomed Biotechnol* 2011: 640610.

- Matranga, C. and P. D. Zamore (2007). "Small silencing RNAs." *Curr Biol* 17(18): R789-93.
- McKee, M. D. and A. Nanci (1996). "Osteopontin: an interfacial extracellular matrix protein in mineralized tissues." *Connect Tissue Res* 35(1-4): 197-205.
- Meijer, G. J., J. D. de Bruijn, et al. (2007). "Cell-based bone tissue engineering." *PLoS Med* 4(2): e9.
- Meissner, A. (2010). "Epigenetic modifications in pluripotent and differentiated cells." *Nat Biotechnol* 28(10): 1079-1088.
- Melchior, F. (2000). "SUMO--nonclassical ubiquitin." *Annu Rev Cell Dev Biol* 16: 591-626.
- Mercer, T. R., M. E. Dinger, et al. (2009). "Long non-coding RNAs: insights into functions." *Nat Rev Genet* 10(3): 155-159.
- Merkle, F. T. and A. Alvarez-Buylla (2006). "Neural stem cells in mammalian development." *Curr Opin Cell Biol* 18(6): 704-709.
- Meshorer, E. and T. Misteli (2006). "Chromatin in pluripotent embryonic stem cells and differentiation." *Nat Rev Mol Cell Biol* 7(7): 540-546.
- Mikkelsen, T. S., M. Ku, et al. (2007). "Genome-wide maps of chromatin state in pluripotent and lineage-committed cells." *Nature* 448(7153): 553-60.
- Ming, G. L. and H. Song (2005). "Adult neurogenesis in the mammalian central nervous system." *Annu Rev Neurosci* 28: 223-250.
- Mishra, R. and F. Karch (1999). "Boundaries that demarcate structural and functional domains of chromatin." *Journal of Biosciences* 24(3): 377-399.
- Miyagi, S., M. Nishimoto, et al. (2006). "The Sox2 regulatory region 2 functions as a neural stem cell-specific enhancer in the telencephalon." *J Biol Chem* 281(19): 13374-13381.

- Miyagi, S., T. Saito, et al. (2004). "The Sox-2 regulatory regions display their activities in two distinct types of multipotent stem cells." *Mol Cell Biol* 24(10): 4207-4220.
- Morgan, H. D., F. Santos, et al. (2005). "Epigenetic reprogramming in mammals." *Hum Mol Genet* 14 Spec No 1: R47-58.
- Mori-Akiyama, Y., H. Akiyama, et al. (2003). "Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest." *Proc Natl Acad Sci U S A* 100(16): 9360-5.
- Moriguchi, T., K. Yano, S. Nakagawa and F. Kaji (2003). "Elucidation of adsorption mechanism of bone-staining agent alizarin red S on hydroxyapatite by FT-IR microspectroscopy." *J Colloid Interface Sci* 260(1): 19-25.
- Mundlos, S. and B. R. Olsen (1997). "Heritable diseases of the skeleton. Part I: Molecular insights into skeletal development-transcription factors and signaling pathways." *FASEB J* 11(2): 125-132.
- Muraglia, A., M. Perera, et al. (2008). "DLX5 overexpression impairs osteogenic differentiation of human bone marrow stromal cells." *Eur J Cell Biol* 87(10): 751-761.
- Murphy, C. L. and J. M. Polak (2002). "Differentiating embryonic stem cells: GAPDH, but neither HPRT nor beta-tubulin is suitable as an internal standard for measuring RNA levels." *Tissue Eng* 8(4): 551-559.
- Murry, C. E. and G. Keller (2008). "Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development." *Cell* 132(4): 661-680.
- Nagy, A., E. Gocza, et al. (1990). "Embryonic stem cells alone are able to support fetal development in the mouse." *Development* 110(3): 815-21.

- Nakashima, K., X. Zhou, et al. (2002). "The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation." *Cell* 108(1): 17-29.
- Nathan, D., K. Ingvarsdottir, D. E. Sterner, G. R. Bylebyl, M. Dokmanovic, J. A. Dorsey, K. A. Whelan, M. Krsmanovic, W. S. Lane, P. B. Meluh, E. S. Johnson and S. L. Berger (2006). "Histone sumoylation is a negative regulator in *Saccharomyces cerevisiae* and shows dynamic interplay with positive-acting histone modifications." *Genes Dev* 20(8): 966-976.
- Nichols, J., B. Zevnik, et al. (1998). "Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4." *Cell* 95(3): 379-91.
- Nieden, N. I., G. Kempka, et al. (2003). "In vitro differentiation of embryonic stem cells into mineralized osteoblasts." *Differentiation* 71(1): 18-27.
- Nielsen, S. J., R. Schneider, U. M. Bauer, A. J. Bannister, A. Morrison, D. O'Carroll, R. Firestein, M. Cleary, T. Jenuwein, R. E. Herrera and T. Kouzarides (2001). "Rb targets histone H3 methylation and HP1 to promoters." *Nature* 412(6846): 561-565.
- Nishikawa, S., L. M. Jakt and T. Era (2007). "Embryonic stem-cell culture as a tool for developmental cell biology." *Nat Rev Mol Cell Biol* 8(6): 502-507.
- Nishimoto, M., A. Fukushima, et al. (1999). "The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2." *Mol Cell Biol* 19(8): 5453-65.
- Niwa, H. (2001). "Molecular mechanism to maintain stem cell renewal of ES cells." *Cell Struct Funct* 26(3): 137-48.
- Niwa, H. (2007). "How is pluripotency determined and maintained?" *Development* 134(4): 635-46.

- Ogbourne, S. and T. M. Antalis (1998). "Transcriptional control and the role of silencers in transcriptional regulation in eukaryotes." *Biochem J* 331 ( Pt 1): 1-14.
- Okabe, S., K. Forsberg-Nilsson, et al. (1996). "Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro." *Mech Dev* 59(1): 89-102.
- Okada, Y., T. Shimazaki, et al. (2004). "Retinoic-acid-concentration-dependent acquisition of neural cell identity during in vitro differentiation of mouse embryonic stem cells." *Dev Biol* 275(1): 124-142.
- Ong, C. T. and V. G. Corces (2011). "Enhancer function: new insights into the regulation of tissue-specific gene expression." *Nat Rev Genet* 12(4): 283-293.
- Ovcharenko, I., M. A. Nobrega, et al. (2004). "ECR Browser: a tool for visualizing and accessing data from comparisons of multiple vertebrate genomes." *Nucleic Acids Res* 32(Web Server issue): W280-6.
- Pan, Y., C. J. Tsai, et al. (2010). "Mechanisms of transcription factor selectivity." *Trends Genet* 26(2): 75-83.
- Park, I. H., R. Zhao, et al. (2008). "Reprogramming of human somatic cells to pluripotency with defined factors." *Nature* 451(7175): 141-6.
- Pavesi, G., F. Zambelli, et al. (2007). "WeederH: an algorithm for finding conserved regulatory motifs and regions in homologous sequences." *BMC Bioinformatics* 8: 46.
- Pesce, M., K. Anastassiadis, et al. (1999). "Oct-4: lessons of totipotency from embryonic stem cells." *Cells Tissues Organs* 165(3-4): 144-52.
- Pesce, M., X. Wang, et al. (1998a). "Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation." *Mech Dev* 71(1-2): 89-98.
- Pevny, L. and M. Placzek (2005). "SOX genes and neural progenitor identity." *Curr Opin Neurobiol* 15(1): 7-13.

- Pevny, L. H. and R. Lovell-Badge (1997). "Sox genes find their feet." *Curr Opin Genet Dev* 7(3): 338-44.
- Phillips, B. W., N. Belmonte, et al. (2001). "Compactin enhances osteogenesis in murine embryonic stem cells." *Biochem Biophys Res Commun* 284(2): 478-84.
- Pittenger, M. F., A. M. Mackay, et al. (1999). "Multilineage potential of adult human mesenchymal stem cells." *Science* 284(5411): 143-147.
- Plachta, N., M. Bibel, K. L. Tucker and Y. A. Barde (2004). "Developmental potential of defined neural progenitors derived from mouse embryonic stem cells." *Development* 131(21): 5449-5456.
- Plath, K., J. Fang, et al. (2003). "Role of histone H3 lysine 27 methylation in X inactivation." *Science* 300(5616): 131-5.
- Ponting, C. P., P. L. Oliver, et al. (2009). "Evolution and functions of long noncoding RNAs." *Cell* 136(4): 629-641.
- Prella, K., N. Zink, et al. (2002). "Pluripotent stem cells - model of embryonic development, tool for gene targeting and basis of cell therapy." *Anat. Histol. Embryol.* 31: 169-186.
- Prior, H. M. and M. A. Walter (1996). "SOX genes: architects of development." *Mol Med* 2(4): 405-412.
- Puchtler, H., S. N. Meloy and M. S. Terry (1969). "On the history and mechanism of alizarin and alizarin red S stains for calcium." *J Histochem Cytochem* 17(2): 110-124.
- Que, J., T. Okubo, et al. (2007). "Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm." *Development* 134(13): 2521-2531.

- Rago, R., J. Mitchen, et al. (1990). "DNA fluorometric assay in 96-well tissue culture plates using Hoechst 33258 after cell lysis by freezing in distilled water." *Anal Biochem* 191(1): 31-34.
- Rassoulzadegan, M., V. Grandjean, et al. (2006). "RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse." *Nature* 441(7092): 469-74.
- Rathjen, J., B. P. Haines, K. M. Hudson, A. Nesci, S. Dunn and P. D. Rathjen (2002). "Directed differentiation of pluripotent cells to neural lineages: homogeneous formation and differentiation of a neuroectoderm population." *Development* 129(11): 2649-2661.
- Rathjen, J. and P. D. Rathjen (2001). "Mouse ES cells: experimental exploitation of pluripotent differentiation potential." *Curr Opin Genet Dev* 11(5): 587-594.
- Reik, W., W. Dean, et al. (2001). "Epigenetic reprogramming in mammalian development." *Science* 293(5532): 1089-93.
- Reik, W. (2007). "Stability and flexibility of epigenetic gene regulation in mammalian development." *Nature* 447(7143): 425-432.
- Ringrose, L. and R. Paro (2004). "Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins." *Annu Rev Genet* 38: 413-43.
- Rizzino, A. and C. Crowley (1980). "Growth and differentiation of embryonal carcinoma cell line F9 in defined media." *Proc Natl Acad Sci U S A* 77(1): 457-461.
- Robertson, E. J. (1987). Embryo-derived stem cell lines. *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. E. J. Robertson. Oxford, UK, IRL Press.: 71-112.
- Robey, P. G., N. S. Fedarko, et al. (1993). "Structure and molecular regulation of bone matrix proteins." *J Bone Miner Res* 8 Suppl 2: S483-487.

- Roth, S. Y., J. M. Denu and C. D. Allis (2001). "Histone acetyltransferases." *Annu Rev Biochem* 70: 81-120.
- Ruthenburg, A. J., C. D. Allis, et al. (2007). "Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark." *Mol Cell* 25(1): 15-30.
- Ryoo, H. M., H. M. Hoffmann, et al. (1997). "Stage-specific expression of *Dlx-5* during osteoblast differentiation: involvement in regulation of osteocalcin gene expression." *Mol Endocrinol* 11(11): 1681-94.
- Sanosaka, T., M. Namihira, et al. (2009). "Epigenetic mechanisms in sequential differentiation of neural stem cells." *Epigenetics* 4(2): 89-92.
- Sanosaka, T., Namihira, M. & Nakashima, K. Epigenetic mechanisms in sequential differentiation of neural stem cells. *Epigenetics : official journal of the DNA Methylation Society* 4, 89-92 (2009).
- Schepers, G. E., R. D. Teasdale, et al. (2002). "Twenty pairs of sox: extent, homology, and nomenclature of the mouse and human sox transcription factor gene families." *Dev Cell* 3(2): 167-70.
- Schuettengruber, B., D. Chourrout, et al. (2007). "Genome regulation by polycomb and trithorax proteins." *Cell* 128(4): 735-45.
- Schulz, W. A. and M. J. Hoffmann (2007). "Transcription factor networks in embryonic stem cells and testicular cancer and the definition of epigenetics." *Epigenetics* 2(1): 37-42.
- Schwartz, P. H., D. J. Brick, et al. (2008). "Differentiation of neural lineage cells from human pluripotent stem cells." *Methods* 45(2): 142-158.
- Serakinci, N., P. Guldberg, et al. (2004). "Adult human mesenchymal stem cell as a target for neoplastic transformation." *Oncogene* 23(29): 5095-5098.

- Shabalina, S. A. and E. V. Koonin (2008). "Origins and evolution of eukaryotic RNA interference." *Trends Ecol Evol* 23(10): 578-87.
- Shen, L. and R. A. Waterland (2007). "Methods of DNA methylation analysis." *Curr Opin Clin Nutr Metab Care* 10(5): 576-581.
- Shiio, Y. and R. N. Eisenman (2003). "Histone sumoylation is associated with transcriptional repression." *Proc Natl Acad Sci U S A* 100(23): 13225-13230.
- Sikorska, M., J. K. Sandhu, et al. (2008). "Epigenetic modifications of SOX2 enhancers, SRR1 and SRR2, correlate with in vitro neural differentiation." *J Neurosci Res* 86(8): 1680-93.
- Sikorski, T. W. and S. Buratowski (2009). "The basal initiation machinery: beyond the general transcription factors." *Curr Opin Cell Biol* 21(3): 344-351.
- Silva, J. and A. Smith (2008). "Capturing pluripotency." *Cell* 132(4): 532-6.
- Sinclair, A. H., P. Berta, et al. (1990). "A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif." *Nature* 346(6281): 240-4.
- Smith, A. (1998). "Cell therapy: in search of pluripotency." *Curr. Biol.* 8: 802–804.
- Smith, A. G., J. K. Heath, et al. (1988). "Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides." *Nature* 336: 688-690.
- Smith, A. G. (1992). "Mouse embryo stem cells: their identification, propagation and manipulation." *Semin Cell Biol* 3(6): 385-99.
- Smith, A. G. (2001). "Embryo-derived stem cells: of mice and men." *Annu Rev Cell Dev Biol* 17: 435-462.
- Solter, D. and J. Gearhart (1999). "Putting stem cells to work." *Science* 283(5407): 1468-1470.

- Stavridis, M. P. and A. G. Smith (2003). "Neural differentiation of mouse embryonic stem cells." *Biochem Soc Trans* 31(Pt 1): 45-49.
- Stolt, C. C., P. Lommes, et al. (2003). "The Sox9 transcription factor determines glial fate choice in the developing spinal cord." *Genes Dev* 17(13): 1677-1689.
- Strahl, B. D., R. Ohba, et al. (1999). "Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in *Tetrahymena*." *Proc Natl Acad Sci U S A* 96(26): 14967-72.
- Strahl, B. D. and C. D. Allis (2000). "The language of covalent histone modifications." *Nature* 403(6765): 41-5.
- Strubing, C., G. Ahnert-Hilger, J. Shan, B. Wiedenmann, J. Hescheler and A. M. Wobus (1995). "Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons." *Mech Dev* 53(2): 275-287.
- Struhl, K. (1998). "Histone acetylation and transcriptional regulatory mechanisms." *Genes Dev* 12(5): 599-606.
- Suter, D. M. and K. H. Krause (2008). "Neural commitment of embryonic stem cells: molecules, pathways and potential for cell therapy." *J Pathol* 215(4): 355-368.
- Suzuki, A., A. Raya, et al. (2006). "Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells." *Proc Natl Acad Sci U S A* 103(27): 10294-9.
- Sved, J. and A. Bird (1990). "The expected equilibrium of the CpG dinucleotide in vertebrate genomes under a mutation model." *Proc Natl Acad Sci U S A* 87(12): 4692-6.
- Szutorisz, H. and N. Dillon (2005). "The epigenetic basis for embryonic stem cell pluripotency." *Bioessays* 27(12): 1286-93.

- Tada, S., T. Era, C. Furusawa, H. Sakurai, S. Nishikawa, M. Kinoshita, K. Nakao, T. Chiba and S. Nishikawa (2005). "Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture." *Development* 132(19): 4363-4374.
- Tai, G., Polak, J. M., Bishop, A. E., Christodoulou, I., and Buttery, L. D. (2004). Differentiation of osteoblasts from murine embryonic stem cells by overexpression of the transcriptional factor osterix. *Tissue Eng* 10, 1456-1466.
- Takahashi, K., K. Tanabe, et al. (2007). "Induction of pluripotent stem cells from adult human fibroblasts by defined factors." *Cell* 131(5): 861-72.
- Takahashi, K. and S. Yamanaka (2006). "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." *Cell* 126(4): 663-676.
- Tang, G., B. J. Reinhart, et al. (2003). "A biochemical framework for RNA silencing in plants." *Genes Dev* 17(1): 49-63.
- Tay, Y., J. Zhang, et al. (2008). "MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation." *Nature* 455(7216): 1124-1128.
- Thomson, J. A., J. Itskovitz-Eldor, et al. (1998). "Embryonic stem cell lines derived from human blastocysts." *Science* 282(5391): 1145-7.
- Thomson, J. A., J. Kalishman, et al. (1995). "Isolation of a primate embryonic stem cell line." *Proc Natl Acad Sci U S A* 92(17): 7844-8.
- Thomson, S., L. C. Mahadevan, et al. (1999). "MAP kinase-mediated signalling to nucleosomes and immediate-early gene induction." *Semin Cell Dev Biol* 10(2): 205-14.
- Thorne, A. W., D. Kmiecik, et al. (1990). "Patterns of histone acetylation." *Eur J Biochem* 193(3): 701-13.

- Tomioka, M., M. Nishimoto, et al. (2002). "Identification of Sox-2 regulatory region which is under the control of Oct-3/4-Sox-2 complex." *Nucleic Acids Res* 30(14): 3202-13.
- Uchikawa, M., Y. Ishida, et al. (2003). "Functional analysis of chicken Sox2 enhancers highlights an array of diverse regulatory elements that are conserved in mammals." *Dev Cell* 4(4): 509-519.
- Uchikawa, M., Y. Kamachi, et al. (1999). "Two distinct subgroups of Group B Sox genes for transcriptional activators and repressors: their expression during embryonic organogenesis of the chicken." *Mech Dev* 84(1-2): 103-20.
- Umlauf, D., Y. Goto, et al. (2004). "Imprinting along the Kcnq1 domain on mouse chromosome 7 involves repressive histone methylation and recruitment of Polycomb group complexes." *Nat Genet* 36(12): 1296-300.
- van de Wetering, M., M. Oosterwegel, et al. (1993). "Sox-4, an Sry-like HMG box protein, is a transcriptional activator in lymphocytes." *EMBO J* 12(10): 3847-54.
- van der Valk, J., D. Brunner, K. De Smet, A. Fex Svenningsen, P. Honegger, L. E. Knudsen, T. Lindl, J. Norberg, A. Price, M. L. Scarino and G. Gstraunthaler (2010). "Optimization of chemically defined cell culture media--replacing fetal bovine serum in mammalian in vitro methods." *Toxicol In Vitro* 24(4): 1053-1063.
- van Houte, L. P., V. P. Chuprina, et al. (1995). "Solution structure of the sequence-specific HMG box of the lymphocyte transcriptional activator Sox-4." *J Biol Chem* 270(51): 30516-24.
- van Leeuwen, F. and B. van Steensel (2005). "Histone modifications: from genome-wide maps to functional insights." *Genome Biol* 6(6): 113.
- Verger, A., J. Perdomo and M. Crossley (2003). "Modification with SUMO. A role in transcriptional regulation." *EMBO Rep* 4(2): 137-142.

- Waddington, C. H. (1942). "The epigenotype." *Endeavour* 1: 18-20.
- Wang, H., L. Zhai, et al. (2006). "Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage." *Mol Cell* 22(3): 383-94.
- Watson, J. D., T. A. Baker, et al. (2008). *Molecular Biology of Gene*. San Francisco, Benjamin Cummings.
- Watt, F. and P. L. Molloy (1988). "Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter." *Genes Dev* 2(9): 1136-43.
- Weber, M., I. Hellmann, et al. (2007). "Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome." *Nat Genet* 39(4): 457-66.
- Weber, M., J. J. Davies, D. Wittig, E. J. Oakeley, M. Haase, W. L. Lam and D. Schubeler (2005). "Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells." *Nat Genet* 37(8): 853-862.
- Wegner, M. (1999). "From head to toes: the multiple facets of Sox proteins." *Nucleic Acids Res* 27(6): 1409-20.
- Wei, L. C., M. Shi, et al. (2002). "Nestin-containing cells express glial fibrillary acidic protein in the proliferative regions of central nervous system of postnatal developing and adult mice." *Brain Res Dev Brain Res* 139(1): 9-17.
- Werner, M. H., J. R. Huth, et al. (1995). "Molecular basis of human 46X,Y sex reversal revealed from the three-dimensional solution structure of the human SRY-DNA complex." *Cell* 81(5): 705-14.

- Werner, M. H. and S. K. Burley (1997). "Architectural transcription factors: proteins that remodel DNA." *Cell* 88(6): 733-6.
- Wiebe, M. S., P. J. Wilder, et al. (2000). "Isolation, characterization, and differential expression of the murine Sox-2 promoter." *Gene* 246(1-2): 383-93.
- Wild, L., J. M. Funes, et al. (2010). "In vitro transformation of mesenchymal stem cells induces gradual genomic hypomethylation." *Carcinogenesis* 31(10): 1854-1862.
- Wiles, M. V. and B. M. Johansson (1999). "Embryonic stem cell development in a chemically defined medium." *Exp Cell Res* 247(1): 241-248.
- Willems, E., I. Mateizel, C. Kemp, G. Cauffman, K. Sermon and L. Leyns (2006). "Selection of reference genes in mouse embryos and in differentiating human and mouse ES cells." *Int J Dev Biol* 50(7): 627-635.
- Williams, R. L., D. J. Hilton, et al. (1988). "Myeloid-leukemia inhibitory factor maintains the developmental potential of embryonic stem-cells." *Nature* 336(6200): 684-687.
- Wilmut, I., A. E. Schnieke, et al. (1997). "Viable offspring derived from fetal and adult mammalian cells." *Nature* 385(6619): 810-813.
- Wilmut, I., A. E. Schnieke, et al. (2007). "Viable offspring derived from fetal and adult mammalian cells." *Cloning Stem Cells* 9(1): 3-7.
- Wilusz, J. E., H. Sunwoo, et al. (2009). "Long noncoding RNAs: functional surprises from the RNA world." *Genes Dev* 23(13): 1494-1504.
- Wobus, A. M. and K. R. Boheler (2005). "Embryonic stem cells: prospects for developmental biology and cell therapy." *Physiol Rev* 85(2): 635-678.
- Wolffe, A. P. (1994). "Architectural transcription factors." *Science* 264(5162): 1100-1.

- Woll, N. L. and S. K. Bronson (2006). "Analysis of embryonic stem cell-derived osteogenic cultures." *Methods Mol Biol* 330: 149-59.
- Wood, H. B. and V. Episkopou (1999). "Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages." *Mech Dev* 86(1-2): 197-201.
- Wright, E., M. R. Hargrave, et al. (1995). "The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos." *Nat Genet* 9(1): 15-20.
- Xu, N., T. Papagiannakopoulos, G. Pan, J. A. Thomson and K. S. Kosik (2009). "MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells." *Cell* 137(4): 647-658.
- Yang, E., E. van Nimwegen, M. Zavolan, N. Rajewsky, M. Schroeder, M. Magnasco and J. E. Darnell, Jr. (2003). "Decay rates of human mRNAs: correlation with functional characteristics and sequence attributes." *Genome Res* 13(8): 1863-1872.
- Yasunaga, M., S. Tada, S. Torikai-Nishikawa, Y. Nakano, M. Okada, L. M. Jakt, S. Nishikawa, T. Chiba, T. Era and S. Nishikawa (2005). "Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells." *Nat Biotechnol* 23(12): 1542-1550.
- Yaworsky, P. J. and C. Kappen (1999). "Heterogeneity of neural progenitor cells revealed by enhancers in the nestin gene." *Dev Biol* 205(2): 309-321.
- Yeom, Y. I., G. Fuhrmann, et al. (1996). "Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells." *Development* 122(3): 881-94.
- Ying, Q. L., J. Wray, et al. (2008). "The ground state of embryonic stem cell self-renewal." *Nature* 453(7194): 519-523.

- Ying, Q. L., M. Stavridis, D. Griffiths, M. Li and A. Smith (2003). "Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture." *Nat Biotechnol* 21(2): 183-186.
- Ying, Q. L. and A. G. Smith (2003). "Defined conditions for neural commitment and differentiation." *Methods Enzymol* 365: 327-341.
- Young, M. F., J. M. Kerr, et al. (1992). "Structure, expression, and regulation of the major noncollagenous matrix proteins of bone." *Clin Orthop Relat Res*(281): 275-294.
- Yuan, H., N. Corbi, et al. (1995). "Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3." *Genes Dev* 9(21): 2635-45.
- Zappone, M. V., R. Galli, et al. (2000). "Sox2 regulatory sequences direct expression of a (beta)-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells." *Development* 127(11): 2367-82.
- Zeng, Y., E. J. Wagner, et al. (2002). "Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells." *Mol Cell* 9(6): 1327-33.
- Zeng, Y., R. Yi, et al. (2003). "MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms." *Proc Natl Acad Sci U S A* 100(17): 9779-84.
- Zernik, J., K. Twarog, et al. (1990). "Regulation of alkaline phosphatase and alpha 2(I) procollagen synthesis during early intramembranous bone formation in the rat mandible." *Differentiation* 44(3): 207-15.
- Zhao, C., W. Deng, et al. (2008). "Mechanisms and functional implications of adult neurogenesis." *Cell* 132(4): 645-660.

- zur Nieden, N. I., G. Kempka, et al. (2003). "In vitro differentiation of embryonic stem cells into mineralized osteoblasts." *Differentiation* 71(1): 18-27.