

***Investigating a Conserved Role
for Nanog in Primordial Germ Cell
Specification***

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RESEARCH ABSTRACT

The specification of Primordial Germ Cells (PGCs) in early mammalian development is a fundamental process of development, critical for, maintaining the germ line. There are two methods by which PGCs are specified in the animal kingdom. Firstly, through the predetermined method involving maternally inherited germplasm, and secondly through the induction of pluripotent precursors in a process termed epigenesis. Embryoid Bodies (EBs) can be used to recapitulate early developmental processes of mammalian development, allowing the dissection and better understanding of critical processes. This project will aim to try and better understand the role of the homeobox transcription factor *Nanog* in specifying PGCs through the process of epigenesis. Mouse *Nanog* over-expression in EBs has been shown to increase PGC specification, assayed through RT-PCR analysis. The functional activity of ancestral *Nanog* is hypothesised to be conserved through the major trunk of evolution in organisms that specify PGCs through the induced method of epigenesis. To test this hypothesis, ancient *Nanog* orthologs were isolated from different positions of the Deuterostome superfamily and over-expressed in EBs. Their effects on enhancing PGC specification were then quantified through quantitative RT-PCR and western blot analysis. The results from this project show that the over-expression of ancient *Nanog* orthologs in mES cells can enhance the process of PGC specification, by increasing the PGC associated markers *Dazl* and *Vasa*. This suggests a possible conservation of functional *Nanog* activity in organisms that specify PGCs through epigenesis, and also that pluripotency is an ancient trait.

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RESEARCH ABSTRACT

The specification of Primordial Germ Cells (PGCs) in early mammalian development is a fundamental process of development, critical for, maintaining the germ line. There are two methods by which PGCs are specified in the animal kingdom. Firstly, through the predetermined method involving maternally inherited germplasm, and secondly through the induction of pluripotent precursors in a process termed epigenesis. Embryoid Bodies (EBs) can be used to recapitulate early developmental processes of mammalian development, allowing the dissection and better understanding of critical processes. This project will aim to try and better understand the role of the homeobox transcription factor *Nanog* in specifying PGCs through the process of epigenesis. Mouse *Nanog* over-expression in EBs has been shown to increase PGC specification, assayed through RT-PCR analysis. The functional activity of ancestral *Nanog* is hypothesised to be conserved through the major trunk of evolution in organisms that specify PGCs through the induced method of epigenesis. To test this hypothesis, ancient *Nanog* orthologs were isolated from different positions of the Deuterostome superfamily and over-expressed in EBs. Their effects on enhancing PGC specification were then quantified through quantitative RT-PCR and western blot analysis. The results from this project show that the over-expression of ancient *Nanog* orthologs in mES cells can enhance the process of PGC specification, by increasing the PGC associated markers *Dazl* and *Vasa*. This suggests a possible conservation of functional *Nanog* activity in organisms that specify PGCs through epigenesis, and also that pluripotency is an ancient trait.

CHAPTER 1: INTRODUCTION

1.1 Pluripotency

A stem cell's "potency" can be defined as its potential to differentiate and produce cell types from different lineages. As differentiation proceeds down a "one-way street", a stem cell's potency is diminished, becoming increasingly more restricted by epigenetic events, which include the repression of pluripotency associated genes (Kim. J et al., 2008). Repression of pluripotency genes is coupled with the activation of cell lineage specific genes. The ordering of stem cells according to their potency is seen to be ordered in a hierarchical structure.

In mammals, the ability of a cell to produce all the cell types required to build an organism, including the extra-embryonic tissue, is termed "totipotent". These unrestricted cells have been identified in the early embryo at the 4-cell (Morula) stage. As differentiation proceeds, the ability to produce extra-embryonic tissue is lost but the multi-lineage potential to form cells of all three embryonic germ layers is retained. This ability to produce all cell types of both the embryo and associated germline is termed "pluripotent". Pluripotent cells are found in a subset of cells in the developing mammalian blastula (**Figure. 1**).

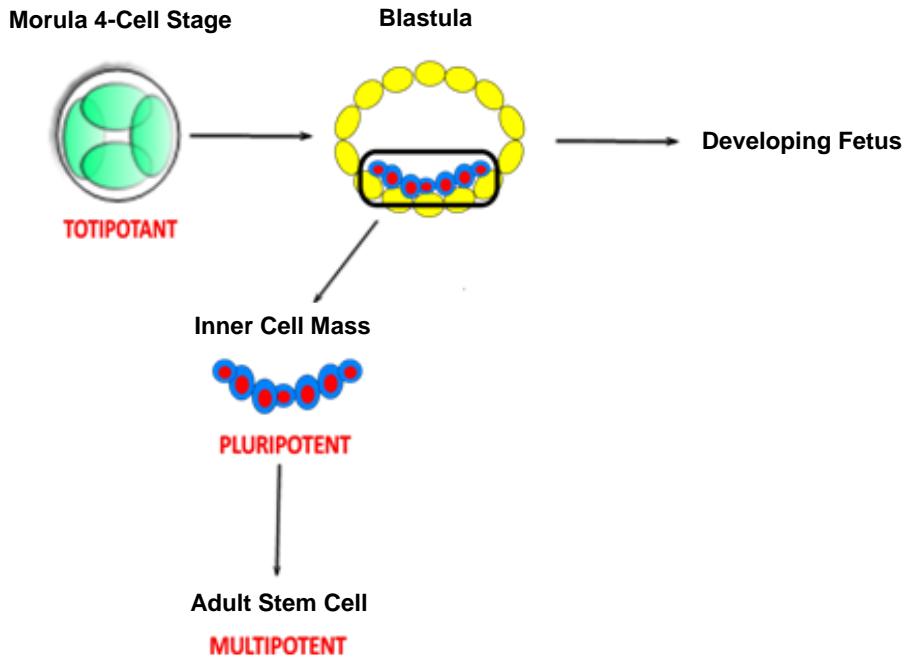


Figure 1: Hierarchy of stem cell potential. Stem cells can be characterised in a hierarchical structure with the most “potent” cells being at the “top of the tree”. As differentiation proceeds, cells become more restricted and cell potency is diminished. Pluripotent cells have the potential to produce cells from all the three germ layers: the endoderm, mesoderm and ectoderm. They are however not able to produce extra-embryonic tissue, such as the placenta.

1.2 Embryonic Stem Cells

In the 1960s, a germ cell cancer cell line isolated from a teratocarcinoma (germ cell tumour) was found to display close similarity to the cells of the Inner Cell Mass (ICM) of a developing mouse. These cancer germ cells were successfully grown in culture as cancer stem cells.

Being derived from a cancer cell line, however, meant that the cancer germline stem cells harboured genetic abnormalities. Despite this, they were initially used to model early mouse development. This work identified the need to isolate the unrestricted potential of cells in the ICM of developing mouse embryos. In the early 1980s, cells of the ICM were captured in culture and termed “Mouse Embryonic Stem Cells” (mES cells) (Martin. G. R, 1980), (Evans. M. J and Kaufman. M. H, 1981) (**Figure. 3A**).

In 1998, Human Embryonic Stem Cells (hES cells) were successfully isolated and cultured in defined media on fibroblast cell feeder layers (Thomson. J. A et al., 1998) (Figure. 3B).

ES cells are defined as being “pluripotent”, possessing the ability to both:

1. self-renew indefinitely in an undifferentiated state
2. differentiate down different cellular lineages; producing adult cell types from all three germ layers, that is, ectoderm, mesoderm and endoderm, as well as germ cells

The possession of these two defining properties is what makes ES cells of great importance and use.

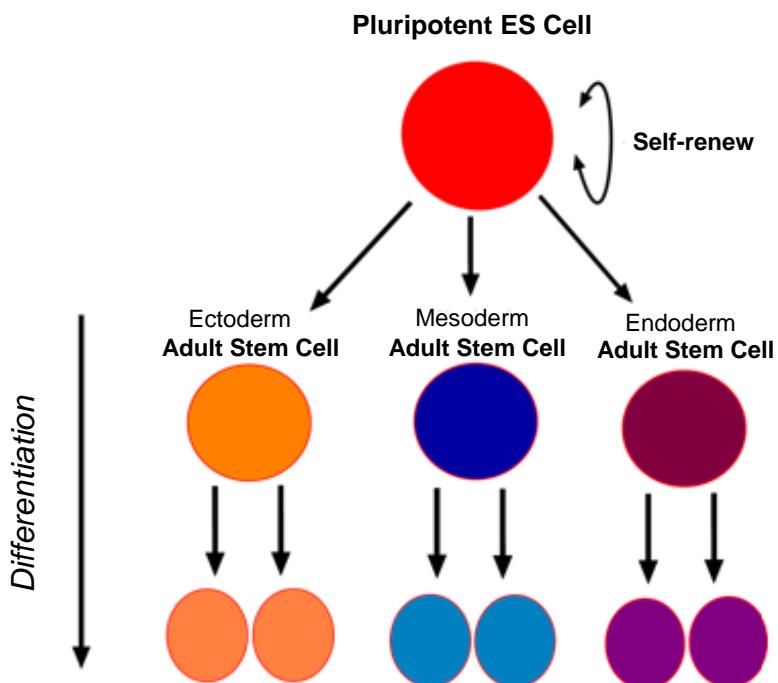


Figure 2: Potential of pluripotent ES cells to divide and self-renew. ES cells possess the ability to derive different cellular lineages of the three germ cell layers, whilst retaining the ability to self-renew.

The ability of ES cells to both self-renew and to differentiate down cellular lineages means that ES cells are distinct from more differentiated and restricted types of adult multi-potent stem cells, for example Neuronal Stem Cells (NSCs) (**Figure 2**). Undifferentiated ES cells can be cultured in defined media for unrestricted periods of time making them “immortal”. Culturing ES cells *in vitro* arrests the normal developmental process seen *in vivo*. This makes the isolation and culture of ES cells distinct from the culture of whole animal embryos, which do not arrest when isolated and continue to progress forward with development. The ability to culture large populations of undifferentiated ES cells has made them an attractive target for a number of applications. Before the full potential of ES cells can be fully harnessed, control mechanisms and underlying cellular regulation need to be characterised.

In mES culture, media is supplemented with the cytokine Leukaemia Inhibitory Factor (LIF) and Bone Morphogenic Proteins (BMPs), allowing cells to remain in an undifferentiated state in cell culture flasks (Ying. Q. L et al., 2003), (Smith. A. G et al., 1988). In hES cell culture, FGF and activin are required along with fibroblast feeder cell layers (Vallier. L et al., 2005). LIF has been shown to drive ES cell self-renewal by activating the transcription factor STAT3 (Ying. Q. L et al., 2003), involved in downstream target gene expression. However, LIF alone has been shown to be insufficient to maintain self-renewal and is dependent on interplay with BMPs to preserve the multi-lineage potential and other associated ES cell properties (Ying. Q. L et al., 2003). The need for BMPs in culturing ES cells resides within the activation of the *Id* genes, a member of the basic helix-loop-helix (bHLH) family, via the Smad pathway (Hollnagel. A et al., 1999).

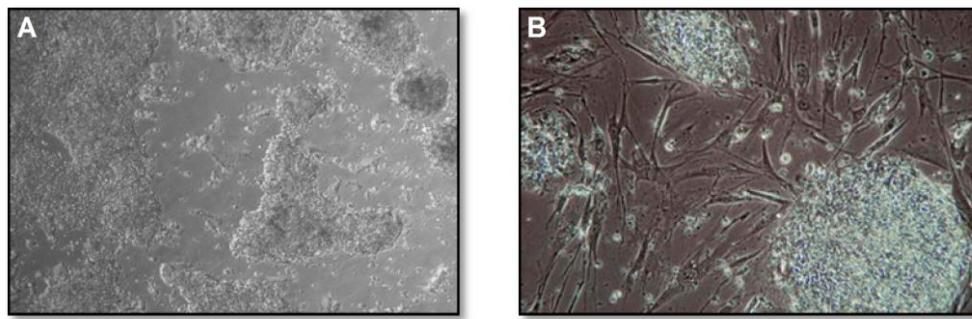


Figure 3: ES cell culture *in vitro*. (A) *In vitro* cultured mES cells (B) cultured hES cells on fibroblast feeder cell layers (Miller. J, 2006)

Assaying for undifferentiated ES cells can be achieved by using Alkaline Phosphotase (AP) staining, which can be used as a measure of ES cell self-renewal. A high level of AP is a characteristic marker of undifferentiated ES cells and is therefore a useful tool in identifying specific pluripotent colonies. As differentiation of ES cells proceeds, AP levels decrease.

Another assay that is used for the characterisation of ES cells exploits their unique ability to form non-cancerous tumours, termed “teratomas”. Teratomas are tumours that are formed from tissues or organs from all three germ layers (Baker. M, 2009). As the ability to produce all cell types of the three germ layers is characteristic of ES cells, teratoma formation serves as a valuable diagnostic tool to study differentiation.

1.3 Nanog

Nanog is a member of the homeobox transcription factor family Homeobox factors play an important role in the control of development. The study of *Nanog* allows the opportunity to uncover and dissect the control mechanisms underlying the maintenance of pluripotency in ES cells, in which *Nanog* has been shown to be a key player (Silva. J et al., 2009). *Nanog* and its role in pluripotency were discovered independently by two separate research groups.

One used *in silico* subtraction studies (Mitsui. K et al., 2003b) which identified *Nanog* as a potential ES cell associated transcription factor. Further work in the form of gene deletion studies then showed that *Nanog* was required to maintain epiblast identity, whilst also being involved in the regulation of pluripotency.

Parallel studies identified *Nanog*'s role in pluripotency by selecting cDNA from an ES cell cDNA library, followed by testing for specific cDNAs which allowed the process of self-renewal to occur in the absence of LIF (Chambers. I et al., 2003a). This showed *Nanog* to be a pluripotency associated transcription factor, involved with enhancing the process of self-renewal of undifferentiated ES cells, making them "immortal" (Mitsui. K et al., 2003a), (Chambers. I et al., 2003b). *Nanog* over-expression is characterised by its ability to maintain ES cells in the absence of cytokines, such as, LIF (Yates. A and Chambers. I, 2005). Further to the control of ES cells, *Nanog* has been found in pluripotent cells of embryos, as well as in derivative ES cells and in the developing germline of mammals (Chambers. I et al., 2003a).

ES cell and NSC fusion experiments have shown that the developmental programming imposed on differentiated cells can be erased (Silva. J et al., 2006), (Wong. C. C et al., 2008). In stem cell fusion assays, *Nanog* promotes pluripotent gene activation; meaning that pluripotency is a phenotypically dominant trait conferred to the somatic cell nucleus through the action of *Nanog* (Silva. J et al., 2006). It has also been noted that *Nanog* deletion in early embryo development is characterised as being lethal; the embryo is arrested in development prior to implantation (Chambers. I et al., 2007), (Mitsui. K et al., 2003b).

In 2006, it was shown that fully differentiated mouse skin fibroblasts could be reversed to achieve the pluripotent characteristics reminiscent of ES cells. The production of reprogrammed Induced Pluripotent Stem (iPS) cells demonstrates the importance that *Nanog* plays in ES cells.

The first mouse iPS cells produced were reprogrammed with four core factors; *Oct-4*, *Sox-2*, *Klf4* and *c-myc* (Takahashi. K and Yamanaka. S, 2006). The four core transcription factors were introduced to differentiated fibroblast cells by an engineered retrovirus. After a period of culturing the cells, pluripotency assays confirmed the reprogrammed state of the iPS cells. This work was then extended a year later to produce reprogrammed human iPS cells from differentiated human fibroblasts (Takahashi. K et al., 2007). Surprisingly, *Nanog* was not required as an exogenous factor in the early reprogramming steps of producing iPS cells (Okita. K et al., 2007), (Takahashi. K et al., 2007). It is also noteworthy that the addition of *Nanog* to the four core factors has not been shown to increase the efficiency of iPS cell reprogramming.

Although *Nanog* was not one of the four core factors used in early reprogramming, the switch from differentiated cell to iPS cell is achieved by the activation of the resident *Nanog* gene. For this reason, *Nanog* has been termed the “gatekeeper of pluripotency”, whose function is required to achieve ground state pluripotency (Silva. J et al., 2009). Screening for *Nanog* activation in early reprogrammed cells enables selection for fully reprogrammed iPS cells (Okita. K et al., 2007). These *Nanog*-selected iPS cells can contribute to adult chimeras and also possess the ability to contribute to the germline, a hallmark of pluripotency. Without *Nanog* activation, partially reprogrammed cells would remain in an intermediate non-viable and non-pluripotent state.

Nanog expression is required to produce both germ cells, which transmit heritable characteristics to offspring, and other non-sex cells termed “somatic cells” (soma), which comprise the organs of adults. *Nanog* has been shown to be highly expressed in PGCs of mouse embryos at Embryonic day (E) 11.5 and E12.5 (Yamaguchi. S et al., 2005), at a time in which there are dramatic epigenetic changes (Chambers. I et al., 2007). The role of *Nanog* in germ cell development is demonstrated by the fact that germ cells do not develop past stage E11.5 in the absence of *Nanog* (Chambers. I et al., 2007), suggesting a role in germ cell maturation.

Genital ridges of *Nanog*-null subject mice were dissected and stained for pluripotency associated factors, such as, *Oct-4* as well as *MvhI*, a mouse VASA homolog and a marker of mature germ cells. The results showed co-localisation of pluripotency associated factors and germ cell specific markers in wild-type mice, but PGCs derived from *Nanog* null ES cells could not mature to the stage of *Vasa* expression. To support the evidence that *Nanog* is required in germ cell maturation, homologous recombination of one *Nanog* allele was shown to restore functionality and consequently germ cell maturation (Chambers. I et al., 2007).

It has been suggested (Silva et al. 2008) that temporary absence of *Nanog* in ES cells does not affect their self-renewal capacity, however, a predisposition to differentiation is increased (**Figure. 4**). It was found that transient *Nanog* expression in ES cells opened a “window of opportunity” in which differentiation increased, however, not forcing ES cells to do so. The same study showed that undifferentiated ES cells may lose *Nanog* expression and then subsequently reacquire it. Also, *Nanog*-null ES cells were able to contribute to various cellular lineages, including liver, neuronal, heart muscle and kidney. These results show that multi-lineage potential is not lost in the absence of *Nanog* however a lack of mature PGCs is observed.

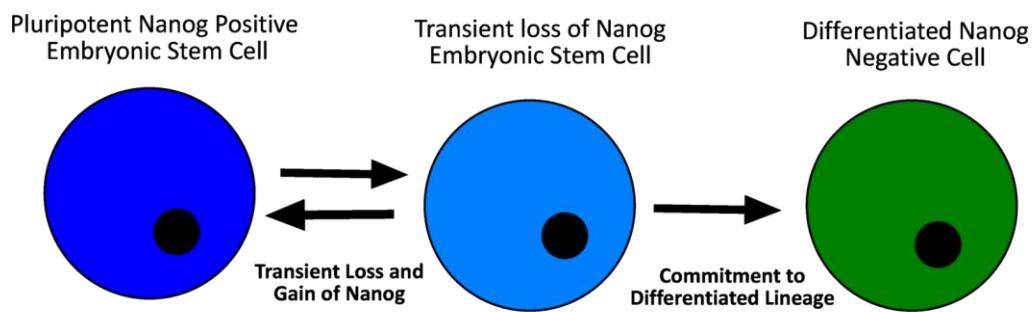


Figure 4: Nanog expression can be lost and subsequently reacquired. A model to show that *Nanog* expression may be lost in undifferentiated ES cells and then subsequently regained. A “window of opportunity” is seen during transient *Nanog* expression in which cell differentiation is seen to occur at increased levels (Adapted from (Chambers. I et al., 2007))

1.4 Forms of Nanog

The *Nanog* gene is conserved in evolution. *Nanog* homologs have been identified at the same positions on the chromosomes of chimpanzees (Fairbanks. D. J and Maughan. P. J, 2006) and amongst other species, such as chicken (Lavial. F et al., 2007), zebrafish (Camp. E et al., 2009), mouse (Mitsui. K et al., 2003a). It is thought that the origins of the human *NANOG* gene predate the branching of the human species, back through evolution.

Nanog can exist in two forms, firstly as a monomer in species such as axolotl and also in the form of a dimer in mammals. The cause of this variation lies in the tryptophan repeat (WR) domain, present at the C-terminal domain (Wang et al., 2008), which is present in the mammalian dimer form of *Nanog*, but not in the *Nanog* monomer form. The WR repeat domain is responsible for driving the dimerisation of *Nanog*. Studies have shown that the dimer form of *Nanog* found in mammals regulates ES cell proliferation (Ma. T et al., 2009). However, the repression of ES cell differentiation can be achieved by the monomeric ancestral form of *Nanog* (Dixon. J. E et al., 2010). It has been suggested that the tryptophan WR repeat domain found in the *Nanog* dimer evolved from the ancestral monomer to allow epiblast expansion in mammals.

1.5 Other Pluripotency Associated Factors

A critical factor associated along with *Nanog* in the maintenance of pluripotency in ES cells is the homeobox transcription factor *Oct-4*, also known as *POU5F1* (Niwa. H et al., 2000) (van den Berg et al., 2010). In *Oct-4* gene knockdown experiments differentiation is observed, demonstrating its critical role in ES self-renewal (Hough. S. R et al., 2006). As a result, *Oct-4* expression is used as a marker of pluripotent cells. In mammalian development, *Oct-4* is expressed strongly in all of the cells in the ICM, as opposed to *Nanog* which is confined to a subset of these cells (Silva. J et al., 2009).

In undifferentiated ES cells, ChIP analysis has shown that the *Oct-3/4* histones are highly acetylated, i.e. strongly expressed but as differentiation proceeds, the promoter is slowly deacetylated (Feldman. N et al., 2006). Work by (Liang. J et al., 2008) has shown that *Nanog* and *Oct-4* interact, communicating with distant repression complexes to regulate gene transcription. The repression complex GCNF has been shown to play a role in the repression of *Oct-4* in somatic cells during the process of early embryonic development (Gu. P et al., 2005).

Cdx2 is also a key homeobox transcription factor. It is involved in the development of the placenta during embryogenesis (Niwa. H et al., 2005). Trophectoderm is the first cell lineage that develops during mammalian embryo development, due to the repression of pluripotency associated genes. *Cdx2* and the pluripotency associated transcription factor *Oct-4*, have been shown to be reciprocal inhibitors (Kuckenberg. P et al., 2010). This leads to the possibility that the first differentiation event in mammalian development is under the control of *Cdx2* and *Oct-4* (Niwa. H et al., 2005)

(Figure. 5).

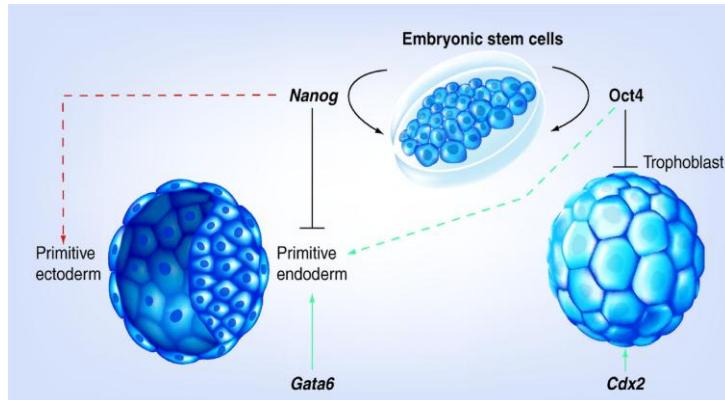


Figure 5: Transcriptional regulation of ES cells. *Nanog* and *Oct-4* transcription factor expression are involved in the maintenance of pluripotency in ES cells. Over expression of *Nanog* has been shown to cause the reversion to a primitive ectodermal form (red dashed arrowed line). *Gata6* acts as a *Nanog* antagonist in forcing differentiation of extra embryonic lineages (Niwa. H et al., 2005). [Reproduced from Cell, Volume 123, Issue 5, 2nd December 2005, “Interaction between Oct3/4 and Cdx2 Determines Trophectoderm Differentiation” with permission from Elsevier]

1.6 Pluripotency Networks

During germline development, there is seen to be dramatic epigenetic erasure, unique PGCs, followed by the re-establishment of epigenetic modifications (Allegrucci. C et al., 2005). As germ cells mediate germline transmission, they are considered to be “immortal”, as they are the link between generations (**Figure. 6**). In an evolutionary sense, the purpose of the somatic cells (soma) is to facilitate the passing on of this heritable information from one generation to the next.

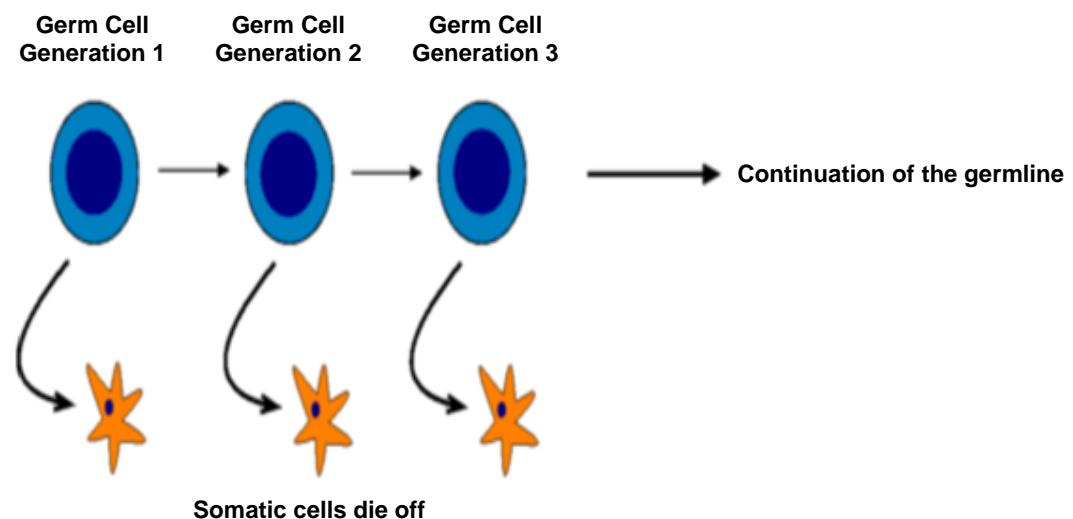


Figure 6: Continuation of the germline. Germ Cells are involved in passing on heritable information from generation to generation and the continuation of the germline. Somatic cells exist evolutionary to support and facilitate transfer of this heritable information to offspring and die off at each generation

In terms of germ cell specification during early development, it is possible to split the Animal Kingdom into two distinct groups (Bachvarova. R. F et al., 2004). There are two ways in which Primordial Germ Cells (PGCs), the precursors to mature haploid gametes (sex cells), can be established during early development (Johnson. A. D et al., 2003b):

1. Specification through germplasm (Predetermined Method)

One way to specify PGCs is through the action of germplasm, as initially proposed by August Weismann. Maternally inherited germplasm functions by inhibiting extracellular signals and restricting differentiation of a subset of cells during development, which go on to turn germline (Houston. D. W and King. M. L, 2000). The evolution of germplasm has been shown to have occurred several times in different species independently (Johnson. A. D et al., 2003b, Johnson. A. D et al., 2003a). As germplasm evolved as a means to specify PGCs, it is hypothesised there was a loss of need, and consequent deletion, of the *Nanog* gene in frogs (Dixon et al., 2010). This idea has been proposed as a reason for the lack of a *Nanog* gene in organisms that specify PGCs through the predetermined method, utilising germplasm.

The “Weismann Barrier” is a term that is used to refer to the restricted one-way transmission of genetic information from the germ cells to the soma; genetic information cannot travel in the opposite direction. This system of germ cell formation is present in species such as the amphibian *Xenopus laevis*, the fruit fly *Drosophila melanogaster*, the nematode *C. elegans*, to name just a few.

2. Specification through Epigenesis (Induced Method)

The second way of specifying germ cells during development is through the process of epigenesis. This is achieved by the induction of pluripotent precursors (Extavour. C. G and Akam. M, 2003) and is thought to involve the action of the *Nanog* transcription factor. This system is utilised by chordates and amniotes, including salamanders and mammals, and has been proposed to be conserved through the trunk of chordate evolution (Johnson. A. D et al., 2003a).

In mammals PGCs are induced early in gastrulation, that is, the process that initiates the formation of the three embryonic germ layers. PGCs are internalised along with the future extra-embryonic mesoderm in the early posterior primitive streak, followed by specification (Bachvarova et al., 2009). There exists strong evidence that a process of inductive germ cell specification, similar to that in mammals, also occurs in turtles and some other reptiles (Bachvarova et al., 2009).

During the development of amniotes, it has been shown that the PGCs appear before the formation of the gonad in the posterior trunk; their final destination. This results in a period of time in which the PGCs are located outside the gonads, requiring migration and colonisation of the gonad (Bachvarova et al., 2009). In contrast, in urodeles the PGCs appear relatively late (Bachvarova. R. F et al., 2004), and throughout development maintain a position close to precursors of the somatic cells of the gonad so that migration is not required (Bachvarova et al., 2009). In lampreys, early development of germ cells is highly similar to that of urodeles, suggesting this is the primitive vertebrate process.

Amphibians diverged around two hundred and fifty million years ago producing two distinct lineages, urodeles, such as the axolotl and anurans, such as xenopus. Urodeles have more traits conserved with mammals than do anurans; one example is the conservation of *Nanog* in urodeles and mammals. (Nieuwkoop. P. D, 1969) showed the differences between anurans and urodele embryos. He noted that mesoderm formation was different between the two lineages: anurans have a double-layered blastula compared to the single-layer seen in urodeles. There is also a different method of germ cell specification: anurans inherit maternal germplasm whilst urodeles have an induced mechanism of PGC formation. Nieuwkoop's classic experiments demonstrated the ability of axolotl animal caps to produce PGCs when exposed to inducing signals from the mesoderm. The same PGC induction cannot be achieved for *Xenopus* embryos, suggesting that the mechanism of PGC specification is conserved from urodeles to mammals (Johnson et al., 2003).

| | <i>Nanog</i> | germplasm |
|-------------------|--------------|-----------|
| Urodeles | + | - |
| Acorn Worm | + | - |
| Mouse | + | - |
| Amphioxus | + | - |
| Sturgeon | + | - |
| Zebrafish | - | + |
| Birds | - | + |
| Anurans | - | + |
| Drosophila | - | + |

Figure 7: Methods of specifying PGCs. A table to show a selection of species and the variation in PGC specification during early development. A “+” in the *Nanog* column indicates that particular species group utilises *Nanog* and the process of epigenesis to specify PGCs and A “+” in the germplasm column indicates that particular species doesn't have a *Nanog* gene

1.7 Germ Cell Production *In Vitro*

In vitro, it is possible to derive PGCs through Embryoid Body (EB) intermediates (Toyooka. Y et al., 2003). EBs can be created from the aggregation of mES cells in a “hanging drop”, which causes mES cells to cluster. EBs can then be collected from hanging drops and cultured *in vitro*, where they differentiate spontaneously. EBs serve as a powerful tool as they mimic many early developmental processes of mammalian development. However, as ES cells are pluripotent and have multi-lineage potential, the EBs are composed of a wide variety of differentiated cell types, which form in a disorganised manner. This is in contrast to the tightly regulated processes involved in normal embryonic development. Despite this, EBs can be used to dissect early developmental processes. Specified PGCs can be isolated from differentiating EBs and embryonic germ cell lines.

George Daley and colleagues (Geijsen. N et al., 2004) showed that the methylation patterns of both the Igf2r and H19 genes were erased in the isolated embryonic germ cells, as is seen in the normal germ cell lineage.

1.8 Germ Cell Specification Factors

Dazl (deleted in azoospermia-like), a germ cell specific RNA binding protein, is a key regulator in early germ cell specification (Kee. K et al., 2009). The related genes *DAZ* and *BOULE* mediate germ cell development and progression to mature haploid gametes. The interplay between *Dazl*, *DAZ* and *BOULE* allows the development and maturation of PGCs to mature sperm and egg cells. Over-expression of *Dazl* was seen to suppress the activity of *Nanog* and induced germ cell nuclear antigen in mES cells (Kee. K et al., 2009). *Dazl* knockdown resulted in lower expression levels of the germ cell specific genes *Stella*, *Mvh1* (mouse Vasa homolog) and *Prdm1* (*Blimp1*).

Blimp1 knock-out experiments have identified roles in germ cell specification during the process of early development (Ohinata et al., 2005). It was shown in the same study that *Blimp1* functions as a transcriptional repressor by blocking extracellular signals, e.g. from cytokines, in much the same way as germplasm functions. This prevents a subset of cells from “turning somatic”, setting the germline apart from other cell types. Consequently, *Blimp1* null embryos lack PGC development (Ohinata et al., 2005). The RNA binding protein Lin 28 has been identified as a key regulator of PGC formation during development (West. J. A et al., 2009). RNA interference (RNAi) was used to screen candidate genes for effects of PGC development in *Stella* (also called *Dppa3*) expressing mES lines. *Blimp1* has the ability to rescue PGCs in Lin 28-deficient cells and therefore demonstrated its importance in PGC specification (West. J. A et al., 2009).

1.9 Developmental Constraint and Constraint Release

PGC specification by germplasm is correlated with greater species variation within a lineage than is possible through the induced method of PGC specification. This is because germplasm allows germ cells to function independently of signals from the soma, i.e. the somatic cells don't influence germ cells. This creates an environment where mutations and alterations to the somatic cells do not affect the transmission of heritable information through the germline. This is proposed as the reason for large variation and diversity seen in species that utilise the germplasm method of germ cell specification (Crother. B. I et al., 2007). It is more advantageous in terms of species diversity and variation to have a weak association between the germline and soma; this weak association is seen in germplasm specification of germ cells. Mammals and other organisms specifying germ cells through induction from pluripotent precursors have a much tighter connection between the germline and the soma. Any large mutations in the somatic cells has an influence on the germ cells and consequently the transmission of the heritable information through the germline (Crother. B. I et al., 2007). A two-step process has been proposed for identifying constraints and constraint release processes in macroevolution (Crother. B. I et al., 2007). Constraints are associated with primitive traits whilst constraint release was linked to derived traits (this proposal can be applied to the case of PGC speciation where the primitive trait, i.e. *Nanog*, is a constraint and the derived germplasm trait is the release).

CHAPTER 2: WORKING HYPOTHESIS

It has been proposed that the evolution of germplasm creates a permissive condition for the loss of *Nanog*. This has been proposed as a reason why *Nanog* was deleted from the frog genome (Dixon. J. E et al., 2010). The *Nanog* homeobox transcription factor is present in organisms that specify PGCs through epigenesis and proposed to be conserved through evolution. Mammals are thought to have evolved from a lineage of reptiles resembling the axolotl (salamander); as a result they are expected to share the same functional *Nanog* activity (Dixon. J. E et al., 2010).

Unpublished work by Dixon et al. has shown that the over-expression of mouse *Nanog* in mES cells serves to increase the number of PGCs seen in EB differentiation experiments. These results lead onto the intriguing question of whether the ability of *Nanog* in increasing PGC specification has been conserved in ancient *Nanog*. If ancient *Nanog* over-expression in increasing the induction of PGCs has been conserved, it would show that in fact *Nanog* genes function in the process of PGC specification and subsequently has been conserved through the main trunk of evolution.

CHAPTER 3: AIMS OF RESEARCH

Question:

"Is the ability of mouse *Nanog* over-expression to increase PGC specification conserved in ancient *Nanog* orthologs?"

Mouse *Nanog* over expression in mES cells has been shown to increase the level of PGC induction in EBs compared to the induction level seen EBs formed from control cell lines (James Dixon and Andrew Johnson, unpublished). The purpose of my work was to test whether the increase in PGC specification caused by over-expression of *Nanog* is a conserved trait, i.e. can ancient *Nanog* orthologs also induce an increase in PGC specification in EBs. To test this hypothesis mES cell lines over-expressing ancient *Nanog* orthologs will be tested for PGC specification.

Induction of PGCs will be measured firstly by quantitative RT-PCR for the germ cell markers *Dazl* and *Vasa*, whilst also monitoring the pluripotency and germline associated markers *Nanog* and *Oct-4* against a mouse β -*Actin* housekeeping gene control. Gene expression levels in the ancient *Nanog* over-expression EBs will be compared to the control levels found in cells containing an empty control vector. Western blotting will be used to confirm *Vasa* expression specifically.

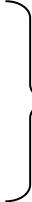
1. I produced pure populations of ancient *Nanog* ortholog over-expression mES lines utilising the pSIN vector and reaffirmed that mouse *Nanog* over-expression increased PGC specification (James Dixon and Andrew Johnson, unpublished)

2. I produced EBs from ancient *Nanog* over expression mES lines and test for PGC induction compared with rates observed in control pSIN-Empty produced EBs

CHAPTER 4: MATERIALS AND METHODS

4.1 Real Time PCR

TaqMan REAL Time PCR (RT-PCR) was carried out on template cDNA to assay target gene expression. Individual target gene master mixes were made up in a controlled sterile hood and were constituted of:

| | |
|---|--|
| <i>10μl TaqMan Gene Expression Master Mix</i> |  <i>per 20μl individual RT-PCR reaction</i> |
| <i>1μl Target Gene Primer + Probe Mix</i> | |
| <i>1μl Template cDNA</i> | |
| <i>8μl Nuclease Free dH₂O</i> | |

Template cDNA was added to master mixes on RT-PCR mixing plates (Thermo Scientific) before 20μl aliquots were transferred onto RT-PCR reading plates (Applied Biosystems). The RT-PCR reading plate were then sealed with optical adhesive film (Applied Biosystems), centrifuged to remove any air bubbles and to collect as the base of the tube followed by loading and analysis for forty cycles of amplification on an Applied Biosystems 7500 PCR System.. Triplicate assays were carried out for each sample to obtain an average expression level.

4.2 Transformation

Fifty microlitres of competent E. Coli 5-a cells (NEB) were thawed on ice and transferred into a sterile 1.5ml eppendorf tube. 1-2μl of plasmid DNA was then added to the competent cells and mixed by pipetting before incubation on ice for 30 minutes. The mixture was then heat shocked at 42°C for 42 seconds in a pre-heated water bath.

Next, the mixture was snap chilled on ice for 5 minutes. 200 μ l of SOC media was then added and mixed by pipetting, followed by incubation for one hour at 37°C in a shaker incubator to amplify transformed competent cells.

4.3 Ampicillin Plating of Transformed Competent Cells

Three hundred millilitres of solid Mu agar (low salt concentration) was heated in a microwave until melted and left to cool. 2.5g of ampicillin sodium salt (SIGMA) was then dissolved in a 25ml 50:50 ratio consisting of 12.5ml distilled water and 12.5ml of 100% ethanol to produce a working concentration. To the cooled Mu agar the ampicillin salt working solution was added to produce a final concentration of 100 μ g/ml. The Mu Agar was then poured into sterile petri dishes under flame and left to set overnight.

Bacterial cultures were plated (usually 10-20 μ l) with 40 μ l of 20mg/ml X-Gal, using a glass spreader that has been sterilised in ethanol. Incubation is at 37°C for 16 hours (overnight). X-Gal was used to screen for successfully transformed colonies.

4.4. P-GEMT Easy Cloning

The p-GEMT easy vector (Promega) was used to clone amplified PCR products with restriction enzyme sites on either end of the amplicon. Cloning of PCR amplified inserts into the pGEMT Easy vector along with control insert DNA was carried out as per the Promega's instructions.

| | <i>Standard</i> | <i>Control</i> |
|--|-----------------|-----------------|
| | <i>Reaction</i> | <i>Reaction</i> |
| <i>2X Rapid Ligation Buffer</i> | <i>5μl</i> | <i>5μl</i> |
| <i>pGEM-T Easy Vector (50ng)</i> | <i>1μl</i> | <i>1μl</i> |
| <i>PCR Insert</i> | <i>Xμl</i> | - |
| <i>Control DNA Insert</i> | - | <i>2μl</i> |
| <i>T4 DNA Ligase</i> | <i>1μl</i> | <i>1μl</i> |
| <i>Nuclease-Free Water to 10μl Total</i> | | |
| <i>Volume</i> | <i>10μl</i> | <i>10μl</i> |

(Adapted from Promega pGEMT Easy Technical Manual)

The ligation reaction was then carried out overnight at 16°C to obtain the highest ligation efficiency. An EcoRI or NotI digest was then used to release the PCR amplicon from the p-GEMT easy vector.

4.5 Plasmid DNA Extraction

QIAprep Spin DNA extractions were carried out to isolate plasmid DNA from bacterial cultures. Extraction was carried out per Qiagen's technical instructions. DNA adsorbed onto the spin column silica membrane and finally washing and elution DNA with either Elution Buffer (Qiagen) or TE buffer (10mM Tris-HCl pH 7.5; 0.05M EDTA) to prevent magnesium-dependant enzymatic cutting of extracted DNA.

4.6 RNA Extraction

RNA extraction from mES cells and EBs was carried out firstly by using QIAshredder (Qiagen) to lyse cells and remove cell debris. An RNeasy kit (Qiagen) was then used on the lysate to adsorb RNA onto the spin column membrane before carrying out an in-column DNase treatment (Promega) to remove any contaminating DNA by incubating at 37°C for one hour.

4.7 Nanodrop Quantification of RNA and DNA

Quantification of the DNA concentration was carried out by using the Nanodrop Spectrophotometer (ND-1000). For DNA quantification, the 260:280 ratio, an indication of sample purity, would be 1.8 for a pure sample. For RNA quantification, the 260:280 ratio was 2.0 for a sample with no contamination.

4.8 Template cDNA Synthesis

Complementary DNA (cDNA) template synthesis was then carried using Superscript III Reverse Transcriptase (Invitrogen) on each of the eight time point EB sample RNAs. The cDNA synthesis reaction used to create template DNA was:

RNase-free dH₂O

200µg Random Hexamer Primers

10 mM dNTP Mix (Invitrogen)

400ng extracted RNA

--65°C for 5 minutes---

5x First Strand Buffer (Invitrogen)

0.1M DTT (Invitrogen)

RNaseOut (Invitrogen)

Superscript III RT (Invitrogen)

--25°C for 5 minutes---

--50°C for 60 minutes---

--70°C for 15 minutes---

To check the integrity of the newly synthesised template cDNA, standard PCR was carried using RedTaq ready mix (Sigma) on the mouse β -*Actin* housekeeping gene. Successful cDNA synthesis was observed by a product band at 302bp after electrophoresis on a 1% agarose gel.

4.9 Agarose Gel Electrophoresis

A 1% agarose gel was made up by dissolving 1g of Ultra-Pure agarose (Invitrogen) in 100ml of a working stock solution of 1xTAE (Tris-acetate-EDTA) and heating in a microwave. To the dissolved agarose, 2 μ l of Ethidium Bromide (EtBr) was added. An appropriate sized molecular marker ladder was also used for molecular size determination. The gel running buffer concentration that used was 1x TAE.

For an RNA analysis gel, sample RNA and 6x loading buffer were heated to 75C for five minutes and then loaded onto a 1% agarose gel. RNA integrity was assessed by two bands representing the two RNA sub-units 28S and 18S; a respective 2:1 ratio in band intensity indicated intact RNA.

4.10 Gel Extraction

Gel extraction was carried out on DNA samples in a 1% agarose gel. A sharp blade was used to excise the band from the gel. The extracted band was then subject to purification with a QIAquick Gel Extraction Kit (Qiagen), following the technical manuals instructions. After extraction, 1 μ l of the purified DNA sample was run on a 1% agarose gel and then visualised for appropriate sized bands.

4.11 Total Protein Extraction

Total protein was extracted from EBs using RIPA buffer. The addition of SDS (Sodium Dodecyl Sulphate) and sodium deoxycholate to the RIPA buffer increases the denaturing properties. RIPA buffer was chosen for the fact that is particularly useful in the extraction of nuclear proteins; a factor attributable to the increased denaturing ability. Another advantage of the RIPA buffer when conducting Western blots is the fact that a preferable low background reading can be obtained. RIPA buffer consists of:

Tris 50mM

Sodium Chloride (NaCl) 150mM

Sodium Dodecyl Sulphate (SDS) 0.1%

Sodium Deoxycholate 0.5%

Triton X 100 or NP40 1%

PMSF Protease Inhibitor

EBs were first washed with 1x sterile PBS. 30 μ l of 1x RIPA buffer was added to samples and pipetted up and down repeatedly to lyse cells. Samples were then vortexed on full speed for 30 seconds to re-suspend and lyse any remaining cells. Samples were then centrifuged on full speed in a cold room for ten minutes to pellet and remove cell debris.

Supernatant containing total protein was then extracted and transferred to a clean and sterile eppendorf tube whilst the cell debris pellet was discarded.

Tissue sample protein extraction was carried out using a pre-sterilised pestle and mortar, grinding tissue samples in liquid nitrogen before the addition of 1x RIPA buffer. The protease inhibitors in the RIPA buffer stop endogenous protein digestion by protease enzymes of mechanical cellular breakdown. Centrifugation was carried out to separate the three phases: fat (top layer), desired protein (middle), cell debris pellet (bottom). The protein phase was collected and stored at -80°C.

4.12 BIO-RAD Protein Assay for Protein Quantification

The BIO-RAD Protein Assay is a modified Bradford Assay that gives a colorimetric measurement of protein concentration in a protein sample. The standard curve consisted of 2-14 μ g/ml BSA measured at 595nm absorbance on a spectrophotometer. To measure the concentration of the samples, 800 μ l of dH₂O was mixed with 1 μ l of extracted protein sample in an eppendorf tube. 200 μ l of BIO-RAD dye was added and mixed by inversion. The samples were measured against the standard curve to obtain a reading of the protein concentration (μ g/ μ l).

4.13 Western blot

An 8% Polyacrylamide gel was used to analyse protein from extracted EB time point samples. The 8% polyacrylamide gel and 5% stacking gel were made according to Maniatis Laboratory Manual Second Edition (Maniatis. T et al., 1989).

For 5ml 8% Polyacrylamide Gel:

Distilled water: 2.3ml

30% acrylamide mix: 1.3ml

1.5M Tris (pH 8.8): 1.3ml

10% SDS: 0.05ml

10% ammonium persulphate: 0.05ml

TEMED (Fisher Scientific) 0.006ml

For 2ml Stacking/Resolving Gel:

Distilled water: 1.4ml

30% acrylamide mix: 0.33ml

1.5M Tris (pH 6.8): 0.25ml

10% SDS: 0.02ml

10% ammonium persulphate: 0.02ml

TEMED (Fisher Scientific) 0.004ml

BIO-RAD gel casts, gel clamps and running docks were used to construct the gel.

After gel polymerisation, gel wells were washed with sterile, distilled water to remove excess acrylamide, before being transferred to a gel dock filled with 1x running buffer (30 g of Tris base; 144.0 g of glycine; 10 g of SDS). After protein quantification, preparation of protein samples was carried out on ice. 20 μ g of Protein was brought to a volume of 10 μ l with distilled water and mixed with 10 μ l of 1x loading dye. 100 μ l of stock 2x loading dye (0.1% SDS; 0.25M glycine; 25mM Tris-HVL pH 8.3) was mixed with 20 μ l 1M DTT to make a working 1x loading dye solution. The protein and loading dye was then boiled for 10 minutes at 95°C, and then centrifuged to collect evaporated sample. 20 μ l of sample was applied to the gel which was run at 100V for one hour.

Immunoblot PVDF membrane (BIO-RAD) was soaked in methanol for one minute then transferred to 1x transfer buffer (25% methanol; 24mM Tris-HCl; 153mM glycine) for ten minutes. The polyacrylamide gel was also soaked in 1x transfer buffer along with two sheets of blot paper (BIO-RAD). Components were then assembled as follows: blot paper, PVDF membrane, polyacrylamide gel and finally another sheet of blot paper.

Semi-dry gel transfer to PVDF membrane was carried out at 300mA for 30 minutes. Following transfer, the PVDF membrane was blocked by incubating it at 4°C overnight in 5% dehydrated low fat milk in 1x PBS (27nm Potassium Chloride, 14.7mM Potassium Phosphate monobasic, 1.38M Sodium Chloride, 80.6mM Sodium Phosphate dibasic (Invitrogen)) and 0.1% Tween-20. The PVDF membrane was then washed for three 10 minute cycles in 1x PBS and 0.1% Tween-20.

Primary antibody incubation was carried out overnight at 4°C in an appropriate dilution of antibody 5% low fat milk in 1x PBS and 0.1% Tween-20 in a sealed bag. The PVDF membrane was then washed for four 10 minute cycles in 1x PBS and 0.1% Tween-20. Secondary Horseradish Peroxidise (HRP) linked antibody incubation of the PVDF membrane was then carried out for two hours on a gentle agitation rocker. The PVDF membrane was then washed for five 10 minute cycles in 1x PBS and 0.1% Tween-20.

Exposure of the PVDF membrane was then carried out by first preparing ECL-detection reagent (GE Healthcare). This consisted of mixing a 40:1 ration of solution A to solution B respectively. ECL-detection solution was then applied onto the PVDF membrane and incubated for five minutes under aluminium foil cover to prevent light exposure. ECL-detection solution was then drained and the PVDF membrane transferred to a dark room.

Under red light, chemiluminescence film (GE Healthcare) was placed over the PVDF membrane and left to expose for an appropriate length of time.

Exposed chemiluminescence film was then processed automatically using an SRX-201 Xograph. Protein transferred PVDF membranes were stored in 1x PBS and 0.1% Tween-20 at 4°C. Stripping of probed PVDF membranes was carried out using 1x glycine stripping buffer (0.2M Glycine, 0.05% Tween-20, pH 2.5) for one hour at 60°C in a sealed plastic bag.

4.14 HEK 293T Cell Virus Production

Day 1:

Lentivirus production was carried out using the HEK 293T cell line. The HEK 293T cells were plated on 10cm petri dishes (Sterilin) at a density of 8x10⁶ six hours before transfection. Plating six hours before transfection was done to allow the HEK 293T cells to attach to the petri dish surface and distribute evenly.

Five hundred microlitres of serum-free DMEM/F12 (no serum/antibiotic) was pipetted into a 1.5ml eppendorf tube and add 30µl FuGene6 transfection reagent (Roche), taking care to avoid the FuGene6 coming into contact with the plastic sides of the tube. The transfection mixture was incubated at room temperature for 5 minutes flicking the tube to mix the transfection mixture. The appropriate concentrations of plasmid DNA were then added at the concentrations:

Backbone Vector DNA: 10µg

psPAX2 packaging DNA: 7.5µg

pMD2-G envelope DNA: 2.5µg

Incubate the transfection mixture with added plasmid DNA for fifteen minutes and mix by flicking to allow the FuGene6 and plasmid DNA to form a complex.

Finally, add the transfection mixture to 6ml of HEK 293T cell media and add to pre-plated HEK 293T cells overnight.

Day 2:

Change the media on each plate of HEK 293T cells from transfection media to 7ml of KSR-replacement mES media.

Day 3:

Passage a confluent wild-type mES T25 flasks in a 1:6 split from a onto three 0.1% gelatinised six well plates; one plate per transfected construct.

4.15 Lentiviral Transduction of mES cells

Day 4:

Collect the virus media from the HEK 293T cells (7ml from each 10cm dish) and filter with a 45 micron filter. To the 7ml of collected virus media, add 4 μ g/ml Polybrene. Add 1ml of virus media to each well of the 0.1% gelatinised six well plates containing mES cells plated previously.

Day 5:

After transduction of the mES cells overnight, replace with freshly collected virus media and leave for a second round of transduction.

Day 6:

Remove the second batch of virus media and replace with fresh KSR-replacement mES media prior to passaging the six well plates and scaling up to 0.1% gelatinised flasks to maintain stable cultured of transduced cells.

4.16 Embryoid Bodies

EBs were created using the “hanging drop” technique which relies on the force of gravity to create a ball of ES cells at the base of a hanging drop. To create the EBs, an 80% confluent T25 flask of mES cells was passaged with 0.25% Trypsin and then a cell count carried out to determine the volume of cell suspension was needed to create a defined number of EBs, consisting of 1000 mES cells per 20 μ l hanging drop. A multi-channel pipette was used to create approximately fifty equally spaced hanging drops on the lid of a 10cm petri dish. PBS was poured into the base of the petri dish to prevent the hanging drops from drying out while in the incubator at 37°C at 5% CO₂.

The hanging drops were left to form for three days until non-necrotic balls of mES cells could be seen in a stereomicroscope. EBs were then collected with a P200 Gilson pipette by looking down a stereomicroscope. After collection, EBs were cultured in EB media, in non-treated and non-attachment 60mm polystyrene dishes with the culture media changed every three days. Time point sample EBs were then washed twice with sterile cold 1x PBS and stored at -80°C.

CHAPTER 5: CELL LINES AND CULTURING

TECHNIQUES

5.1 CGR8 Wild Type mES Cell Line and Culture Media

The wild-type mES cell line that was used in this research was CGR8 (ECACC 07032901). The mouse CGR8 ES Cell line is characterised by the fact that it is feeder-free, i.e. it does not require fibroblast feeder cells to survive. The mES cells will be grown and cultured on 0.1% gelatinised flasks with media containing the addition of the cytokine leukaemia inhibitory factor (LIF) at 100ng/ml (Chemicon).

A high density of mES cells was maintained (1:3/1:4 dilution on passaging) when the cells are grown to optimise the cell cultures and to better mimic the *in vivo* cellular environment. The cells were checked daily for correct morphology to assay whether any differentiation had taken place. Mouse ES cells may take on neuronal-like morphology if differentiation has taken place. Cell positioning was also checked, as normal mES cells cluster and aggregate whereas unhealthy ES cells appear isolated.

Mouse ES cells need fresh medium every day after a 1:3 dilution and every other day after 1:4 dilution. This is to stop nutrient depletion and acidification. A low passage number of cells will be used for this study as genetic malformations accumulate with increasing passage and cell age, causing a loss in cell line quality. A common characteristic is the accumulation of an extra chromosome 12; the chromosome that the *Nanog* gene is located on. This would affect the results that were produced, meaning that a low passage cell culture is desired.

A freeze medium containing 80% DMEM/F12; 10% DMSO and 10% FCS is used when storing the mES cell lines.

CGR8 mES Media:

DMEM/F12 (Gibco)
15% Fetal Calf Serum (Sigma)
2mM Glutamine (Sigma)
1% Penicillin/Streptomycin (Sigma)
0.1mM Non-Essential Amino Acids (Sigma)
0.1mM (x1000) β-Mercaptomethanol (BME) (Sigma)
Leukaemia Inhibitory Factor 100ng/ml (Chemicon)

5.2 Embryoid Body Culturing Media

Embryoid Body Differentiation Media:

DMEM/F12 (Gibco)
15% KSR-Replacement (Gibco)
2mM Glutamine (Sigma)
1% Penicillin/Streptomycin (Sigma)
0.1mM Non-Essential Amino Acids (Sigma)

5.3 HEK 293T Cells and Culturing Media

The HEK 293T cells are stored in liquid nitrogen and then thawed to be grown in a T25 flask. At a 1:10 dilution, the T25 flasks will need passaging approximately every three days but the medium changed every day. Media changed consists of aspirating used medium and replacing with 15ml fresh HEK 293T culture medium. A freeze medium containing 80% DMEM/F12; 10% DMSO and 10% FCS is used when storing the HEK 293T cells.

HEK 293T Cell Media:

DMEM/F12 (Gibco)

10% Fetal Calf Serum (Sigma)

2mM Glutamine (Sigma)

1% Penicillin/Streptomycin (Sigma)

0.1mM Non-Essential Amino Acids (Sigma)

5.4 Transduced mES Cell Line and Culturing Media

Transduced mES cells were produced by the lentiviral transduction of wild type CGR8 cells. Like the wild-type CGR8 cell line, the transduced mES cells were grown and cultured on 0.1% coated gelatin flasks in the presence of KSR-replacement media supplemented with leukaemia inhibitory factor (LIF). A high density culture was maintained (1:3/1:4 dilution on passaging).

KSR-Replacement ES Cell Media:

DMEM/F12 (Gibco)

15% KSR-Replacement (Gibco)

2mM Glutamine (Sigma)

1% Penicillin/Streptomycin (Sigma)

0.1mM Non-Essential Amino Acids (Sigma)

0.1mM (x1000) β -Mercaptomethanol (BME)

Leukaemia Inhibitory Factor 100ng/ml (Chemicon)

pSIN Transduced KSR-Replacement ES Cell Media:

DMEM/F12 (Gibco)

15% KSR-Replacement (Gibco)

2mM Glutamine (Sigma)

1% Penicillin/Streptomycin (Sigma)

0.1mM Non-Essential Amino Acids (Sigma)

0.1mM (x1000) β -Mercaptomethanol (BME)

Leukaemia Inhibitory Factor 100ng/ml (Chemicon)

1 μ g/ml Puromycin

CHAPTER 6: TESTING CONSERVATION OF ANCIENT NANOG IN PGC SPECIFICATION

To address the question, “Is the ability of *Nanog* over-expression to increase PGC induction conserved in ancient *Nanogs*”, ancient *Nanog* over-expression cell lines were utilised. Previous work has shown that mouse *Nanog* over-expression in EBs increases the expression of PGC markers when analysed through RT-PCR (James Dixon and Andrew Johnson, unpublished). The aim of the study was to produce EBs via hanging drops from mES cell lines over-expressing *Nanog* ortholog from lower chordates and then compare gene expression levels to those seen in EBs produced from mES cells transduced with a control vector. The derivation of PGCs from EB intermediates has been achieved in various previous studies (Hubner et al., 2003), (Nayernia. K et al., 2006).

Mouse ES cell lines containing lower chordate *Nanog* over-expression trans-genes were obtained from Dr James Dixon and Dr Andrew Johnson. The ancient Nanog orthologs were isolated by firstly using the axolotl *Nanog* protein sequence for alignment with EST databases to isolate putative orthologs from lower chordate species. Their identity as true *Nanog* orthologs was confirmed elsewhere (Johnson and Dixon, unpublished).

6.1 pSIN-EF2-Nanog-Puromycin Over-Expression Vector

The ancient *Nanog* over-expression lines were created by Dr James Dixon (Institute of Genetics, University of Nottingham) by lentiviral transduction of wild type CGR8 mES cells with the pSIN-EF2-*Nanog*-Puromycin vector (Addgene plasmid #16578). Using the lentivirus pSIN vector allows for efficient, stable gene delivery and also expression in desired mES Cell lines, driven by the constitutive EF1- α promoter.

The ancient *Nanog* cDNA orthologs that were sub-cloned into the pSIN-vector and subsequently over-expressed in transduced mES cell lines. The ancient *Nanogs* used in this experiment were:

1. pSIN-Axolotl (*Ambystoma mexicanum*)
2. pSIN-Mouse (*Mus musculus*)
3. pSIN-Sturgeon (*Acipenser oxyrinchus*)
4. pSIN-Acorn Worm (*Saccoglossus kowalevskii*)
5. pSIN-Amphioxus V1 (*Branchiostoma floridae* Vent-1)
6. pSIN-Amphioxus V2 (*Branchiostoma floridae* Vent-2)

A control cell line was used to which gene expression and PGC induction could be compared. The control cell line was made by transducing mES cells with a pSIN- EF2-Puromycin vector lacking an insert (pSIN-Empty). Using this line as a control, any effect of lentiviral transduction and puromycin selection could be standardised.

pSIN-EF2-Nanog-Puro Plasmid Map:

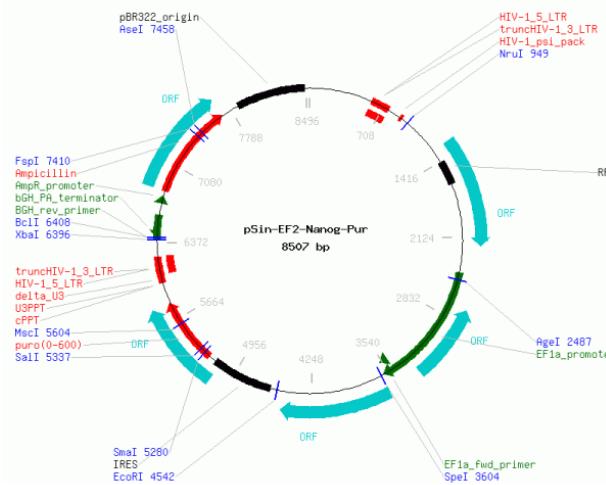


Figure 8: pSIN-EF2-Nanog-Puromycin construct. The pSIN construct (Addgene plasmid #16578) used in the *Nanog* over-expression experiments (Plasmid Map adapted from Addgene)

6.2 Ancient Nanog Nanodrop Quantification

After pSIN plasmids were transformed into competent E. Coli 5-α cells (NEB) and amplified, plasmid DNA was isolated from bacterial solutions by using a DNA isolation protocol (for full procedure see methods). Below is a table of the measurements obtained from the Nanodrop quantification of all six ancient *Nanogs*.

| Cell Line | ng/μl | 260:280 |
|-------------------|--------------|----------------|
| pSIN-Axolotl | 152.30 | 1.87 |
| pSIN-Mouse | 158.90 | 1.86 |
| pSIN-Sturgeon | 217.80 | 1.88 |
| pSIN-Acorn Worm | 115.70 | 1.90 |
| pSIN-Amphioxus V1 | 90.00 | 1.82 |
| pSIN-Amphioxus V2 | 87.40 | 1.81 |

Table 1: Ancient Nanog plasmid DNA quantification. Nanodrop quantification of the plasmid DNA of the six ancient *Nanog* over-expression vectors. The optimised purity ratio for DNA at 260:280 is 1.8; the readings obtained fall very close to this figure indicating that there is no or very little contamination of the ancient Nanog samples.

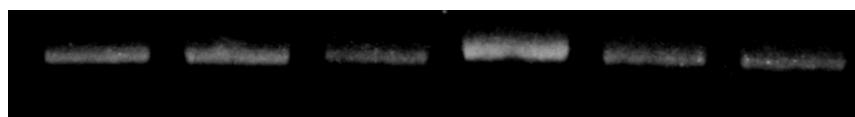


Figure 9: Ancient Nanog Gel Electrophoresis. 1% agarose gel electrophoresis of ancient Nanog plasmid DNA after Miniprep isolation (Qiagen). 1 μl of DNA was loaded into each well. Confirmation was bands at appropriate size of approximately 8496bp. 1: pSIN-Empty, Lane 2: pSIN-Axolotl, Lane 3: pSIN-Mouse, Lane 4: pSIN-Sturgeon, Lane 5: pSIN-Acorn Worm, Lane 6: pSIN-Amphioxus V1, Lane 7: pSIN-Amphioxus V2

Plasmid DNA was isolated for all of the six ancient *Nanog* over-expression vectors; positive conformation was by product bands at the appropriate molecular size of 8469bp on a 1% agarose gel, corresponding to the size of the pSIN vector and *Nanog* trans-gene insert.

The packaging plasmid, psPAX2 (Addgene plasmid #12260), along with the envelope plasmid, pMD2-G (Addgene plasmid #12259) are needed for functional lentiviral production in conjunction with the pSIN backbone vector.

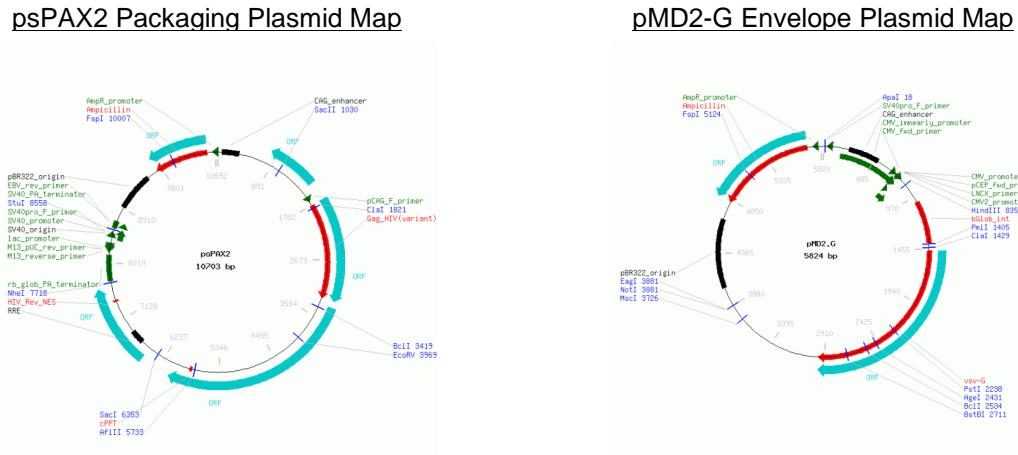


Figure 10 (left) and Figure 11 (right): Packaging and Envelope plasmids. psPAX2 packaging plasmid) and pMD2-G envelope plasmid were used for functional lentiviral production with the appropriate backbone vector in HEK 293T cells (Plasmid Maps adapted from Addgene)

6.3 Embryoid Body Time Point Collection

The transduced ancient *Nanog* over-expression mES lines were first puromycin selected to produce pure cell lines. Pure ancient *Nanog* mES lines were then used to make EBs through the hanging drop method, at one thousand mES cells per 20 μ l drop. Non-necrotic EBs were then isolated from hanging drops three days after formation (day 0), and plated in 60mm non-treated plates (sterilin). A differentiation media consisting of mES media without the addition of the cytokine LIF causes spontaneous EB differentiation. Differentiation was carried out for a period of ten days, changing the EB media every third day. EB time point samples were taken on days 5 and 10 after formation. These intervals were chosen as an early time point, before PGCs are specified, and a late time point, at which any increase in PGC specific markers could be quantified.

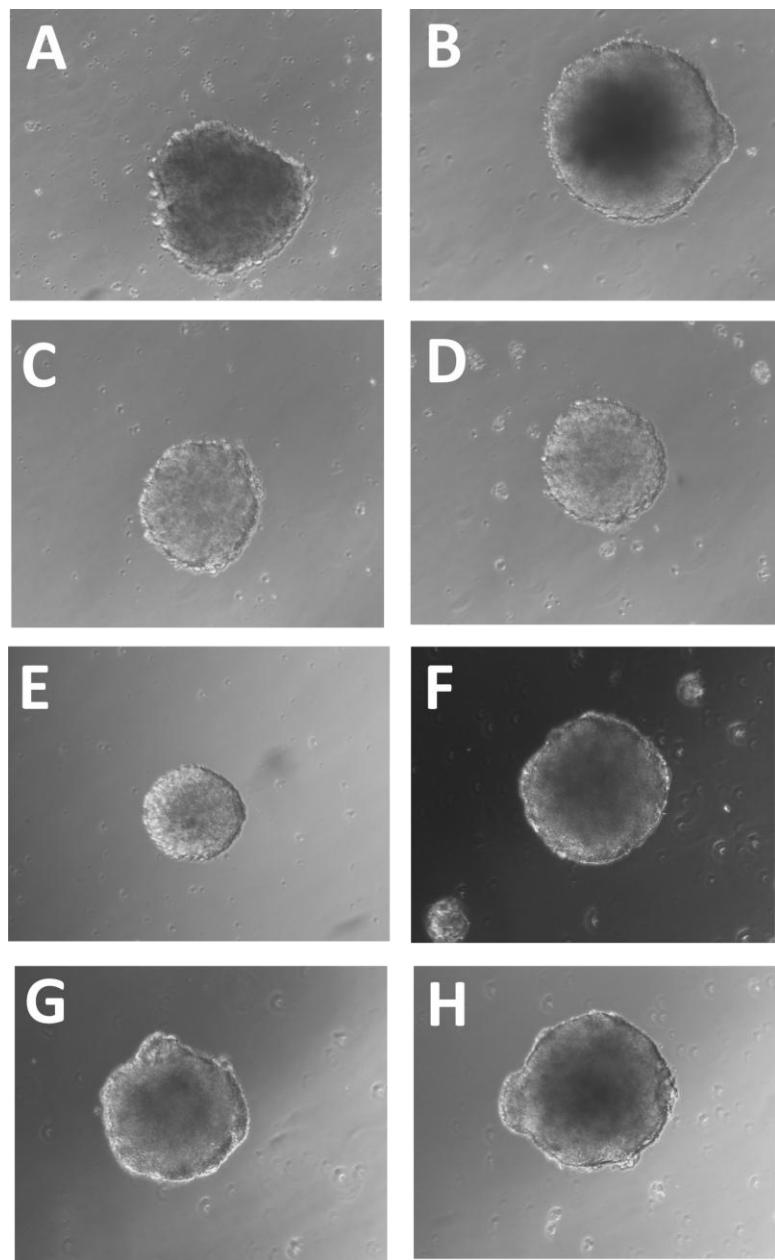


Figure 12: Control and Ancient *Nanog* over-expression Embryoid Bodies at day 0. Embryoid Bodies created from 1000 mES cells per 20 μ l hanging drops collected at day 0 and cultured in EB media (no LIF). A: CRG8 wild type; B: pSIN-Empty, C: pSIN-Axolotl; D: pSIN-Mouse; E: pSIN-Sturgeon; F: pSIN-Acorn Worm, G: Amphioxus V1; H: pSIN-Amphioxus V2

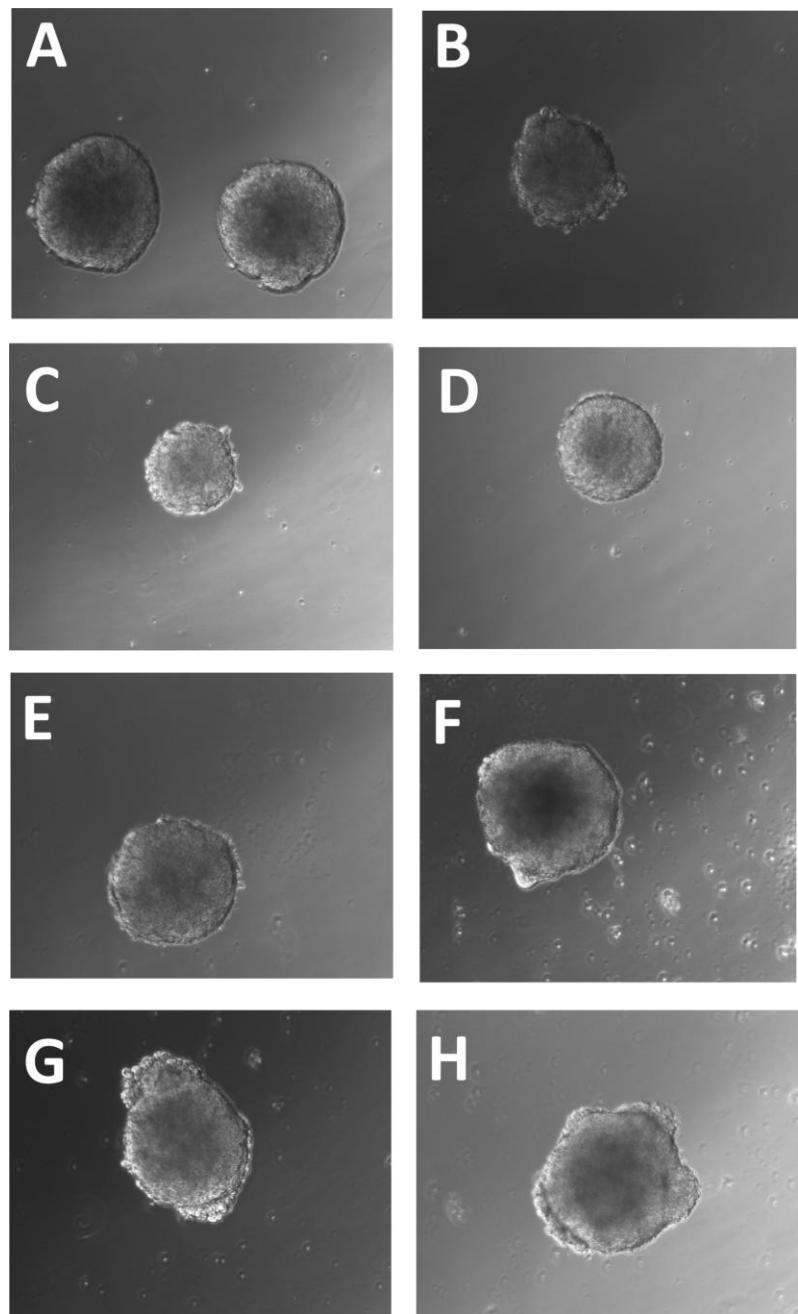


Figure 13: Control and Ancient *Nanog* over-expression Embryoid Bodies at day 5. Embryoid Bodies created from 1000 mES cells per 20 μ l hanging drops collected at day 5 and cultured in EB media (no LIF). A: CRG8 wild type; B: pSIN-Empty, C: pSIN-Axolotl; D: pSIN-Mouse; E: pSIN-Sturgeon; F: pSIN-Acorn Worm, G: Amphioxus V1; H: pSIN-Amphioxus V2

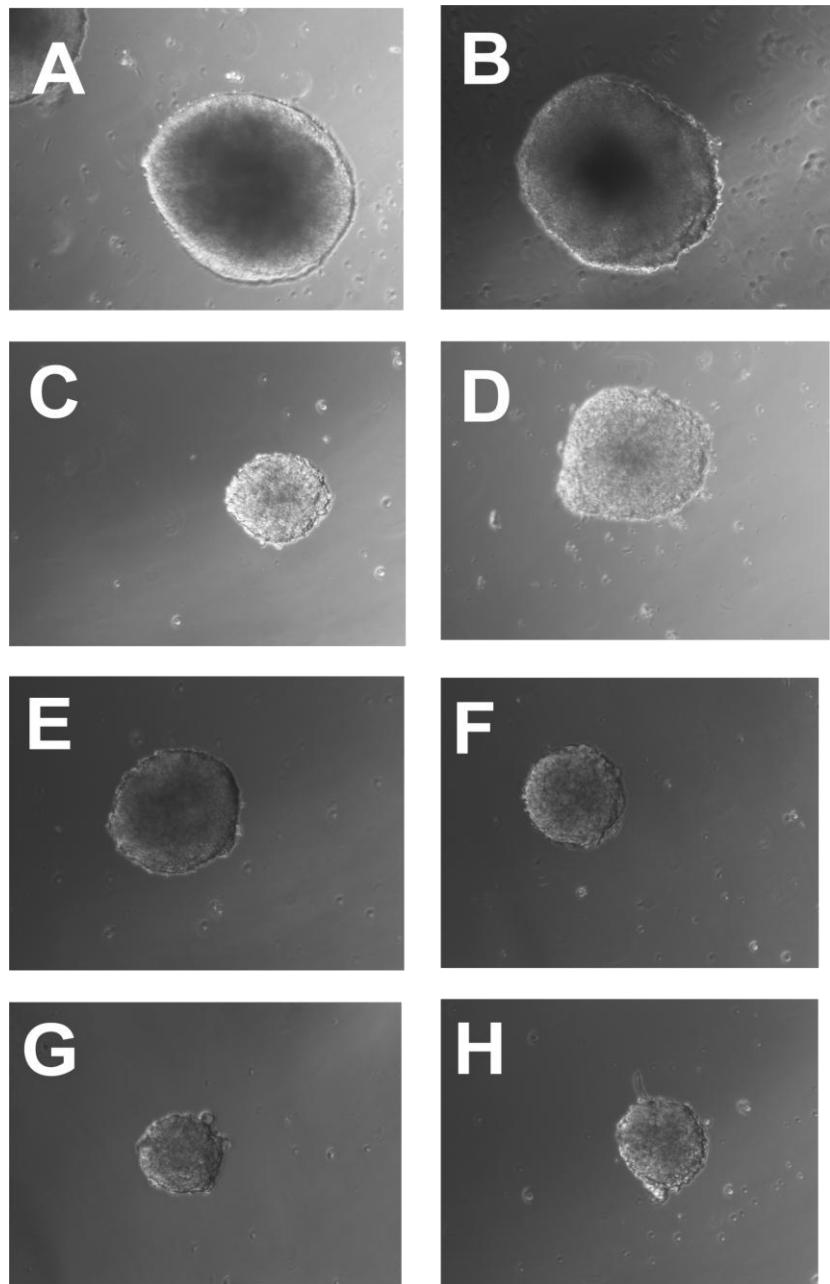


Figure 14: Control and Ancient *Nanog* over-expression Embryoid Bodies at day 10. Embryoid Bodies created from 1000 mES cells per 20 μ l hanging drops collected at day 10 and cultured in EB media (no LIF). A: CRG8 wild type; B: pSIN-Empty, C: pSIN-Axolotl; D: pSIN-Mouse; E: pSIN-Sturgeon; F: pSIN-Acorn Worm, G: Amphioxus V1; H: pSIN-Amphioxus V2

6.4 RNA Extraction and cDNA Synthesis from EB Time Points

RNA was extracted from EB time point samples by firstly using a QIAshredder (Qiagen) and then an RNeasy RNA Extraction Kit (Qiagen). Briefly, EBs were washed with sterile 1xPBS, lysed, homogenised using the QIAshredder (Qiagen) and then passed through the RNA isolation column to trap the RNA in a silica membrane. DNase treatment was then carried out on the RNA isolation column using DNasel (Ambion), to remove any remaining double stranded DNA contamination. RNA was then denatured and subsequently run on a 1% agarose gel to check the integrity, indicated by two distinct bands representing 28S and 18S ribosomal RNA at a 2:1 intensity ratio respectively. Isolated RNA was then stored at -80°C.

| <i>Cell Line</i> | <i>ng/µl</i> | <i>260:230</i> |
|-------------------------|---------------------|-----------------------|
| pSIN-Empty | 119.50 | 2.00 |
| pSIN-Axolotl | 114.6 | 2.01 |
| pSIN-Mouse | 99.00 | 2.02 |
| pSIN-Sturgeon | 123.70 | 2.04 |
| pSIN-Acorn Worm | 88.60 | 1.92 |
| pSIN-Amphioxus V1 | 111.70 | 2.06 |
| pSIN-Amphioxus V2 | 104.40 | 2.06 |

Table 2: Embryoid Body RNA quantification at day 5. After RNA was extracted from EBs at day 5, RNA, was quantified and checked for purity using a Nanodrop

| Cell Line | ng/μl | 260:230 |
|-------------------|-----------------------------|----------------|
| pSIN-Empty | 104.70 | 1.96 |
| pSIN-Axolotl | 125.20 | 1.96 |
| pSIN-Mouse | 93.40 | 1.93 |
| pSIN-Sturgeon | 112.10 | 1.95 |
| pSIN-Acorn Worm | 88.60 | 1.92 |
| pSIN-Amphioxus V1 | 90.20 | 1.92 |
| pSIN-Amphioxus V2 | 63.20 | 1.94 |

Table 3: Embryoid Body RNA quantification at day 10. After RNA was extracted from EBs at day 10, RNA, was quantified and checked for purity using a Nanodrop

After RNA was extracted, cDNA synthesis was then carried out on 400ng of RNA using Superscript III Reverse Transcriptase (Invitrogen). The integrity of the synthesised cDNA was checked by PCR amplification using mouse β -Actin primers with a positive and negative (no-RT) control. Successful cDNA synthesis was observed by a product band at 302bp after electrophoresis on a 1% agarose gel.

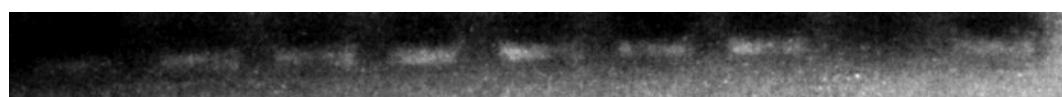


Figure 15: 1% agarose gel electrophoresis to check cDNA integrity. PCR amplification was carried out with mouse β -Actin primers. 0.5 μ l of cDNA synthesised from the RNA extracted from ancient Nanog over-expression EBs was loaded into each well and the appropriate band at 302bp visualised. Lane 1: pSIN-Empty, Lane 2: pSIN-Axolotl, Lane 3: pSIN-Mouse, Lane 4: pSIN-Sturgeon, Lane 5: pSIN-Acorn Worm, Lane 6: pSIN-Amphioxus V1, Lane 7: pSIN-Amphioxus V2, Lane 8: negative control (no-RT), Lane 9: positive control

6.5 RT-PCR Analysis of EB Time Point Samples

RT-PCR was carried out on the ancient *Nanog* over-expression cDNA for the pluripotency associated gene markers *Nanog* and *Oct-4*. The early PGC associated marker *Dazl* was assayed along with *Vasa*, a marker of mature migratory PGCs. A mouse β -*Actin* housekeeping control was used to normalise levels of gene expression. Gene expression assay kits consisting of primer and probe mixes were obtained from Applied Biosystems and RT-PCR carried out according to the manufacturer's instructions. Accession number for the Applied Biosystems primer and probe kits can be seen below:

Nanog, Mm02384862_g1

Oct-4, Mm00658129_gH

Actin, Mm02619580_g1

Dazl, Mm00515630_m1

Vasa, Mm00802445_m1

The Applied Biosystems TaqMan Gene Expression 7500 system was used to quantify levels of endogenous marker gene expression. To compare levels of gene expression, mouse β -*Actin* expression from the pSIN-Empty control cell line was used. Relative changes in gene expression, (RQ), were calculated for each individual gene marker relative to β -*Actin*. Consequently, β -*Actin* expression from the pSIN-Empty cell line was assigned an RQ value of 1.0. To analyse the RT-PCR data, Ct (cycle threshold) values of the genes of interest were compared to a normalised control gene in EB time point samples.

The threshold level was set to intersect a point at which the RT-PCR amplification is in the exponential phase. The amplification for each particular gene marker was analysed in triplicate, to obtain a mean Ct value.

If there was a clear outlying amplification value for a particular triplicate, the anomalous result was omitted and consequently a duplicate analysis was carried out.

The comparative Ct approach, or $2^{-\Delta\Delta Ct}$, was used to compare Ct values for each gene marker (Bookout and Mangelsdorf, 2003). The Ct value of the gene of interest was normalised to the Ct value of the control reference, that is, *β-Actin* expression from the pSIN-Empty control cell line. This produced a quantitative value for the fold change in relative gene expression, or ΔCt :

$$\Delta Ct = Ct(\text{sample}) - Ct(\text{reference})$$

$$\Delta\Delta Ct = \Delta Ct(\text{sample}) - \Delta Ct(\text{reference})$$

Positive and negative error bars for the relative gene expression (RQ) values were calculated from the standard deviation of the relative gene expression.

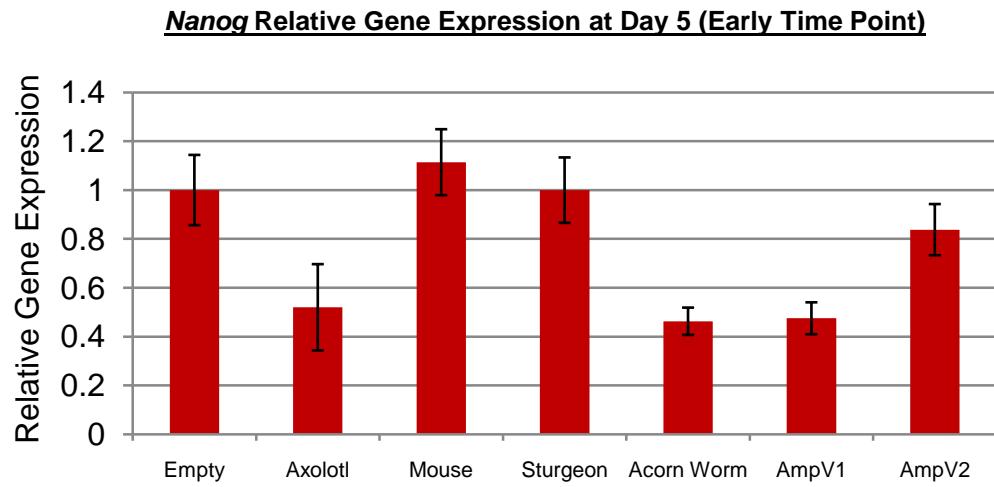


Figure 16A: Nanog gene expression at day 5. Nanog relative gene expression analysis (y axis) from EB time point samples at day 5 early time point. Relative gene expression was calculated using the 2^{-ΔΔCt} method. All expression levels are normalised to mouse β -Actin from the pSIN-Empty control cell line at day 5. The individual cell lines are indicated on the x-axis. Error bars were calculated from the standard deviation of the relative gene expression

| Cell Line | RQ Value | RQ Standard Deviation |
|-------------------|----------|-----------------------|
| pSIN-Empty | 1.00 | 0.504 |
| pSIN-Axolotl | 0.52 | 0.620 |
| pSIN-Mouse | 1.20 | 0.474 |
| pSIN-Sturgeon | 1.00 | 0.470 |
| pSIN-Acorn Worm | 0.46 | 0.196 |
| pSIN-Amphioxus V1 | 0.48 | 0.230 |
| pSIN-Amphioxus V2 | 0.80 | 0.368 |

Figure 16B: Nanog gene expression values and standard deviation RQ at day 5.

Data table of relative Nanog gene expression at day 5, showing relative gene expression (RQ) normalised to mouse β -Actin gene expression in the pSIN-Empty control at day 5

Oct-4 Relative Gene Expression at Day 5 (Early Time Point)

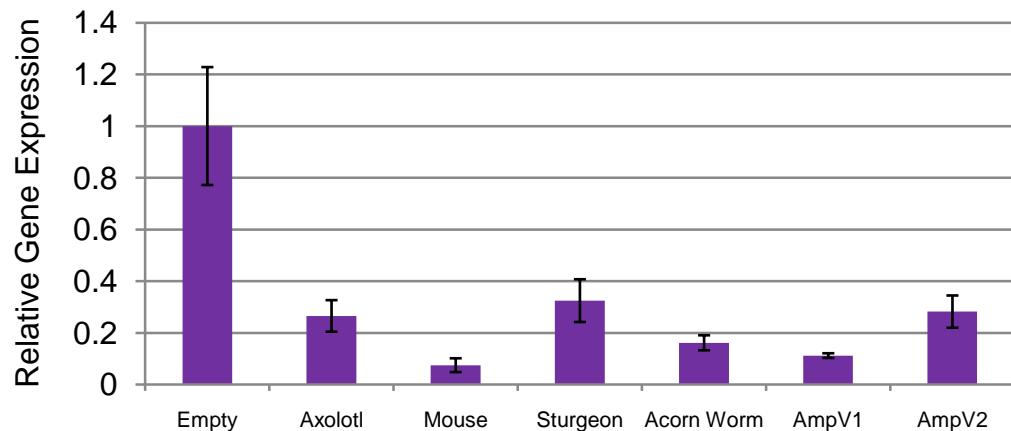


Figure 17A: Oct-4 gene expression at day 5. Oct-4 relative gene expression analysis (y axis) from EB time point samples at day 5 early time point. Relative gene expression was calculated using the 2 method. All expression levels are normalised to mouse β -Actin from the pSIN-Empty control cell line at day 5. The individual cell lines are indicated on the x-axis. Error bars were calculated from the standard deviation of the relative gene expression

| Cell Line | RQ Value | RQ Standard Deviation |
|-------------------|----------|-----------------------|
| pSIN-Empty | 1.00 | 0.228 |
| pSIN-Axolotl | 0.27 | 0.0609 |
| pSIN-Mouse | 0.075 | 0.0268 |
| pSIN-Sturgeon | 0.32 | 0.0827 |
| pSIN-Acorn Worm | 0.16 | 0.0290 |
| pSIN-Amphioxus V1 | 0.11 | 0.0091 |
| pSIN-Amphioxus V2 | 0.28 | 0.0827 |

Figure 17B: Oct-4 gene expression values and standard deviation RQ at day 5.

Data table of relative Oct-4 gene expression at day 5, showing relative gene expression (RQ) normalised to mouse β -Actin gene expression in the pSIN-Empty control at day 5

Dazl Relative Gene Expression at Day 5 (Early Time Point)

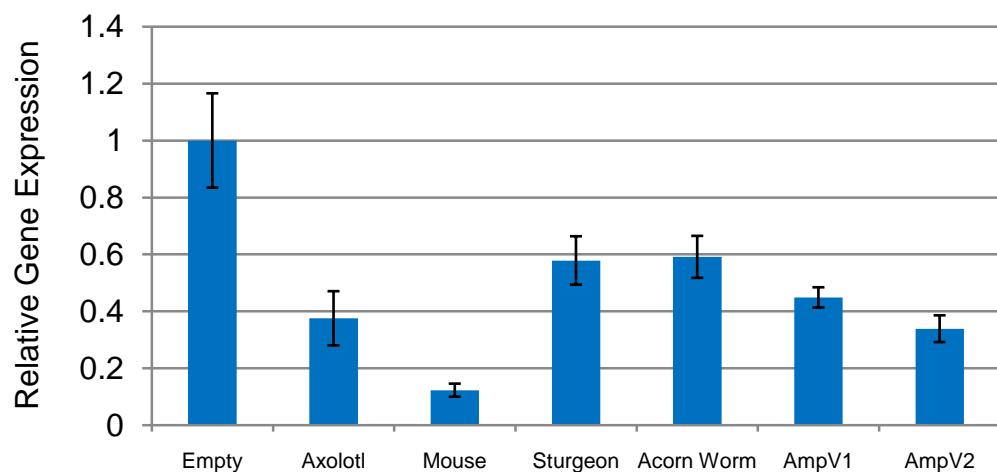


Figure 18A: Dazl gene expression at day 5. Dazl relative gene expression analysis (y axis) from EB time point samples at day 5 early time point. Relative gene expression was calculated using the 2^{-ΔΔCt} method. All expression levels are normalised to mouse β -Actin from the pSIN-Empty control cell line at day 5. The individual cell lines are indicated on the x-axis. Error bars were calculated from the standard deviation of the relative gene expression

| Cell Line | RQ Value | RQ Standard Deviation |
|-------------------|----------|-----------------------|
| pSIN-Empty | 1.00 | 0.166 |
| pSIN-Axolotl | 0.38 | 0.095 |
| pSIN-Mouse | 0.12 | 0.023 |
| pSIN-Sturgeon | 0.58 | 0.085 |
| pSIN-Acorn Worm | 0.59 | 0.074 |
| pSIN-Amphioxus V1 | 0.45 | 0.036 |
| pSIN-Amphioxus V2 | 0.34 | 0.047 |

Figure 18B: Dazl gene expression values and standard deviation RQ at day 5. Data table of relative Dazl gene expression at day 5, showing relative gene expression (RQ) normalised to mouse β -Actin gene expression in the pSIN-Empty control at day 5

From the RT-PCR data generated, it can be seen that *Nanog* expression at day 5 was slightly higher in the EBs produced from the pSIN-Mouse *Nanog* over-expression cell line, with an RQ value of 1.20 (RQ SD +/- 0.474) relative to the control. Gene expression levels of pSIN-Sturgeon EBs showed very similar levels of *Nanog* gene expression with an RQ value of 1.00 (RQ SD +/- 0.470). *Nanog* was seen to have reduced levels of expression in the Amphioxus V2 produced EBs with an RQ value calculated at 0.80 (RQ SD +/- 0.368). Levels of *Nanog* expression were found to be at a lower level but fairly consistent in the other EBs collected at day 5 produced from pSIN-Axolotl, pSIN-Amphioxus V1 and pSIN-Acorn Worm, with a normalised RQ values of 0.52 (SD +/- 0.620), 0.48 (RQ SD +/- 0.230) and 0.46 (RQ SD +/- 0.196). In all cases *Nanog* levels in the test cell lines was within two fold of those in controls.

Oct-4 gene expression levels were found to be highest in the control EBs, RQ 1.00 (RQ SD +/- 0.228). Reduced levels of *Oct-4* expression were found in the pSIN-Sturgeon, pSIN-Amphioxus V2 and pSIN-Axolotl produced EBs, with respective RQ values calculated at 0.32 (RQ SD +/- 0.0827), 0.28 (RQ SD +/- 0.0623) and 0.27 (RQ SD +/- 0.0609). The pSIN-Acorn Worm EBs exhibited relative expression levels of 0.16 RQ (RQ SD +/- 0.0290). The lowest levels of *Oct-4* gene expression was seen in the pSIN-Amphioxus V1 and pSIN-Mouse produced EBs, with relative RQ values of 0.11 (RQ SD +/- 0.0091) and 0.075 (RQ SD +/- 0.0268) respectively.

Analysis of EBs collected at the early time point, showed that the levels of *Dazl* gene expression were highest in the control EBs, with an RQ value at 1.00 (RQ SD +/- 0.166). Values obtained for pSIN-Acorn Worm and pSIN-Sturgeon display similar levels of normalised *Dazl* gene expression at a RQ value of 0.59 (RQ SD +/- 0.074) and 0.58 (RQ SD +/- 0.085) respectively. Relative expression levels were found to be similar in EBs collected for pSIN-Amphioxus V1, pSIN-Axolotl, and pSIN-Amphioxus V2 with RQ values of 0.45 (RQ SD +/- 0.036), 0.38 (RQ SD +/- 0.095) and 0.34 (RQ SD +/- 0.047). The lowest levels of expression were seen in EBs formed from the pSIN-Mouse *Nanog* over-expression cell line with a normalised *Dazl* expression level of 0.12 (RQ SD +/- 0.023).

Nanog Relative Gene Expression at Day 10 (Late Time point)

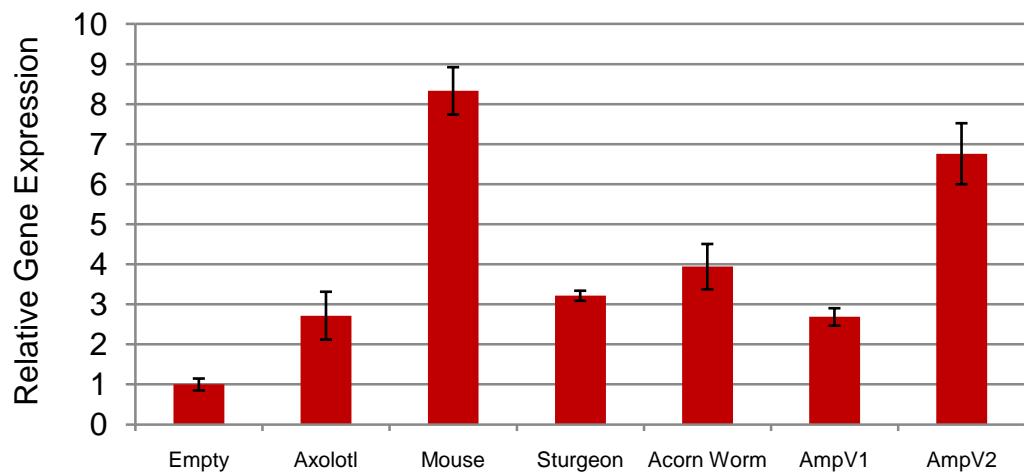


Figure 19A: Nanog gene expression at day 10. Nanog relative gene expression analysis (y axis) from EB time point samples at day 10 early time point. Relative Gene Expression was calculated using the 2^{-ΔΔCt} method. All expression levels are normalised to mouse β -Actin from the pSIN-Empty control cell line at day 10. The individual cell lines are indicated on the x-axis. Error bars were calculated from the standard deviation of the relative gene expression

| Cell Line | RQ Value | RQ Standard Deviation |
|-------------------|----------|-----------------------|
| pSIN-Empty | 1.00 | 0.148 |
| pSIN-Axolotl | 2.72 | 0.598 |
| pSIN-Mouse | 8.33 | 0.591 |
| pSIN-Sturgeon | 3.22 | 0.126 |
| pSIN-Acorn Worm | 3.94 | 0.567 |
| pSIN-Amphioxus V1 | 2.69 | 0.217 |
| pSIN-Amphioxus V2 | 6.76 | 0.762 |

Figure 19B: Nanog gene expression values and standard deviation RQ at day 10. Data table of relative Nanog gene expression at day 10, showing relative gene expression (RQ) normalised to mouse β -Actin gene expression in the pSIN-Empty control at day 10

Oct-4 Relative Gene Expression at Day 10 (Late Time point)

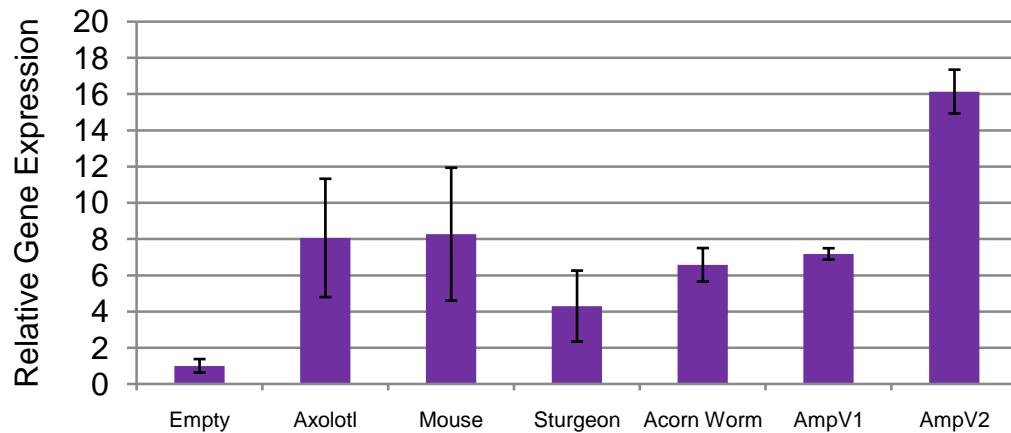


Figure 20A: Oct-4 gene expression at day 10. Oct-4 relative gene expression analysis (y axis) from EB time point samples at day 5 early time point. Relative gene expression was calculated using the 2^{-ΔΔCt} method. All expression levels are normalised to mouse β -Actin from the pSIN-Empty control cell line at day 10. The individual cell lines are indicated on the x-axis. Error bars were calculated from the standard deviation of the relative gene expression

| Cell Line | RQ Value | RQ Standard Deviation |
|-------------------|----------|-----------------------|
| pSIN-Empty | 1.00 | 0.373 |
| pSIN-Axolotl | 8.06 | 3.264 |
| pSIN-Mouse | 8.26 | 3.665 |
| pSIN-Sturgeon | 4.30 | 1.960 |
| pSIN-Acorn Worm | 6.58 | 0.923 |
| pSIN-Amphioxus V1 | 7.17 | 0.315 |
| pSIN-Amphioxus V2 | 16.20 | 1.210 |

Figure 20B: Nanog gene expression values and standard deviation RQ at day 10. Data table of relative Oct-4 gene expression at day 10, showing relative gene expression (RQ) normalised to mouse β -Actin gene expression in the pSIN-Empty control at day 10

Dazl Relative Gene Expression Levels at Day 10 (Late Time Point)

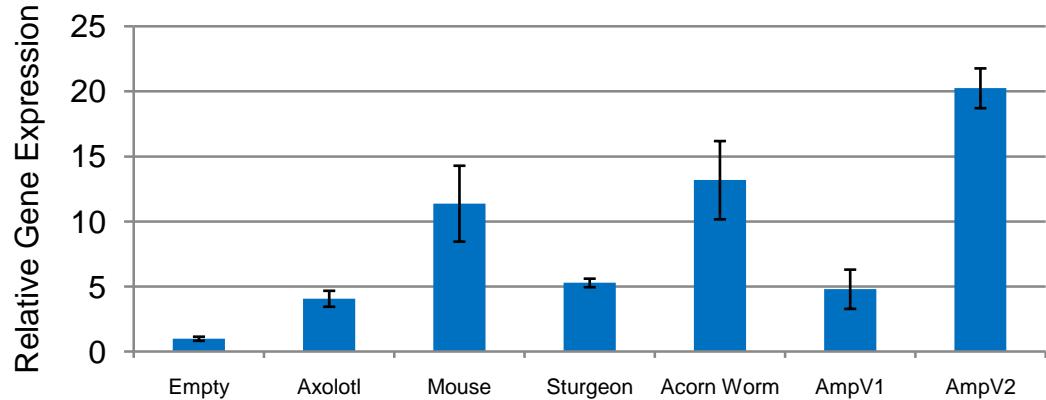


Figure 21A: Dazl gene expression at day 10. Dazl relative gene expression analysis (y axis) from EB time point samples at day 5 early time point. Relative gene expression was calculated using the 2^{-ΔΔCt} method. All expression levels are normalised to mouse β -Actin from the pSIN-Empty control cell line at day 10. The individual cell lines are indicated on the x-axis. Error bars were calculated from the standard deviation of the relative gene expression

| Cell Line | RQ Value | RQ Standard Deviation |
|-------------------|----------|-----------------------|
| pSIN-Empty | 1.00 | 0.157 |
| pSIN-Axolotl | 4.07 | 0.613 |
| pSIN-Mouse | 11.38 | 2.917 |
| pSIN-Sturgeon | 5.29 | 0.329 |
| pSIN-Acorn Worm | 13.19 | 3.006 |
| pSIN-Amphioxus V1 | 4.81 | 1.509 |
| pSIN-Amphioxus V2 | 20.25 | 1.529 |

Figure 21B: Dazl gene expression values and standard deviation RQ at day 10.

Data table of relative Dazl gene expression at day 10, showing relative gene expression, normalised to mouse β -Actin gene expression in the pSIN-Empty control at day 10

Vasa Relative Gene Expression at Day 10 (Late Time point)

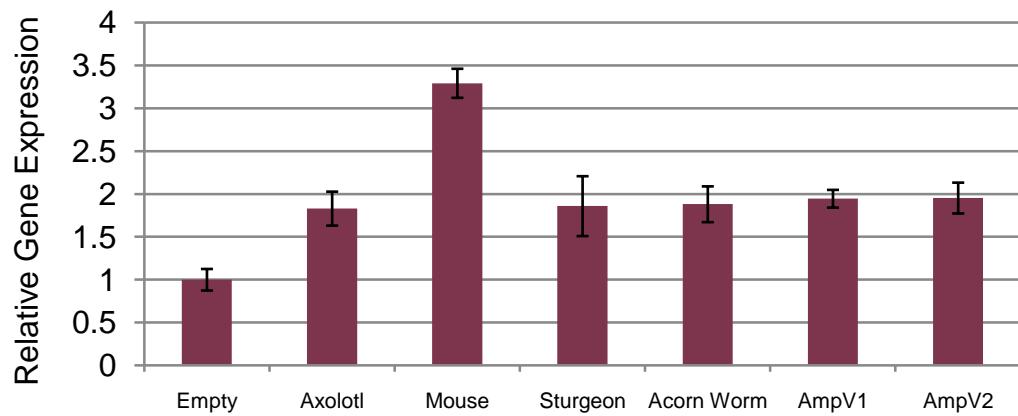


Figure 22A: Vasa gene expression at day 10. Vasa relative gene expression analysis (y axis) from EB time point samples at day 5 early time point. Relative Gene Expression was calculated using the 2^{-ΔΔCt} method. All expression levels are normalised to mouse β -Actin from the pSIN-Empty control cell line at day 10. The individual cell lines are indicated on the x-axis. Error bars were calculated from the standard deviation of the relative gene expression

| Cell Line | RQ Value | RQ Standard Deviation |
|-------------------|----------|-----------------------|
| pSIN-Empty | 1.00 | 0.126 |
| pSIN-Axolotl | 1.83 | 0.198 |
| pSIN-Mouse | 3.29 | 0.169 |
| pSIN-Sturgeon | 1.86 | 0.349 |
| pSIN-Acorn Worm | 1.88 | 0.209 |
| pSIN-Amphioxus V1 | 1.94 | 0.103 |
| pSIN-Amphioxus V2 | 1.95 | 0.180 |

Figure 22B: Vasa gene expression values and standard deviation RQ at day 10.

Data table of Relative Vasa gene expression at day 10, showing relative gene expression (RQ), normalised to mouse β -Actin gene expression in the pSIN-Empty control at day 10

When *Nanog* gene expression levels were analysed at late stage day 10 time point, the normalised pSIN-Empty cell line exhibited the lowest levels of expression with a baseline RQ value of 1.00 (RQ SD +/- 0.148). On a whole, the overall trend of the normalised *Nanog* gene expression levels was observed to increase in all of the ancient pSIN-*Nanog* cell lines. The pSIN-Mouse cell line was seen to show the highest levels of normalised *Nanog* gene expression with an RQ value of 8.33 (RQ SD +/- 0.591). This was then followed by the quantitative pSIN-Amphioxus V2 *Nanog* gene expression level, with a normalised RQ value of 6.76 (RQ SD +/- 0.762). Expression levels for EBs produced from pSIN-Acorn Worm, pSIN-Sturgeon, pSIN-Amphioxus V1 and pSIN-Axolotl were seen to have comparable levels of normalised *Nanog* gene expression with RQ values of 3.94 (RQ SD +/- 0.567), 3.22 (RQ SD +/- 0.126), 2.69 (RQ SD +/- 0.217) and 2.72 (RQ SD +/- 0.598) respectively.

The lowest levels of *Oct-4* expression in the EBs collected at the late day 10 time point sample were seen to be in the normalised pSIN-Empty control, RQ 1.00 (RQ SD +/- 0.373). Up-regulation in *Oct-4* gene expression in the EBs produced from the cell lines pSIN-Axolotl, pSIN-Mouse and pSIN-Sturgeon, with RQ values calculated to be 8.06 (RQ SD +/- 3.264), 8.26 (RQ SD +/- 3.665 and 4.30 (RQ SD +/- 1.960). Though there was an up-regulation in the expression of *Oct-4*, error bars calculated from the standard deviation of RQ for these particular cell lines were found to be large making accurate quantification of expression levels difficult. The standard error of the RQ is due to the variance of the technical triplicate repeats from the mean Ct value. Relative expression values for pSIN-Amphioxus V1 and pSIN-Acorn Worm were seen to be up-regulated by similar levels, RQ 7.17 (RQ SD +/- 0.315) and 6.58 (RQ SD +/- 0.923) respectively. Robust *Oct-4* gene expression levels were seen in the pSIN-Amphioxus V2 produced EBs. These were by far the highest levels exhibited by the ancient *Nanog* over-expression cell lines, with a relative RQ value calculated at 16.20 (RQ SD +/- 1.210).

As with the relative levels of *Nanog* gene expression levels seen at the day 10 time point samples, the pSIN-Empty cell line produced EBs exhibited the lowest levels of *Dazl* expression when analysed through RT-PCR quantification, with an RQ value of 1.00 (RQ SD +/-0.157). *Dazl* gene expression levels were seen to be significantly increased in the pSIN-Amphioxus V2 ancient *Nanog* over-expression EBs, when compared to the normalised mouse Actin gene expression levels in the pSIN-empty control. The pSIN-Amphioxus V2 EBs showed an RQ value of 20.25 (RQ SD +/- 1.529), a robust *Dazl* gene expression increase. There was also a robust increase in *Dazl* gene expression in both the pSIN-Acorn Worm and pSIN-Mouse produced EBs, with an RQ readout values of 13.19 (RQ SD +/-3.001) and 11.38 (SD+/- 2.917) respectively. Similar levels of *Dazl* expression were seen for both the pSIN-Sturgeon, pSIN-Amphioxus V1 and pSIN-Axolotl over-expression EBs, with relative RQ values of 5.29 (RQ SD +/-0.329), 4.81 (RQ SD +/-1.507) and 4.07 (RQ SD +/-0.617) respectively.

Levels of *Vasa* gene expression were found to be up-regulated in all of the ancient *Nanog* over-expression EBs, compared to the normalised mouse β -*Actin* gene expression of the pSIN-Empty cell line, RQ 1.00 (RQ SD +/- 0.126). It was observed that the highest levels of *Vasa* expression was in the pSIN-Mouse produced EBs, with a relative RQ value of 3.29 (RQ SD +/- 0.169). Levels of *Vasa* gene expression levels were found to present at comparable levels in the other ancient *Nanog* over-expression EBs with RQ values calculated for pSIN-Axolotl, pSIN-Sturgeon, pSIN-Acorn Worm, pSIN-Amphioxus V1 and pSIN-Amphioxus V2 at 1.83 (RQ SD +/- 0.198), 1.86 (RQ SD +/- 0.349), 1.88 (RQ SD +/- 0.209), 1.94 (RQ SD +/- 0.103) and 1.95(RQ SD +/- 0.180) respectively.

6.6 Protein Extraction and Western blot Analysis

I extracted total protein from EBs collected at the final day 10 time point, which was used to assess target protein levels. Protein levels were measured by Western blot analysis. Total protein was isolated from EB time point samples by using RIPA buffer to lyse EBs. Briefly, 30 μ l of 1x RIPA buffer was then added to each sample to lyse cells, followed by vortexing and centrifugation to remove cellular debris. Protein samples were then measured using a BIO-RAD protein assay against a negative reference in a light spectrophotometer at 595nm.

| <i>Cell Line</i> | $\mu\text{g}/\mu\text{l at } 595\text{nm}$ |
|--------------------|--|
| CGR8 W/T | 9.00 |
| pSIN-Empty | 6.80 |
| pSIN-Axolotl | 13.90 |
| pSIN-Mouse | 15.10 |
| pSIN-Sturgeon | 12.40 |
| pSIN-Acorn Worm | 13.70 |
| pSIN-Amphioxus V1 | 16.20 |
| pSIN-Amphioxus V2 | 12.80 |
| Mouse Testis (+ve) | 50.00 |
| Mouse Spleen (-ve) | 22.10 |

Table 4: Embryoid Body total protein quantification at day 10. BIO-RAD protein quantification analysis for EBs collected at the late Day 10 time point. The table includes the protein concentration for positive control (mouse testis) as well as negative control (mouse spleen). Extracted protein was quantified by the measuring against a standard curve at 595nm in a spectrophotometer

A Western blot was carried out to analyse the pSIN-mouse *Nanog* over-expression trans-gene. The transgene was tagged with a C-terminal Myc tag, which could be utilised to detect Nanog protein levels in EBs formed from transduced mES cells. 20 μ g of sample protein were loaded and ran against a rainbow molecular of known weight marker for size determination. Nanog protein is around 34kDa in size.

The Primary Myc-antibodies were obtained from Abcam (Ab #9106) and diluted according to manufacturer's instructions at 1:3000 in 5% dehydrated low fat milk in 1xPBS and 0.1% Tween-20. Horseradish Peroxidise (HRP) conjugated anti-rabbit secondary antibodies (Cell Signalling #7076) and diluted according to manufacturer's instructions at 1:3000 in 5% dehydrated low fat milk in 1x PBS and 0.1% Tween-20.

To compare over-expression levels of the mouse *Nanog* transgene, wild type CGR8 and pSIN-Empty control EBs were also probed. If the mouse *Nanog* transgene is functioning correctly, a significantly more intense band should be seen at the appropriate size when visualised compared to the control pSIN-Empty and wild type CGR8 cell lines, which have no transgene Myc-tag.

Anti-Myc Western blot:



Figure 23: Anti-Myc Western blot for *Nanog* transgene over-expression. Anti-Myc Western blot carried out with anti-Myc primary antibodies along with secondary anti-rabbit HRP-conjugated antibodies exposed for five minutes. Far left: protein isolated from EBs CGR8 wild-type, middle: protein isolated from pSIN-Empty EBs, Far Right: protein isolated from pSIN-Mouse over-expression EBs

The Western blot shows that significant Myc expression protein from pSIN-Mouse over-expressing EBs. Comparison with protein from control lines confirms over-expression of the mouse *Nanog* transgene.

The Vasa homolog *MvhI* (*DDX4*) is specifically expressed in germ cells and as a result, the mouse Vasa will be used as a marker of later stage PGC specification in EB intermediates. Primary anti-*MvhI* antibodies were obtained from Abcam (Ab #13840) and used in conjunction with secondary anti-rabbit antibodies to probe for *MvhI* in late stage (day 10 EB) time point samples. As a positive control to test the anti-*MvhI* antibodies, both mouse and mat ovaries were obtained from sacrificed female CD1 strain. A negative control consisting of mouse spleen was also used to assess correct antibody specificity. Whole tissue sample preparation was carried out before total protein was extracted using mechanical grinding combined with RIPA buffer, to solubilise proteins. 20µg of sample protein were loaded into each lane.

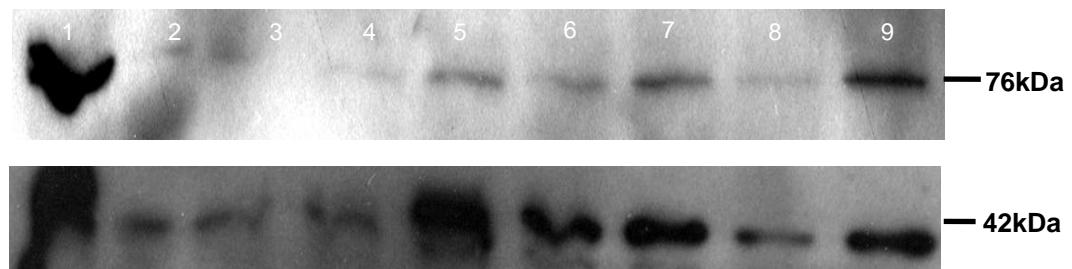


Figure 24 (Above): Ancient Nanog Embryoid Body anti-Vasa Western blot. Anti-Vasa (mouse *MvhI*) Western blot carried out with anti-Vasa primary antibodies along with secondary anti-rabbit HRP-conjugated antibodies to probe protein isolated from late time point day 10 EBs (exposed for five minutes). Lane 1: mouse testis (positive control), Lane 2: mouse spleen (negative control), Lane 3: pSIN-Empty, Lane 4: pSIN-Axolotl, Lane 5: pSIN-Mouse, Lane 6: pSIN-Sturgeon, Lane 7: pSIN-Acorn Worm, Lane 8: pSIN-Amphioxus V1, Lane 9: pSIN-Amphioxus V2. **Figure 25 (Below): MAPK protein loading control.** MAPK loading control using anti-ERK2 primary antibodies along with secondary anti-rabbit HRP-conjugated antibodies. Protein loaded in corresponding lanes

The Western blot shows an intense band at the appropriate molecular weight of 76kDa for mouse Vasa in the positive control (lane 1) and no band visible for the negative control (lanes 2 and 3). There can be bands present. MAPK protein loading control was then carried out to confirm equivalent protein loading using primary ERK-2 antibodies (C-14 Santa Cruz Biotechnologies), diluted according to manufacturer's instructions at 1:2000 in 5% dehydrated low fat milk in 1xPBS and 0.1% Tween-20. Secondary anti-rabbit HRP-conjugated antibodies were then used (Cell Signalling #7076) and diluted according to manufacturer's instructions at 1:3000 in 5% dehydrated low fat milk in 1xPBS and 0.1% Tween-20. Appropriate bands at 42kDa were observed in corresponding lanes.

There was a problem identified whilst carrying out the MAPK loading control on the anti-Vasa Western blot, which resulted in the variance seen in the band intensities. However, due to time restrictions on the project completion, it was not possible to carry out a repeat loading control.

CHAPTER 7: DISCUSSION

7.1 Ancient Nanog Over-expression Enhances PGC Specification

Nanog has been shown to play a key role in the regulation of ES cells and the pluripotent state (Chambers. I et al., 2003a), (Mitsui. K et al., 2003b), (Silva. J et al., 2006),(Silva. J et al., 2009), whilst also being expressed and required for germ cell maturation (Chambers. I et al., 2007). In regards to the foundation work which gave rise to this project, it was reaffirmed in this study that mouse *Nanog* over-expression enhanced the process of PGC specification. This was shown by the increased PGC associated gene marker expression, corroborating with work that was carried out by James Dixon and Andrew Johnson (unpublished). From the RT-PCR gene expression data generated for the ancient *Nanog* over-expression EB time point samples, there is a strong suggestive trend in the data.

In all, gene expression levels in the ancient *Nanog* over-expression EBs were found to display slightly lower levels of expression, however, on a comparable scale with expression levels of the pSIN-Empty control population of EBs. Firstly, at the early EB collection time point, at day five, gene markers for the pluripotency associated factors *Nanog* and *Oct-4* are seen to be at low levels, on a comparable scale to the expression levels seen in the control pSIN-Empty produced EBs. The same trend in results can be seen for the early PGC associated marker *Dazl* when assayed through quantitative RT-PCR at the early stage day 5 time point. These results lead to the conclusion that in the early time point collected EBs, marker gene expression was indicative of the fact that PGCs were yet to be specified.

Another possible explanation to the results obtained for the early time point is the fact that both *Nanog* and *Oct-4* are both expressed in pluripotent cells, as well as being expressed in the germline cells. Remaining pluripotent cells may still be present in the differentiating EBs at the early day 5 time point.

As the time course of EB differentiation progressed to the day 10 time point, it can be clearly seen that target gene expression for both *Nanog* and *Oct-4* assayed by RT-PCR is up-regulated in all of the ancient *Nanog* over-expression cell lines. Western blot analysis probing for the Myc tag on the C-terminal domain of the *Nanog* over-expression transgene confirmed the over-expression in the pSIN-Mouse EBs. It was assumed that the other ancient *Nanogs* were also over-expressed in EBs to the same extent; however, it could not be confirmed as they contained no C-terminal Myc tag. The use of an anti-*Nanog* antibody used in Western blot analysis could be used in further work to confirm the elevated *Nanog* levels in the ancient *Nanog* EBs. Up-regulated *Dazl* and *Vasa* expression relative to the control, suggests PGC induction by the 10 day collection point. Most importantly, the process of PGC specification was apparently enhanced by ancient *Nanog* over-expression. Further, at this late stage of differentiation pluripotent cells would not be expected to be retained in the EBs. Control EBs carrying the pSIN-Empty vector showed the lowest levels of *Dazl* and *Vasa* gene expression at day 10.

The ancient *Nanog* genes showed varying abilities to augment, or induce, PGC specification. It was shown that the axolotl, sturgeon and amphioxus *Nanogs* exhibited the weakest enhancement of PGC specification. There was between a 2.7 (Amphioxus V1) and 8.3 (Mouse) fold relative increase in *Nanog* gene expression observed over the control population of EBs.

In comparison, *Oct-4* gene expression was seen to be up-regulated at between 4.3 (Sturgeon) and 16.2 (Amphioxus V2) fold relative to the control EBs. The same data trend was seen for the early PGC specific marker *Dazl* which showed up-regulation in all of the assayed ancient *Nanog* over-expression lines relative to control levels of between 4.04 fold increase (Axolotl) and 20.3 fold increase (Amphioxus V2).

Vasa gene expression for the ancient *Nanog* over-expression lines was also increased over the pSIN-Empty control. The pSIN-Mouse produced EBs showed the largest increase in *Vasa* expression, with a relative increase calculated at 3.3. *Vasa* expression levels were up-regulated in the other ancient *Nanog* over-expression cell lines by about two fold over the control. The Western blot data generated by probing total protein extracted from the day 10 ancient *Nanog* EBs for *Vasa*, showed no visible levels in the control population of EBs. As there was low amount of non-specific background in the Western blot, computational analysis of relative band intensities proved problematic; therefore they were analysed visually. Comparable levels of *Vasa* protein were seen for the pSIN-Axolotl, pSIN-Sturgeon and pSIN-Amphioxus V1 produced EBs; termed baseline levels for visual analysis. There were increases seen in the *Vasa* protein levels observed in the EBs produced from the pSIN-Mouse and pSIN-Acorn Worm cell lines of approximately twofold over base line levels. There was however, a significant up-regulation seen in *Vasa* protein levels detected in the pSIN-Amphioxus V2 produced EBs. The increase in *Vasa* protein levels was seen to be at approximately a threefold visual increase over the base line levels of *Vasa* protein levels; however, more accurate levels were not able to be quantified because of problems with computational analysis. These results reaffirmed the corresponding RT-PCR data generated *Vasa* gene expression at the late stage day 10 time point.

As the Applied Biosystems primers used detected endogenous levels of gene expression, it is clear that ancient *Nanog* over-expression in EBs was shown to up-regulate endogenous gene levels. Further work into looking at solely endogenous levels of *Nanog* gene expression could use TaqMan RT-PCR primers targeted to the 3' UTR region, meaning that only endogenous levels of *Nanog* would be detected through RT-PCR analysis.

The ancient *Nanog* over-expression cell lines that are of particular interest are those of the pSIN-Amphioxus V2 and pSIN-Acorn Worm. In EBs from these cell lines, *Nanog*, *Oct-4*, *Dazl* and *Vasa* gene expression were found to be up-regulated.

The high levels of both *Nanog* and *Oct-4* gene expression in ancient *Nanog* over-expressing cell lines correlate with the robust increase that was seen in the PGC associated gene marker *Dazl* at the late stage day 10 time point. In the pSIN-Amphioxus V2 produced EBs, levels of *Vasa* gene expression were found to be at a two fold increase when analysed through quantitative RT-PCR, but were found to be at approximately a threefold visual increase over baseline *Vasa* protein levels when analysed through Western blot. In the pSIN-Acorn Worm produced EBs, *Nanog*, *Oct-4* and *Dazl* were found to be up-regulated by normalised gene expression levels of 4, 6.6 and 13.2 respectively. Again, as was seen in the pSIN-Amphioxus V2, high levels of *Nanog* and *Oct-4* correlated with a robust increase in the level of the PGC associated *Dazl* gene marker. Levels of *Vasa* gene expression were found to correlate with probed levels of *Vasa* protein through Western blot analysis, which were found to be at a two fold increase over baseline levels.

It is also noteworthy that Amphioxus V1 (*Vent-1*) over-expression in EBs proved to be inefficient in the process of enhancing PGC specification. This is in stark contrast with Amphioxus V2 (*Vent-2*), which was the most efficient in inducing PGCs from EBs. This data therefore suggest that Amphioxus *Vent-2* has functional similar to *Nanog* in the process of PGC specification, suggesting this function is conserved. Interestingly, these data suggest that the basal function of *Nanog* in chordates is in the specification of PGCs, with other related functions evolving over time.

(Dixon. J. E et al., 2010), showed that *Nanog* genes from axolotl, and other lower chordates, are expressed as a monomer, in contrast to the dimers formed by mouse *Nanog* and human *NANOG*. My study shows that monomeric forms of *Nanog*, lacking the WR repeat domain, can function to enhance the process of PGC specification. This finding complements studies showing that monomeric Nanogs can interact with molecules in the mammalian pluripotency network (Dixon. J. E et al., 2010).

One possible reason for the low levels of *Vasa* gene expression and *Vasa* protein levels observed could be the late stage at which *Vasa* is turned on in normal development. Studies by (Fujiwara. Y et al., 1994) have shown that *Mvh1* (mouse *Vasa*), is detectable at 12.5d.p.c. Further work by (Toyookaa. Y et al., 2000) has shown through whole mount staining that there are no *Mvh1*-positive cells detectable between 7.5-9.5 d.p.c., however between 10.5-11.5d.p.c. *Mvh1*-positive cells were detectable in the undifferentiated genital ridges. As the late time point EB collection was at day 10, this may have been too early in PGC development. To test this, EBs could be cultured for longer periods, on the order of weeks rather than days. This approach could also be relevant to the data for *Nanog* and *Oct-4* expression. As *Oct-4* and *Nanog* are markers of pluripotent cells, in addition to PGCs, PGC specific expression might be more accurately determine at later stages. It is impossible to be certain that the expression we see is a result of enhanced PGC production in the EBs, or simply higher expression from resident PGCs. To determine the numbers of PGCs produced in EBs would require Immunohistochemistry, using antibodies to PGC specific proteins.

An interesting avenue of future work would be to carry out other ancient *Nanog* over-expression EB experiments, such as over-expressing the recently characterised sea anemone *Nanog*. The sea anemone is a diploblast, i.e. it contains the ectoderm and endoderm germ cell layers but lacks mesoderm. Diploblasts evolved before the appearance of triploblastic organisms. Consequently, if enhancement of PGC specification, observed in the pSIN-Amphioxus V2 cell line, could be repeated with *Nanog* orthologs from diploblasts, then it would provide more evidence for the idea that the ancient function of *Nanog* was indeed in germ cell specification and pluripotency, with more modern functions evolving subsequently.

7.2 Biological Replicate of Ancient *Nanog* Over-Expression

As well as extending the findings of this study, biological repeats of the experiments performed are needed to reaffirm the results that were seen. After the completion of the first ancient *Nanog* over-expression experiment, a repeat experiment was carried out. It was decided that the time points at which EBs were collected in repeat procedures should be changed to better capture the EB differentiation time course. It was decided that new EB time point samples were to be collected firstly on Day 0 (EB formation day) and then on day 3, day 6 and day 9 and 14 after EB formation. This would give a better picture of when PGCs are specified in differentiating EBs and allow the gene expression profiles of target genes to be tracked over an extended period of time.

However, it was found that insufficient RNA could be collected from many of the time point EB samples. Problems were then encountered with quantitative RT-PCR analysis. Template cDNA produced from RNA extracted from the ancient *Nanog* over-expression EBs could not be amplified. Correct template cDNA production was seen through the utilisation of mouse β -*Actin* primers and standard PCR amplification, however no amplification could be obtained for any of the gene markers through RT-PCR. Due to time constraints on project completion, there was no data available for the biological repeat of the ancient *Nanog* over-expression experiment at the time of thesis completion; however, replicate experiments are currently underway.

7.3 Final Conclusion and Summation of Work

In relation to the initial aims set out in this project and the hypothesis on which it is based, it has been shown that the over-expression of ancestral *Nanog* orthologs in mES cells can function to enhance the process of PGC specification. This study provides evidence for the hypothesis that the functional activity of *Nanog* is conserved through some species of deuterostomes and also that pluripotency is an ancient trait.

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