CHAPTER 3 *NANOG* overexpression does not affect the number of PGCs but affects dorsoventral patterning

3.1 Introduction

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 reaching the genital ridge beyond the E11.5 stage (Chambers et al., 2007). So we hypothesize that it has an equivalent function in zebrafish germline development. In order to address this question, we misexpressed human *NANOG* and mouse *Nanog* mRNA in zebrafish embryos. We used mouse *Nanog* and human *NANOG* in this project because no clear orthologue of *Nanog* in zebrafish was apparent at the time. Although a *Nanog* homolog has been cloned from medaka recently and a putative zebrafish *nanog* has been identified, the degree of the similarity is overall very low compared with human *NANOG* and mouse *Nanog* (Camp et al., 2009). Nevertheless, functional studies with the putative zebrafish *nanog* are warranted, such as gain-of-function overexpression and loss-of-function knockdown with morpholinos. In zebrafish, *vasa* RNA and *nanos* RNA are components of germ plasm and markers of primordial germ cells (Yoon et al., 1997; Braat et al., 1999; Köprunner et al., 2001). Therefore we used *nanos* probe to detect primordial germ cells in embryos injected with human *NANOG* mRNA and in uninjected control embryos. We find that *NANOG*

overexpression does not affect the number of PGCs. Overexpression of either human *NANOG* or mouse *Nanog*, however, leads to dorsalized changes in the morphology of zebrafish embryos and suggests defects in dorsoventral patterning.

3.2 Materials

Special Equipment

Needle pippet puller	KOPF
Glass capillaries with inner filament	WPI
Microloader	Eppendorf
Microscope	Leica
Microscope	ZEISS

Chemicals

2% phenol red

mineral oil

human *NANOG* and mouse *Nanog* mRNA (kept at -80 degree)

nanos probe (obtained from Kong Jun)

Tricaine (MS-222)

3.3 Methods

Microinjection and whole mount in situ

Wild-type zebrafish embryos were collected and injected at the one-cell or two-cell stage with four sets of *NANOG/Nanog* mRNA, 50 pg human *NANOG* mRNA, 100 pg human *NANOG* mRNA, 50 pg mouse *Nanog* mRNA and 100 pg mouse *Nanog* mRNA on the same day. Injection of 50 pg and 100 pg mouse *Nanog* was repeated once on another day. Injection of 50 pg and 100 pg human *NANOG* was repeated for four times on four other days (one set of 50 pg human *NANOG* and one set of 100 pg human *NANOG* injected on one day). Embryos injected with *NANOG/Nanog* mRNA were classified into groups according to their morphology and counted. They were anesthetised with tricaine (0.016%) for photograph.

Whole mount in situ hybridisation with *nanos* probe was performed to detect the presence of primordial germ cells (Köprunner et al., 2001). In situ hybridization was performed according to the standard protocol (Thisse et al., 1993). The number of stained primordial germ cells was counted under microscope.

T-tests

As the number of PGCs is independent in *NANOG*-injected and uninjected control embryos and is normally distributed, we performed a two-tailed t-test with two samples assuming unequal variances to compare the number of PGCs in *NANOG*-injected and uninjected control embryos. In this chapter, a difference with p value smaller than 0.05 was considered significant.

3.4 Results

3.4.1 Germ line development

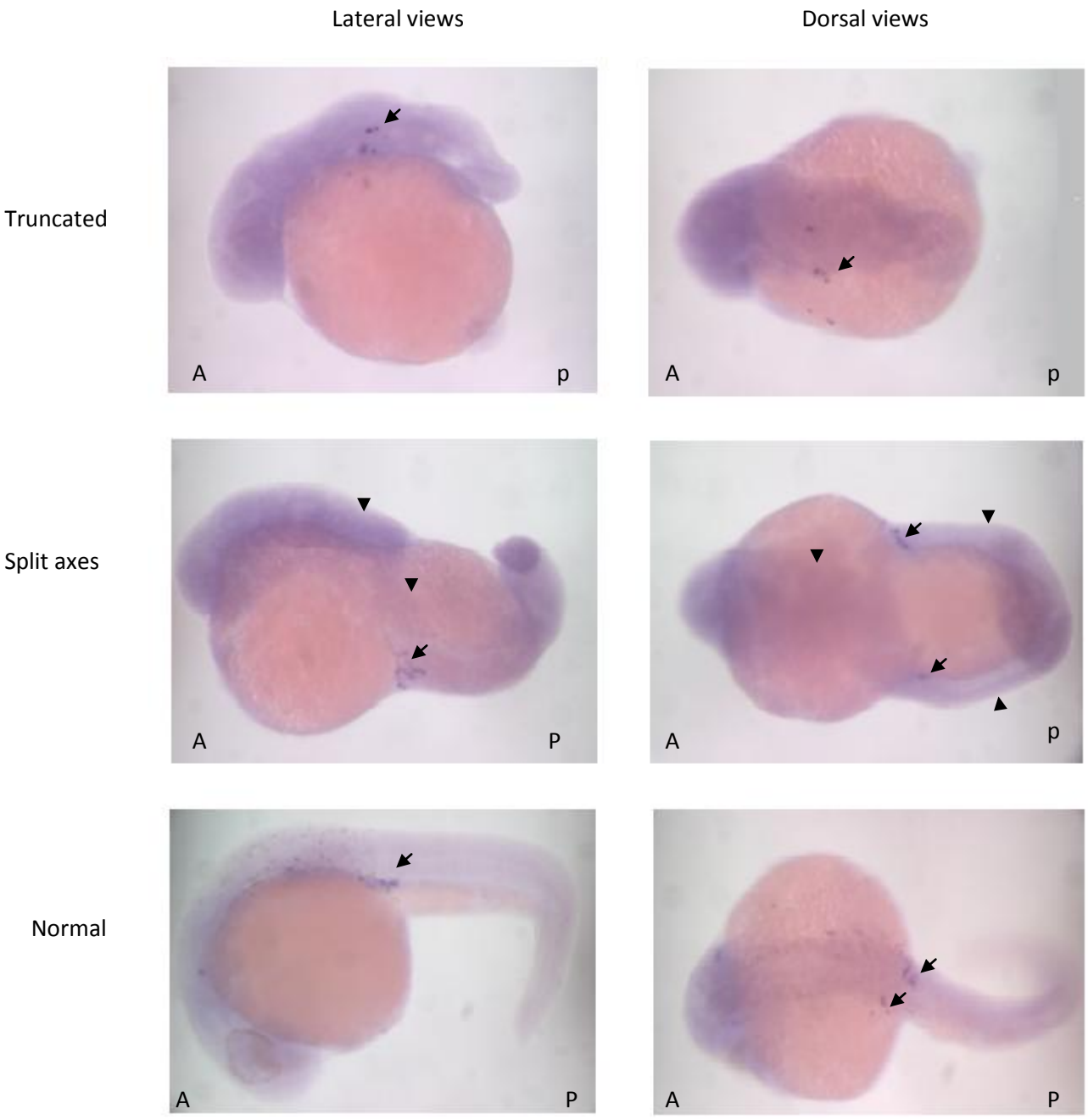
Number of primordial germ cells

Embryos injected with 100pg human *NANOG* could be classified into three categories according to their phenotype at 26-somite stage, truncated tail, split axes and normal (Figure 17a and 17b). As discussed by Köprunner et al. (2001), *nanos*-expressing cells are primordial germ cells. In order to assess the effects of overexpression of human *NANOG* mRNA on zebrafish germ line development, the number of primordial germ cells in three categories of *NANOG*-injected embryos was counted compared with the number of PGCs in uninjected control embryos (Figure 17a and 17b; Figure 18a and 18b; Table 1; Figure 19).

T-tests

Results of t-tests with two samples assuming unequal variances show that p values of all the comparisons in the number of PGCs (*NANOG*-injected embryos with normal phenotype vs uninjected control embryos, *NANOG*-injected embryos with split axes vs uninjected control embryos, and *NANOG*-injected embryos with truncated phenotype vs uninjected control embryos) are bigger than 0.05 (Table 2), indicating that the number of primordial germ cells doesn't change significantly in *NANOG*-injected embryos compared with that in uninjected control embryos.

a


Lateral views
Dorsal views
Truncated
Split axes
Normal
A
p
A
p
A
P
A
p
A
P
A
P

b

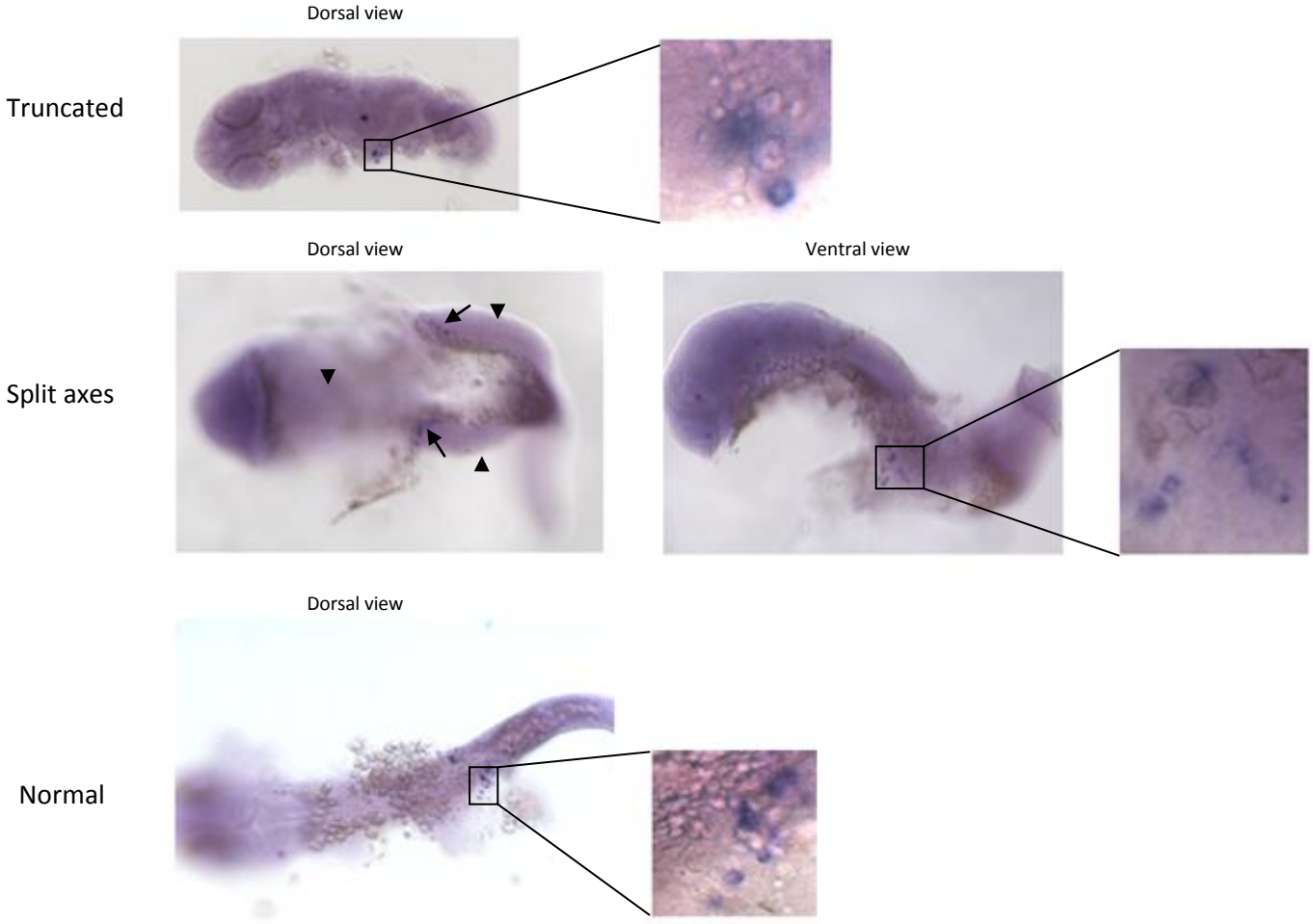
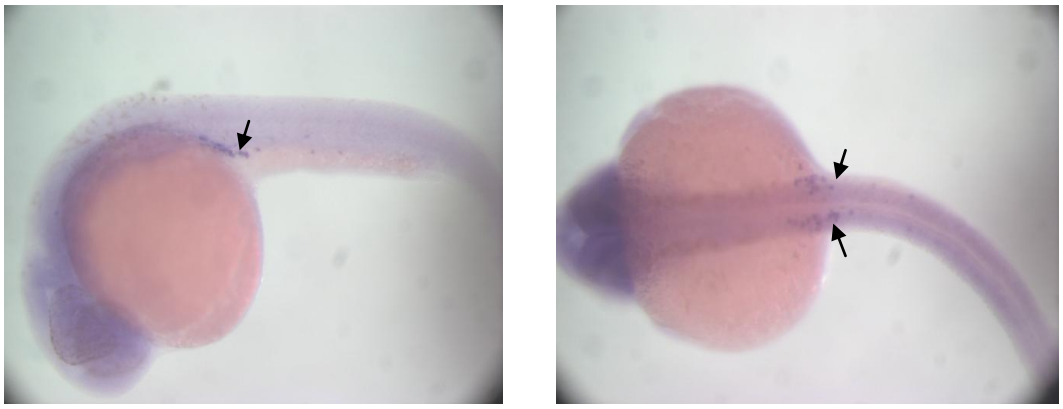


Figure 17. Whole mount in situ of *nanos* at 26-somite stage in embryos injected with 100 pg human *NANOG* mRNA. a, lateral views and dorsal views of *in situ* embryos. b, flattened embryos with yolks removed. In rectangulars are primordial germ cells. Arrows point to primordial germ cells. Arrowheads point to axes.

a



b

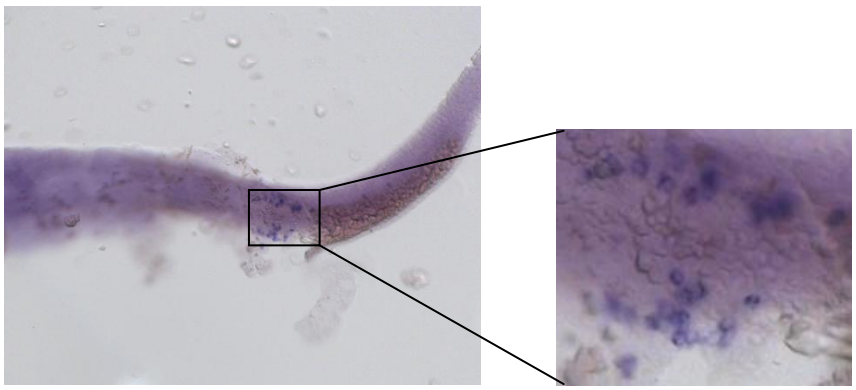
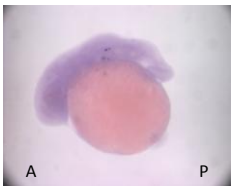
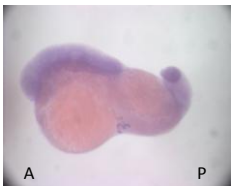
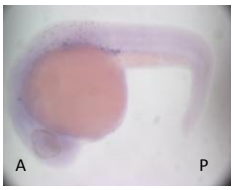
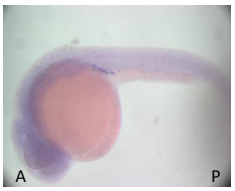


Figure 18. Whole mount in situ of *nanos* at 26-somite stage in uninjected control embryos. a, a lateral view and a dorsal view of an *in situ* embryo. Arrows point to primordial germ cells. b, a flattened embryo with the yolk removed. In the rectangular are primordial germ cells.

Table 1. Number of primordial germ cells in *NANOG*-injected embryos and uninjected control embryos

	Number of primordial germ cells in each embryo for two independent injections	Mean	Standard Deviation	Morphology
Human *NANOG* 100 pg	12,15	13.5	2.1	Truncated 
	10,8,12,15,11,6,8,10,9,16,21,22,12	12.3	4.9	Split axes 
	14,15,12,7,14,17	13.2	3.4	Normal 
Uninjected	15,17,15,13	15	1.6	Normal 

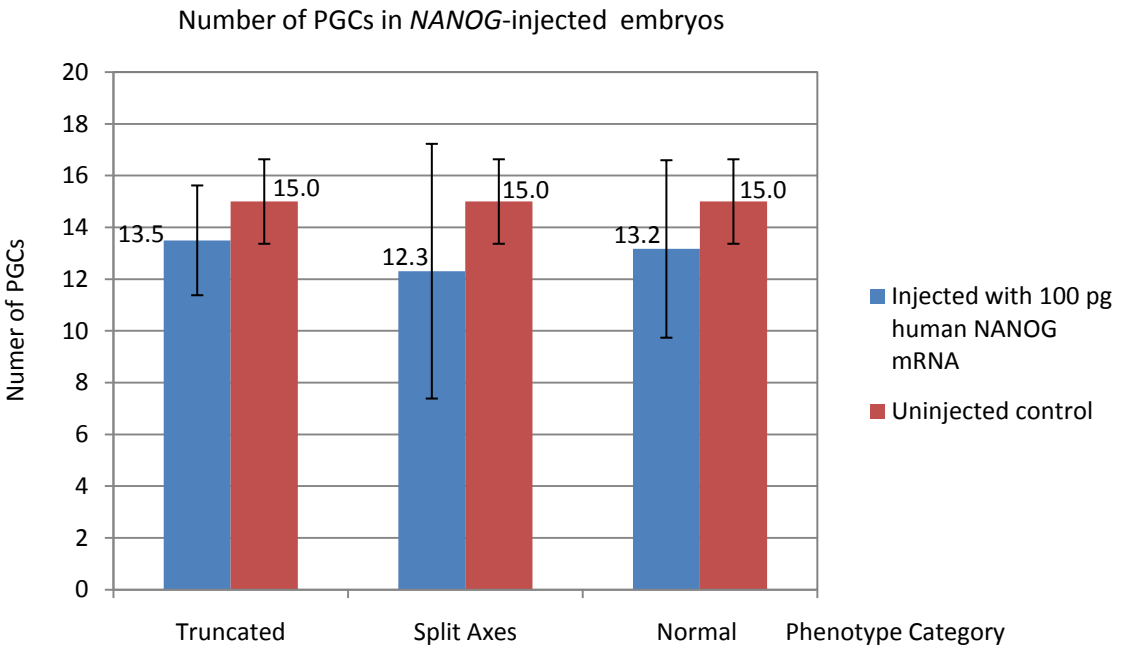


Figure 19. Number of PGCs in *NANOG*-injected embryos compared with uninjected control embryos

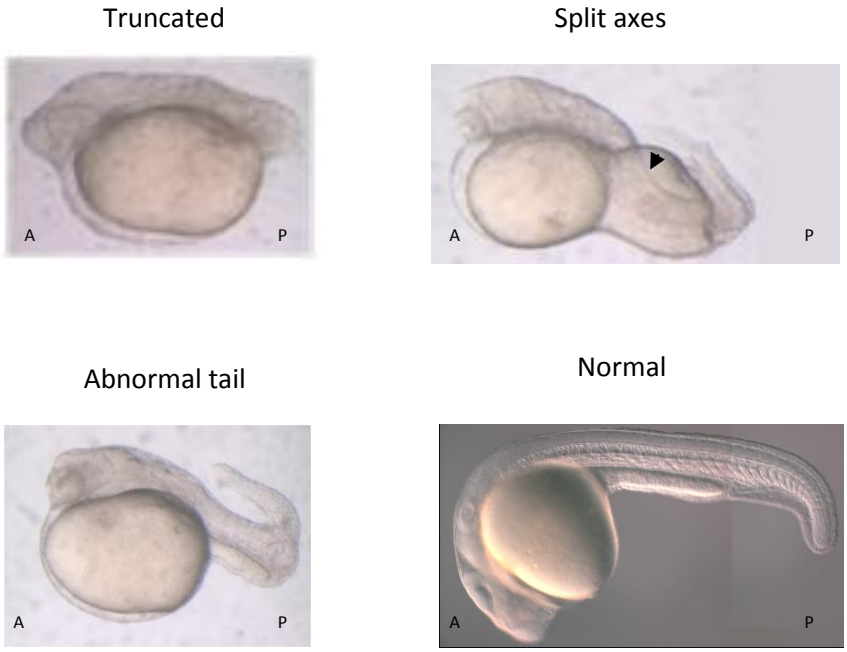
Table 2. p values of rejecting significant changes in the number of PGCs in three categories of *NANOG*-injected embryos according to their phenotype

	p value
NANOG-injected embryos with truncated-tail phenotype vs uninjected control embryos	0.489747
NANOG-injected embryos with split axes vs uninjected control embryos	0.111738
NANOG-injected embryos with normal phenotype vs uninjected control embryos	0.292814

3.4.2 Dorsoventral patterning

We next tested the effects of mouse *Nanog* and human *NANOG* on zebrafish development. Morphology of zebrafish embryos injected with human *NANOG* mRNA and mouse *Nanog* mRNA was recorded (Figure 20a and 20b). There are four categories of phenotype at 26-somite stage, truncated tails (truncated), split axes in the posterior trunk (split axes), abnormal tail and normal (Figure 20a and 20b). It suggests that overexpression of human *NANOG* mRNA or mouse *Nanog* mRNA causes a defect in dorsoventral patterning. Table 3 illustrates the dorsalized changes in the morphology of zebrafish embryos injected with 50 pg and 100 pg human *NANOG* and mouse *Nanog* mRNA and their number. There is a clear concentration-dependent effect with higher doses giving severer truncations and split axes. Among the sixty nine embryos injected with 50 pg human *NANOG* mRNA, 25% are with abnormal tail and 75% are with normal phenotype. The total percentage of abnormal embryos rose up to 56% when we increased the dose of human *NANOG* mRNA up to 100 pg. 18% of embryos are with truncated tails and 38% are with split axes. Among the sixteen embryos injected with 50 pg mouse *Nanog* mRNA, 81% are with split axes and 19% are with normal phenotype. When the dose rises up to 100pg, there are 23% of embryos with truncated phenotype, 10% with split axes and 49% with abnormal tails.

a



b

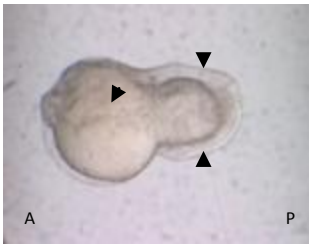
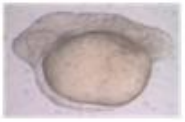
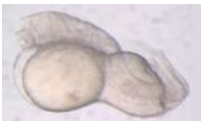
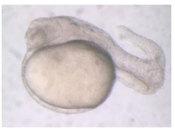
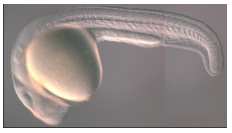


Figure 20. Zebrafish embryos injected with human *NANOG* or mouse *Nanog* mRNA at 26-somite stage. a, phenotypes of *NANOG*-injected or *Nanog*-injected embryos at 26-somite stage. b, a dorsal view of an embryo with split axes at 26-somite stage. Arrowheads point to axes.

Table 3. Changes in morphology of zebrafish embryos after injection of *NANOG*/*Nanog*

Morphology	Truncated 	Split Axes 	Abnormal tail 	Normal 	Total Number
Human *NANOG* 50pg	--	--	17	52	69
Human *NANOG* 100pg	7	--	15	17	39
Mouse *Nanog* 50pg	--	13	--	3	16
Mouse *Nanog* 100pg	9	4	19	7	39