Chapter 5 Conclusions

Nanog has been found to be essential in promoting mouse germ line development (Chambers et al., 2007). In order to investigate whether it impacts zebrafish germ line development, we overexpressed mammalian *NANOG/Nanog* mRNA in zebrafish (Chambers et al., 2003; Mitsui et al., 2003). Human *NANOG* and mouse *Nanog* were used in this thesis because when we started there was no clear orthologue of human *NANOG* or mouse *Nanog* in zebrafish. We find that *NANOG* overexpression in zebrafish embryos doesn't change the number of primordial germ cells but affects dorsoventral patterning. The work presented in this thesis shows the first investigation of microinjecting mammalian *NANOG/Nanog* mRNA into zebrafish embryos, studying the effects of overexpressing mammalian *NANOG/Nanog* mRNA in zebrafish embryos on germ line development and dorsal-ventral patterning. The construction of hsnanog-pCS2+ and mnanog-pCS2+ and in vitro transcription of human *NANOG* and mouse *Nanog* mRNA have been presented in detail, followed by the discussion of dorsalized changes in the morphology of zebrafish embryos and no effect on the number of primordial germ cells after injection with *NANOG* mRNA. Quantitative RT-PCR (Freeman et al., 1999) was used to quantify expression of five genes involved in dorsal-ventral patterning, and t-tests were performed to investigate the changes in these genes' expression. It is found that overexpression of mammalian *NANOG/Nanog* mRNA significantly upregulates expression of the dorsal gene *goosecoid* (Stachel et al., 1993; Schulte-Merker et al., 1994) and significantly downregulates expression of the ventral gene *wnt8a* (Lekven et al.,

2001;Ramel and Lekven, 2004), which is consistent with the dorsalized phenotypes in *NANOG*/*Nanog*-injected embryos.

The investigation presented in this thesis contributes to our understanding of the effects of NANOG/Nanog on some zebrafish developmental features, and demonstrates that microinjection represents a valuable approach for studying *NANOG/Nanog* function. The results show that overexpression of mammalian *NANOG*/*Nanog* mRNA by microinjection leads to significant changes in the phenotype of the zebrafish embryos (25% and 56% of embryos show significant dorsalization in their phenotype after injection with 50pg and 100pg human *NANOG*; 81% and 82% of embryos show significant dorsalization in their phenotype after injection with 50pg and 100pg mouse *Nanog*), causing the mis-specification of fates along the dorsal-ventral axis, rather than verifying the original hypothesis that *NANOG* overexpression would lead to the overproduction of primordial germ cells in the zebrafish embryos (no significant change in the number of primordial germ cells can be observed following injection of human *NANOG* mRNA). However, previous studies in other model organisms such as mice show that *Nanog* is essential for specification of germ cells in their embryos (Chambers et al., 2007). There are three possible reasons that might lead to the difference between the experimental result and the original hypothesis: firstly, the transcription factor Nanog may function in different ways during the developmental processes of different model organisms (Chambers et al., 2007; Yamaguchi et al., 2009; Sánchez-Sánchez et al., 2010); Secondly, germ cells are specified by inheriting germ plasm in zebrafish (Raz, 2003), which is different from the way they are specified in mammals (Saga, 2008; Ewen and Koopman, 2010); thirdly, the difference between the technologies, such as exogenous induction, knockout and microinjection, that have been used in the experiments to study *Nanog* in the model organisms may lead to these different results (Chambers et al., 2003; Mitsui et al., 2003; Chambers et al., 2007; Yamaguchi et al., 2009) .

In order to further investigate the function of NANOG/Nanog, several approaches can be taken to supplement the current experiments or take the research a step further. One of them is to transfect dissociated zebrafish embryonic cells with *NANOG/Nanog* and score for *vasa* positive cells (Knaut et al., 2000). Another one is to inject the *piggyBAC* vector (Ding et al., 2005) carrying *NANOG/Nanog* gene into zebrafish embryos and score for *vasa* positive cells (Knaut et al., 2000). These two methods could be used to study whether *NANOG/Nanog* has an equivalent function in sustaining pluripotency as it does in human and mouse embryonic stem cells and the inner cell mass (Chambers et al., 2003; Mitsui et al.,2003; Chambers et al., 2007). However, these two methods are more time-consuming than microinjection. Also, more genes involved in dorsal-ventral patterning could be selected to investigate the function of NANOG/Nanog in the processes of zebrafish development. Moreover, only two sets of *NANOG/Nanog* mRNA injection dosage have been used in the current experiment because of the time constraint, more sets of injection dosage could be tested so that the effects of *NANOG/Nanog* overexpression might be further investigated. The estimation of gene expression is not ideal in this thesis because some of the data fall beyond the range of data points used for linear regression. Accuracy of the measurement could be improved by diluting cDNA samples of experimental replicates of embryos injected with human *NANOG* or mouse *Nanog* and control replicates of uninjected embryos.

The result that *NANOG* overexpression doesn't lead to a significant change in the number of primordial germ cells in zebrafish, supports the view that zebrafish animal cap, including somatic cells and primordial germ cells, doesn't develop from a pluripotent ground state. Frog, which has similar mechanism of primordial germ cell specification with zebrafish, doesn't have any *NANOG*/*Nanog* homologue in its genome (Hellsten et al., 2010). In contrast, Nanog has been identified as a regulator of pluripotency in many organisms

initiating somatic and germ cell development from ground state pluripotency, suggesting the conserved pluripotency network from urodele amphibians to mammals (Dixon et al., 2010).

As a homeodomain protein, NANOG/Nanog may function as a dominant negative form of Vox or Vent, suppressing function of Vox or Vent (Kawahara et al., 2000a; Kawahara et al., 2000b; Imai et al., 2001) and dorsalizing zebrafish embryos. To further investigate mechanism of NANOG/Nanog function, *vox* or *vent* RNA could be injected to test whether it could rescue the defect in dorsoventral patterning caused by *NANOG*/*Nanog* overexpression. RNA extracted from different stages of *NANOG*/*Nanog*-injected embryos could be sequenced to give a transcriptome view of the changes caused by *NANOG/Nanog* overexpression (Ryan et al., 2008; Wang et al., 2009). It could be compared with the transcriptome of zebrafish embryos injected with *vox* or *vent* dominant negative forms. Moreover, chromatin immunoprecipitation combined with sequencing could give information about NANOG/Nanog binding sites in zebrafish genome, which would be helpful for studying how NANOG/Nanog functions (Johnson et al., 2007; Jothi et al., 2008).

At the time we started the work, there was no clear orthologue of *Nanog* in zebrafish. A putative zebrafish *nanog* (Zgc: 193933) has been proposed recently (Camp et al., 2009). Similar approaches could be used to study the function of the potential zebrafish *nanog*. Zebrafish *nanog* mRNA could be overexpressed to study whether it has effect on the number of zebrafish PGCs and dorsoventral patterning. On the other hand, it is also meaningful to introduce morpholinos of the putative zebrafish *nanog* to interrupt its endogenous expression for loss-of-function studies. According to the result of this thesis, no significant change in the number of zebrafish PGCs would be expected for gain-of-function or loss-of-function studies of zebrafish *nanog*. One possibility is that overexpressing zebrafish *nanog* would dorsalize embryos, while a morpholino knockdown would cause a ventralized phenotype.

However, a potential medaka *Nanog* has been identified, but its overexpression doesn't cause any specific change in phenotype (Camp et al., 2009), in contrast with the defect in dorsoventral patterning caused by overexpressing mammalian *NANOG*/*Nanog* in zebrafish described in this thesis. No effect of medaka *Nanog* overexpression might be attributed to the absence of WR domain in it, which mediates dimerization of mammalian NANOG/Nanog (Mullin et al., 2008; Wang et al., 2008). Therefore, mammalian NANOG/Nanog or medaka Nanog with WR domain inserted could be overexpressed in medaka to further these studies.

Follow-up studies on function of zebrafish *nanog* including rescue with *vox* or *vent* mRNA, RNA-seq (Ryan et al., 2008; Wang et al., 2009) and Chip-seq (Johnson et al., 2007; Jothi et al., 2008) could be used to investigate its interaction with other transcription factors as well as with zebrafish genome at different stages of zebrafish development.

As has been discussed in the previous chapters, several other transcription factors such as OCT4, SOX2, LIN28 and KLF4 appeal to researchers because of their special functionalities including promoting pluripotency (Yu et al., 2007). Further research using similar technologies as presented in this thesis could also be carried out to investigate the function of these important transcription factors in the developmental processes of zebrafish.