CHAPTER 1 Introduction

1.1 Transcription factors and Nanog

Transcription factors control the transfer of genetic information from DNA to mRNA and are essential for regulation of gene expression in many important biological processes (Karin, 1990; Latchman, 1997). Genes are upregulated or downregulated by transcription factors through their binding to specific sequences of DNA by different kinds of mechanisms, including inhibiting RNA polymerase from binding to DNA, catalyzing biochemical modifications of histone proteins, and recruiting coactivator or corepressor proteins to the transcription factor complex (Xu et al., 1999; Narlikar et al., 2002).

A homeobox is a DNA sequence first identified in genes regulating anatomical development in *Drosophila* (McGinnis et al., 1984; Scott and Weiner et al., 1984). The homeobox encodes a highly conserved helix-turn-helix protein domain, the homeodomain, which can bind DNA when expressed (Tullius, 1995). Homeobox genes encode transcription factors which switch on expression of downstream genes (Liang and Biggin, 1998). One class of homeobox genes is *Hox* genes, which specify the anterior-posterior axis and segment identity (Wellik, 2009; Alexander et al., 2009; Iimura et al., 2009). *Antennapedia* in *Drosophila* can cause development of legs instead of antennae (Postlethwait and Schneiderman, 1969). Other classes of homeobox genes include *Barx1*, *Msx1* and *Nkx2* (Hill et al., 1989; Kim and Nirenberg, 1989; Tissier-Seta et al., 1995; Harvey, 1996).

The *Nanog* gene was first isolated by Dr. Ian Chambers et al. (2003) and derives its name from the mythological Celtic land of the ever-young Tir nan Og (Chambers et al., 2003;

Mitsui et al., 2003). It encodes a homeodomain-containing transcription factor found in embryonic stem cells, which plays an important role in sustaining pluripotency (Chambers et al., 2003; Mitsui et al., 2003). It enables embryonic stem cells to produce any somatic cell type or primordial germ cells (Chambers et al., 2003; Mitsui et al., 2003; Chambers et al., 2007), and functions with other transcription factors to maintain embryonic stem cell identity (Nichols et al., 1998; Niwa et al., 2000; Avilion et al., 2003; Chambers and Tomlinson, 2009). Mouse Nanog and human NANOG proteins consist of a Serine-rich N-terminal motif, a homeodomain, and a Tryptophan-rich C-terminus (Chambers et al., 2003; Mitsui et al., 2003). The homeodomain region plays an important role in binding DNA sequences (Tullius, 1995). A maximum of 50% amino acid identity is found over the homeodomain of Nanog with Barx1, Msx1 and members of the NK-2 family (Hill et al., 1989; Kim and Nirenberg, 1989; Tissier-Seta et al., 1995; Harvey, 1996; Chambers et al., 2003). The *Nanog* homeobox is closest to that of the *NK-2* gene family (Kim and Nirenberg, 1989; Harvey, 1996; Mitsui et al., 2003). However, mouse Nanog and human NANOG have valine instead of tyrosine in a conserved position in the homeodomain (Harvey, 1996; Mitsui et al., 2003). Mouse Nanog and human NANOG lack the TN and SD domains, which are conserved in members of the NK-2 family (Harvey, 1996; Mitsui et al., 2003). In addition, the Tryptophan-rich domain (WR domain) in mouse Nanog and human NANOG mediates dimerization (Mullin et al., 2008; Wang et al., 2008).

Overexpression of *Nanog* in mouse embryonic stem cells causes them to self renew in the absence of leukemia inhibitory factor (LIF) (Chambers et al., 2003; Mitsui et al., 2003). Aside from ES cells, Nanog is found in mammalian pluripotent cells and developing germ cells (Chambers et al., 2003; Mitsui et al., 2003). The inner cell mass with *Nanog* deleted fails to mature into pluripotent epiblast, suggesting that Nanog is indispensable for constructing the inner cell mass state (Mitsui et al., 2003; Silva et al., 2009). *Nanog* is

expressed in mouse germ cells during the period of epigenetic erasure and germ line commitment (Yamaguchi et al., 2005) and is thought to mediate germline development (Chambers et al., 2007). It is essential for PGCs to complete colonizing the genital ridge (Chambers et al., 2007). Embryonic stem cells with *Nanog* disrupted can contribute to the germ line but *Nanog*-deficient primordial germ cells stay in the soma rather than migrating to the genital ridge beyond E11.5 (Chambers et al., 2007). It suggests that Nanog has an essential function in construction of the germ cell state (Chambers et al., 2007).

Among numerous transcription factors, some factors have been of recent interest because of their ability to cause otherwise differentiated cells to become pluripotent (Hanna et al., 2010). These induced pluripotent stem cells (iPS) are derived from somatic cells by introducing a defined set of genes (Hanna et al., 2010). They were first generated by Takahashi and Yamanaka in 2006, who used retroviruses to transfect mouse fibroblasts with a selection of genes considered important for embryonic stem cell maintenance. Transgenic expression of these transcription factors has been used to generate induced pluripotent stem cells from human and mouse somatic cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007). Different methods have been used to introduce transcription factors into somatic cells, including retroviral, lentiviral, adenoviral and plasmid transfection (Stadtfeld and Hochedlinger, 2010). These reprogrammed cells are similar to ES cells in their morphology, expressing ES cell marker genes, and forming tumours when injected into mice (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007).

In embryonic stem cells, Nanog, along with Oct3/4 and Sox2, is necessary to promote pluripotency (Nichols et al., 1998; Niwa et al., 2000; Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003). Research by Yu et al. (2007) shows that NANOG is one of the four factors (OCT4, SOX2, NANOG and LIN28) that are sufficient to reprogram human

somatic cells to pluripotent stem cells, which exhibit the essential characteristics of embryonic stem (ES) cells. Aside from the work of Yu et al. in 2007, Takahashi and Yamanaka have tested twenty four factors that maintain pluripotency in embryonic stem cells as initial candidates of key factors inducing pluripotency in somatic cells in 2006. As a result, four factors, Oct3/4, Sox2, Klf4 and c-Myc, were found to be sufficient in reprogramming mouse fibroblasts (Takahashi and Yamanaka, 2006).

It is meaningful to study these important transcription factors including Nanog and induced pluripotent stem cells as they have great potential for biomedical research, disease research and toxicology (Yamanaka, 2009).

Aside from mouse and human, orthologues of *Nanog* have been identified in chick and axolotl (Lavial et al., 2007; Dixon et al., 2010). At the time we started the work, we were not aware of Zgc: 193933 as a potential zebrafish *nanog*, so we decided to work with human *NANOG* and mouse *Nanog* cDNAs. Although recently a *Nanog* homolog has been cloned from medaka and a potential zebrafish *nanog* (Zgc: 193933) has been identified, the similarity is overall very low compared with human *NANOG* and mouse *Nanog* (Chambers et al., 2003； Mitsui et al., 2003; Camp et al., 2009). Moreover, the putative Nanog homeodomain protein in medaka has not been found to regulate pluripotency in ES cells as mammalian NANOG/Nanog, chicken Nanog and axolotl Nanog do (Chambers et al., 2003; Mitsui et al., 2003; Lavial et al., 2007; Dixon et al., 2010). Additionally, human *NANOG*, mouse *Nanog* and chicken *Nanog* enable LIF-independent self-renewal when overexpressed in mouse ES cells, which medaka *Nanog* has not been found to be able to (Chambers et al., 2003; Mitsui et al., 2003; Lavial et al., 2007; Dixon et al., 2010). Axolotl *Nanog* overexpression in mouse embryonic stem cells cannot support self-renewal in the absence of LIF, but its homodimer generated with WR domain inserted is able to rescue LIF-

independent self-renewal (Dixon et al., 2010). There are no studies on the activity of the potential zebrafish *nanog* (Zgc: 193933) to justify it as an orthologue of *Nanog.*

1.2 Overexpression of mammalian *NANOG*/*Nanog* mRNA in zebrafish embryos

During animal development, the germ line serves an important role producing gametes and offspring. Primordial germ cells are stem cells that give rise to the gametes. In many animals, primordial germ cells originate near the gut and migrate to the developing gonads. In the gonads, they undergo mitosis and meiosis as they differentiate into mature gametes. There are different ways of generating the germ line, and one way of germ line formation is by induction (Saffman and Lasko, 1999). In mammals primordial germ cells are induced from epiblast (Ying et al., 2001), and in axolotls they can be specified from primitive ectoderm in response to inducing signals (Sutasurya and Nieuwkoop, 1974; Johnson et al., 2003). It has been proposed recently that ground state pluripotency giving rise to any somatic cell type or primordial germ cells is conserved from urodele amphibians to mammals (Dixon et al., 2010). In zebrafish and frogs, however, primordial germ cells are specified by genetic determinants inherited from maternal stores (Ikenishi et al., 1974; Zust and Dixon, 1975; Houston and King, 2000; Raz, 2002; Raz, 2003).

1.2.1 Germ line development in mammals

In mammals primordial germ cells are induced from pluripotent epiblast cells by signals controlled by zygotic genes of neighbouring cells (Ying et al., 2001; Ying et al., 2002; Saga, 2008).

Early specification of mouse primordial germ cells is completed by induction of regulators including Blimp1 and Bmps such as Bmp2, Bmp4 and Bmp8b (Lawson et al., 1999; Ying et al., 2001; Ying and Zhao, 2001; Saga, 2008). Migration of mouse primordial germ cells relies on receptor-ligand interactions, for example the *C-kit* receptor expressed in the PGCs and its ligand *Steel* expressed by somatic cells along the migratory route (Matsui et al., 1990; Bernex et al., 1996). They are also guided towards the genital ridge by long-distance signalling (Godin et al., 1990). In addition, PGC migration is guided by the interaction between PGCs and extracellular matrix molecules such as Beta1 integrins (Anderson et al., 1999). In mouse, primordial germ cells lacking *Nanog* cannot mature on reaching the genital ridge (Chambers et al., 2007).

1.2.2 Germ line development in zebrafish

In zebrafish, germ line specification occurs in cells that inherit germ plasm from maternal stores (Raz, 2002; Raz, 2003). During the zygote period, maternal germline determinants are located in a special type of cytoplasm, the germ plasm. During early cleavage stages, zebrafish germ plasm is first divided into four blastomeres. The germ plasm in each blastomere is then asymmetrically distributed into only one daughter cell during cell divisions. The cytoplasmic determinants specify primordial germ cells at the late blastula stage. In subsequent divisions of primordial germ cells, germ plasm is symmetrically distributed into

both daughter cells (Yoon et al, 1997; Braat et al., 1999; Knaut et al., 2000; Raz, 2002; Raz, 2003). Primordial germ cells go through six steps of migration in the first 24 hours post fertilization (Weidinger et al., 1999). They are first attracted towards an intermediate target and then migrate to the final target, the gonads (Weidinger et al., 2002).

Zebrafish *vasa* is a germ cell-specific gene and its transcript is a marker for zebrafish germ line. Zebrafish *vasa* RNA is a component of germ plasm and segregates asymmetrically during early cleavage stages. Zygotic *vasa* transcription starts after germ plasm segregation pattern changes (Yoon et al., 1997; Braat et al., 1999; Knaut et al., 2000).

Another important component of zebrafish germ plasm is *nanos* RNA. Similar to *vasa* RNA, maternal *nanos* RNA degrades in somatic cells but specifically stabilizes in PGCs, enabling specific expression of *nanos* in PGCs. Moreover, zygotic Nanos protein is essential for migration and survival of PGCs in zebrafish (Köprunner et al., 2001).

1.2.3 Zebrafish Development

Zebrafish, *Danio rerio*, is an important model organism in developmental studies. The embryo of the zebrafish goes through several periods including zygote, cleavage, blastula, gastrula, segmentation, pharyngula, hatching and early larva in the first three days after fertilization. Cytoplasm streams toward the animal pole and forms blastodisc which is incompletely cleaved into interconnected blastomeres. As the number of blastomeres increases, cell cycles lengthen asynchronously when the embryo enters midblastula transition, and the blastodisc spreads over the yolk, which is completely engulfed at the end of gastrula period. The somites develop during segmentation period. The embryo completes most processes of morphogenesis, cell growth and cell differentiation at the end of hatching period.

The early larva continues to grow rapidly, go through changes such as inflation of the swim bladder and begins to swim about actively (Kimmel et al., 1995).

1.2.4 Dorsal-Ventral Patterning

Dorsal-ventral patterning is an important stage of early zebrafish development. Dorsal-ventral pattern formation is regulated by the opposing effect of ventralizing genes such as the *bmp* gene family (Nikaido et al., 1997) and the *wnt* gene family (Kelly et al., 1995; Lekven et al., 2001; Ramel and Lekven, 2004), and dorsal-specific genes such as *chordin* (Schulte-Merker et al., 1997). Bone morphogenetic proteins (BMPs) are key mediators of dorsal-ventral patterning and are required for the induction of ventral fates in fish and frogs (Nikaido et al., 1997; Schmid et al., 2000). Ventral genes such as *gata1* and the *bmp* genes themselves are activated by BMP signalling (Kishimoto et al., 1997; Schmid et al., 2000; Stickney et al, 2007). A BMP activity gradient, which forms by the interaction between BMPs and BMP antagonists, patterns cell fates along the dorsal-ventral axis (Schier and Talbot, 2005). In this thesis, we use the following five factors which function in dorsal-ventral patterning (Figure 1).

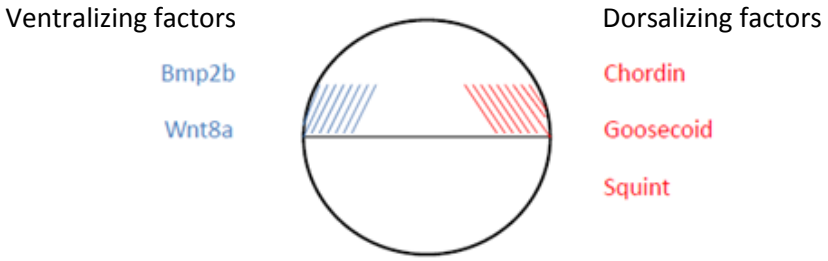

Figure 1. Some zebrafish dorsoventral patterning factors

An important member of the *bmp* gene family is ***bmp2b*** (Martínez-Barberá et al., 1997; Nikaido et al., 1997). It functions as an essential gene during early dorsal-ventral patterning in zebrafish, being required for ventral specification (Kishimoto et al., 1997; Nguyen et al., 1998). Overexpression of *bmp2b* causes expansion of ventral gene expression and reduces dorsal structures such as notochord (Nikaido et al., 1997). Previous studies have shown that mutations in the zebrafish *bmp2b* gene cause a dorsalized phenotype (Kishimoto et al., 1997; Nguyen et al., 1998). In the *swirl* mutant, several processes for primordial germ cell migration are affected, leading to a defect in movement of PGCs toward the dorsal side of the embryo (Weidinger et al., 1999).

Aside from *bmp2b*, *bmp4* and *bmp7* are both important members of the *bmp* family (Martínez-Barberá et al., 1997; Nikaido et al., 1997; Dick et al., 2000; Schmid et al., 2000). Zebrafish *bmp7* is essential for ventral cell specification (Dick et al., 2000; Schmid et al., 2000). A strongly dorsalized phenotype, *snailhouse*, was caused by mutations in *bmp7* (Dick et al., 2000; Schmid et al., 2000). Zebrafish *bmp4* is expressed in ventral cells during late blastula and gastrula stages and specifies ventroposterior cell fates (Nikaido et al., 1997; Stickney et al., 2007). Disrupted development of ventral tissues such as ventral tail fin is found in $bmp4^{st72}$ mutant embryos (Nikaido et al., 1997; Stickney et al., 2007). The dorsalized phenotypes of *swirl* and *snailhouse* mutant embryos can be rescued by *bmp4* (Nguyen et al., 1998). In addition, the role of BMP signalling in dorsoventral patterning changes as zebrafish development proceeds over different stages (Pyati et al., 2005). It is suggested that in zebrafish *bmp2b* and *bmp7* function in early stages of dorsoventral patterning, while *bmp4* together with *bmp2b* regulates late patterning (Stickney et al., 2007).

Homeobox genes *vox* and *vent* encode ventralizing transcriptional factors in early stages of zebrafish dorsoventral patterning (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby

et al., 2000). Expression of *vox* is detected in all blastomeres at the midblastula transition but is downregulated by the late blastula stage (Kawahara et al., 2000a; Melby et al., 2000). Expression of *vent* is detected ventrally at the late blastula stage (Kawahara et al., 2000b; Melby et al., 2000). The asymmetric distribution of Vox and Vent is established by the interaction with their antagonist Bozozok (Kawahara et al., 2000a; Melby et al., 2000). Overexpression of *vox* or *vent* can ventralize zebrafish embryos probably by repressing dorsal genes such as *squint, chordin* and *goosecoid* (Kawahara et al., 2000a; Kawahara et al., 2000b; Imai et al., 2001). In zebrafish, *vox* and *vent* repress dorsal fates of zebrafish redundantly (Imai et al., 2001). Either *vox* or *vent* expression disrupted by antisense morpholinos doesn't lead to any dorsoventral patterning defect, while embryos lacking both *vox* and *vent* display a dorsalized phenotype and an expansion of organizer gene expression (Imai et al., 2001).

Dorsal-specific genes ***chordin*** and ***goosecoid*** are important in dorsoventral patterning, and their expression is dorsally restricted (Stachel et al., 1993; Schulte-Merker et al., 1994; Schulte-Merker et al., 1997). Chordin is a BMP antagonist (Schulte-Merker et al., 1997) and mutations in *chordin* result in a ventralized phenotype (Hammerschmidt et al., 1996). In *chordino* mutant embryos, some PGCs are found in positions posterior to the correct ones, indicating a defect in migration of PGCs (Weidinger et al., 1999). The homeobox gene *goosecoid* was first found in a screen for homeobox genes in a dorsal lip cDNA library of *Xenopus* (Blumberg et al., 1991). It was named *goosecoid* for its similarity with *Drosophila* genes *gooseberry* (Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1984) and *bicoid* (Frohnhöfer and Nüsslein-Volhard, 1986). *Xenopus goosecoid* is important for Spemann's organizer function and microinjection of *goosecoid* mRNA into the ventral side of *Xenopus* embryos induces secondary body axes (Cho et al., 1991). Zebrafish *goosecoid* is expressed on the dorsal side of the embryo and its expression domain defines the prospective shield (Stachel et al., 1993; Schulte-Merker et al., 1994). Lithium treatment causes a

dorsalized phenotype of zebrafish embryos and upregulates *goosecoid* expression (Stachel et al., 1993).

Nodal signals are important factors which induce mesoderm formation and dorsoventral patterning in vertebrate embryos (Zhou et al., 1993; Conlon et al., 1994; Jones et al., 1995; Joseph and Melton, 1997). In zebrafish, a *nodal*-related gene ***squint*** functions in patterning of mesendoderm (Heisenberg and Nüsslein-Volhard, 1997; Erter et al., 1998; Feldman et al., 2000; Dougan et al., 2003). Dorsal mesoderm structures are reduced in *squint*^{cz35} mutant embryos (Feldman et al., 2000; Dougan et al., 2003). Overexpression of *squint* induces dorsal mesoderm structure such as notochord and somites (Erter et al., 1998). It has been shown that Squint specifies the fate of cells directly at a distance as a morphogen does (Chen and Schier, 2001).

Wnt signalling pathway also plays an important role in dorsal-ventral patterning during early development (Christian et al., 1991; Christian and Moon, 1993; Hoppler et al., 1996; Lekven et al., 2001; Ramel and Lekven, 2004). Expression of *Xenopus wnt*8 is ventrally restricted (Christian et al., 1991; Smith and Harland, 1991; Christian and Moon, 1993). Overexpression of *Xenopus wnt8* in organizer cells after the midblastula stage is found to ventralize these cells and overexpression of a dominant negative form of *Xenopus* wnt8 reduces ventral mesodermal tissues, suggesting that *wnt8* specifies ventral fates (Christian et al., 1991; Smith and Harland, 1991; Christian and Moon, 1993; Hoppler et al., 1996).

Aside from dorsoventral patterning, Wnts function in anterior-posterior patterning of the neurectoderm (McGrew et al., 1995; McGrew et al., 1997; Bang et al., 1999). Overexpression of Wnts can posteriorize neural tissue (McGrew et al., 1995; McGrew et al., 1997; Bang et al., 1999), while interfering Wnt signalling increases the expression of anterior

neural genes and reduces the expression of posterior neural genes (McGrew et al., 1997; Hsieh et al., 1999).

Zebrafish ***wnt8a*** is a homologue of *Xenopus wnt8* (Kelly et al., 1995). It is required for ventral specification of mesoderm and also anterior-posterior patterning of the neurectoderm (Lekven et al., 2001; Ramel and Lekven, 2004). Embryos homozygous for *Df(LG14)wnt8*^{w8} with the *wnt8* locus removed exhibit defects in dorsal-ventral mesoderm patterning including an expansion of the shield and defects in anterior-posterior neural patterning (Lekven et al., 2001). Zebrafish Wnt8a is thought to antagonize dorsal organizer (Lekven et al., 2001). It has been suggested that Wnt8a repress the dorsal organizer by regulating early expression of the transcriptional repressors Vent and Vox (Ramel and Lekven, 2004).

1.2.5 Project Aims

Zebrafish, *Danio rerio*, is used as an important model organism for many functional studies, especially for the study of early vertebrate development. With previous studies on Nanog's effect on mouse germ line development (Chambers et al., 2007), we hypothesize that Nanog has an equivalent function in promoting zebrafish germ line development. Although a zebrafish *nanog* sequence has been suggested, there are no studies on the activity of this gene (Zgc:193933) (Camp et al., 2009). At the time we began this work we were not aware of Zgc:193933 as a potential *Nanog* orthologue so we decided to work with human *NANOG* amd mouse *Nanog* cDNAs (Chambers et al., 2003; Mitsui et al., 2003; Camp et al., 2009). We therefore introduce mammalian *NANOG*/*Nanog* into zebrafish embryos. With the study of the changes in germline development and in dorsal-ventral patterning after injecting

NANOG/Nanog mRNA into zebrafish embryos, the effects of overexpression of mammalian *NANOG/Nanog* mRNA on zebrafish embryonic development can be studied. Nevertheless, further studies such as gain-of-function experiments in this thesis with the putative zebrafish *nanog* are warranted to take the study of the effects of *Nanog* on specification of germ line and on dorsal-ventral patterning a step further. Loss-of-function studies such as disrupting endogenous expression of the putative zebrafish *nanog* with morpholinos are worthwhile to supplement studying the function of *nanog* in zebrafish germ line specification and dorsoventral patterning.

The study described in this thesis is to introduce humang *NANOG* and mouse *Nanog* mRNA into zebrafish embryos to study the effects of overexpression of mammalian *NANOG*/*Nanog* mRNA on zebrafish germline development and dorsal-ventral patterning. Figure 2 shows the flowchart of the experiment.

Human *NANOG* and mouse *Nanog* mRNA were injected into zebrafish embryos, and were found to cause changes in morphology. However, microinjection of human *NANOG* into zebrafish embryos doesn't lead to significant changes in the number of primordial germ cells. Moreover, it is found that overexpression of human *NANOG* or mouse *Nanog* mRNA hyperdorsalises zebrafish embryos.

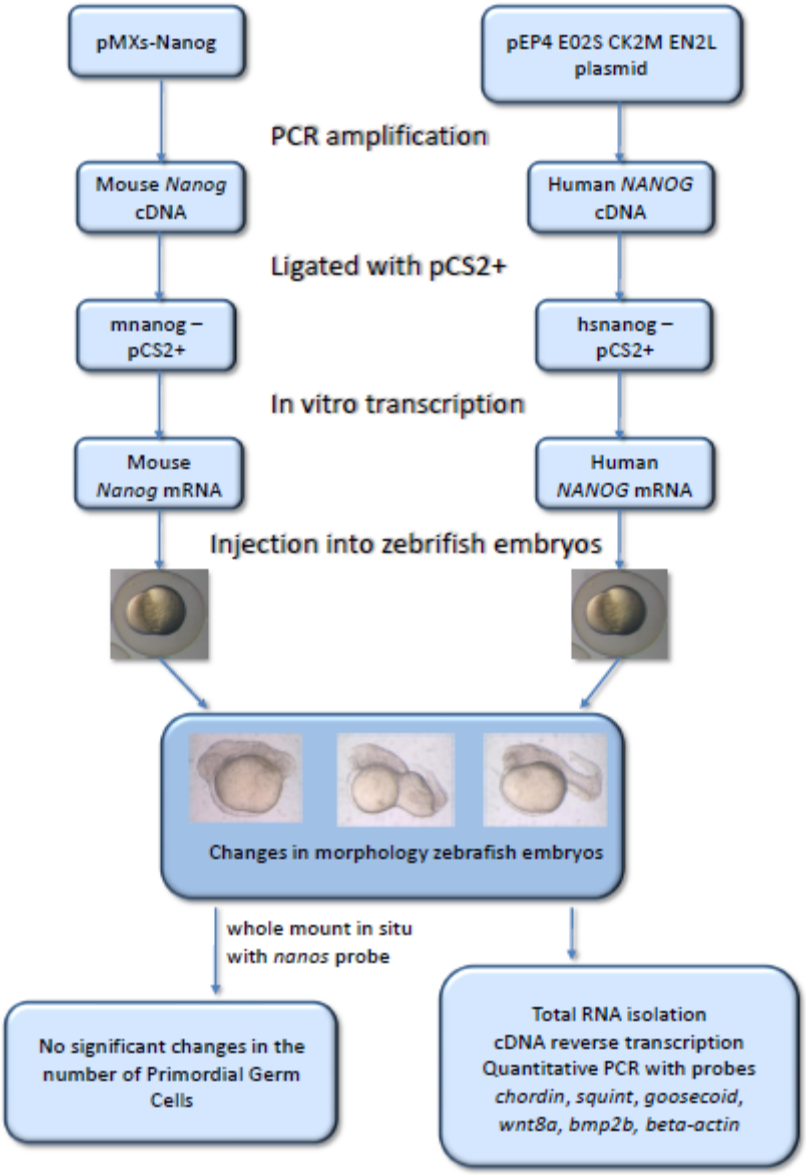

Figure 2. Experimental flowchart

1.3 Chapter Overview

The second chapter discusses PCR amplification of human *NANOG* and mouse *Nanog*, construction of hsnanog-pCS2+ and mnanog-pCS2+ and in vitro transcription of human *NANOG* and mouse *Nanog* mRNA.

The third chapter discusses that injection of human *NANOG* mRNA or mouse *Nanog* mRNA leads to changes in the phenotype of zebrafish embryos but there is no significant change in the number of primordial germ cells.

The fourth chapter discusses total RNA isolation from zebrafish embryos injected with mouse *Nanog* and embryos injected with human *NANOG*, reverse transcription and quantitative RT-PCR assay of expression of five genes involved in dorsal-ventral patterning. It is found that injection of mouse *Nanog* or human *NANOG* mRNA into zebrafish embryos hyperdorsalises zebrafish embryos.

Finally the fifth chapter concludes the work completed in this thesis and discusses what can be done in the future.