CHAPTER 4 Overexpression of mammalian *NANOG/Nanog* hyperdosalises zebrafish embryos

4.1 Introduction

In chapter 3, the morphology of *NANOG/Nanog*-injected embryos suggests a defect in dorsal-ventral patterning (Driever, 1995). Therefore we hypothesize that overexpression of *NANOG/Nanog* would alter expression of some dorsal-ventral genes but we are uncertain about whose expression it would affect (Schier and Talbot, 2005). In order to further investigate the effects of *NANOG/Nanog* on zebrafish dorsoventral patterning, we evaluated the specific effects of *NANOG/Nanog* on expression of three dorsal genes *chordin*, *squint* and *goosecoid* (Stachel et al., 1993; Schulte-Merker et al., 1994; Schulte-Merker et al., 1997; Dougan et al., 2003), and two ventral genes *wnt8a* and *bmp2b* (Kishimoto et al., 1997; Nguyen et al.,1998; Lekven et al., 2001; Ramel and Lekven, 2004) in this chapter. Quantitative RT-PCR has been an efficient method to assess gene expression at transcript levels and therefore was utilized to evaluate whether overexpression of *NANOG/Nanog* significantly changed expression of these five dorsal-ventral genes (Freeman et al., 1999). Simultaneous amplification of reference genes is a common method for normalising quantitative RT-PCR data (Bustin, 2000; Vandesomplete et al., 2002). In zebrafish, *beta-actin* is one of the commonly used normalisers for quantitative RT-PCR (Tang et al., 2007). It is stably expressed in the first few days of zebrafish development and is expressed in most tissues (Tang et al., 2007). It is suitable for zebrafish developmental time course analysis and is stably expressed following experimental manipulation (Tang et al., 2007; McCurley and

Gallard, 2008). Therefore in this chapter it was used to normalise variances in gene expression caused by amount and quality of different samples. We find that overexpression of *NANOG/Nanog* significantly upregulates expression of the dorsal gene *goosecoid* and significangly downregulates expression of the ventral gene *wnt8a,* which is consistent with the dorsalized phenotypes in mammalian *NANOG*/*Nanog*-injected embryos in chapter 3.

4.2 Materials

Special Equipment

NanoDrop	ND-1000 Spectrophotomer
Micropipet puller	KOPF
Glass capillaries with inner filament	WPI
Microloader	Eppendorf
Electrocoating bath	Apollo Instrumentation
Peltier Thermal Cycler	MJ Research
Real-time PCR thermal cycler	

Kits, enzymes and chemicals

KOD Hot Start PCR Kit (Novagen)

Ultra Pure^{TM} Agarose (Invitrogen)

DNA marker HyperLadder I and HyperLadder IV (Bioline)

Ethidium bromide 10 mg/ml (Sigma)

SuperScriptII Reverse Transcriptase

DNase I (RNase-free).

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

5X First strand buffer

10X DNase Buffer

2X Taqman mix

TRIZOL

Isopropanol

70% ethanol

Phenol:Chloroform:isoamyl alcohol

DTT

beta-actin primers

beta-actin taqman probe

chordin taqman probe

squint taqman probe

goosecoid taqman probe

wnt8a taqman probe

bmp2b taqman probe

2% phenol red

mineral oil

4.3 Methods

Microinjection of human *NANOG* and mouse *Nanog*

H725 fish were set up the night before injection. Embryos were collected the next day. Two sets of *NANOG/Nanog* mRNA, 100 pg human *NANOG* mRNA and 100 pg mouse *Nanog* mRNA was injected into embryos at one-cell or two-cell stage on the same day. Twenty embryos injected with *NANOG/Nanog* mRNA were later collected at shield stage and pooled together as one biological replicate. We collected three replicates in total for human *NANOG* mRNA and mouse *Nanog* mRNA each. Another three biological replicates, each with twenty uninjected embryos pooled together in the same way, were used as control.

Total RNA isolation and reverse transcription

The embryos pooled together were frozen on dry ice. Total RNA was isolated from embryos following a standard protocol (Materials and Methods Appendix). Reverse transcription was performed and cDNA was made (Materials and Methods Appendix).

Quantitative RT-PCR

Quantitative RT-PCR is used to quantify expression of five genes, *chordin*, *squint*, *goosecoid*, *wnt8a* and *bmp2b*. Ct is the cycle number required for specific DNA to reach a threshold of amplification, which is detected by fluorescence its taqman probe generates. The more cycles required to reached the threshold means the less the quantity of specific DNA in the initial sample for amplification. Log 10 of the initial concentration of specific DNA in known samples against Ct generates a straight line, which is standard curve (Higuchi et al., 1993). It enables us to assess the quantity of specific DNA in unknown samples. In order to assess the quantities of *chordin*, *sqt*, *goosecoid*, *wnt8a*, *bmp2b* and *beta-actin*, standard curves of *chordin*, *squint*, *goosecoid*, *wnt 8a*, *bmp2b* and *beta-actin* were yielded with linear regression of a plot of log 10 of their quantities against Ct in a series of diluted cDNA mix (undiluted, 1 in4, 1 in 16, 1 in 64, 1 in 256, and 1 in 1024) of three uninjected control replicates. The standard curves were then used to evaluate the quantities of transcripts in total cDNAs of *NANOG/Nanog*-injected replicates and uninjected control replicates. The quantity of *beta-actin* here was used to normalize variances caused by amount and quality of different samples.

T-tests

We aimed to compare mean of normalized quantities of *chordin*, *squint*, *goosecoid*, *wnt8a* and *bmp2b* in cDNA replicates from *NANOG/Nanog*-injected embryos and uninjected control embryos. As the two samples are independent and transcript levels of these genes are normally distributed in *NANOG/Nanog*-njected embryos and uninjected control embryos, we performed a two-tailed t-test with two samples assuming unequal variances to test the difference. Difference in normalized quantities with p value smaller than 0.1 was considered significant in this chapter.

4.3 Results

Total RNA extraction and reverse transcription

Gel electrophoresis of RNA shows that RNA of good quality was obtained, with two bands of about 750 bp and 1,300 bp, representing distribution of RNA conformations (Figure 21). Figure 22 illustrates PCR amplification of *beta-actin* segments from cDNAs of three *NANOG*-injected replicates, three *Nanog*-injected replicates and three uninjected control replicates. The 400 bp *beta-actin* segment can be amplified from all the cDNAs, which indicates that the cDNAs are of good quality and could be used for quantitative RT-PCR assays. All bands are of similar brightness, indicating that *beta-actin* in all the cDNAs are of similar quantity.

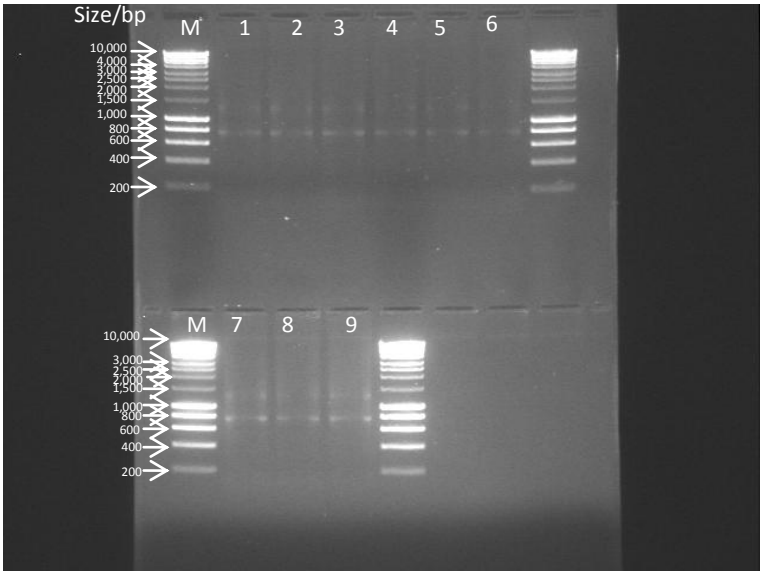

Figure 21. RNA Gel Electrophoresis

1-3, RNA extracted from three replicates of embryos injected with 100 pg human *NANOG*. 4-6, RNA extracted from three replicates of embryos injected with 100 pg mouse *Nanog*. 7-9, RNA extracted from three replicates of uninjected control embryos. M, Marker Hyperladder I. Band pattern of all the nine lanes is the same. There are two bands in each lane, of approximately 750 bp and 1,300 bp. These bands represent distribution of RNA conformation.

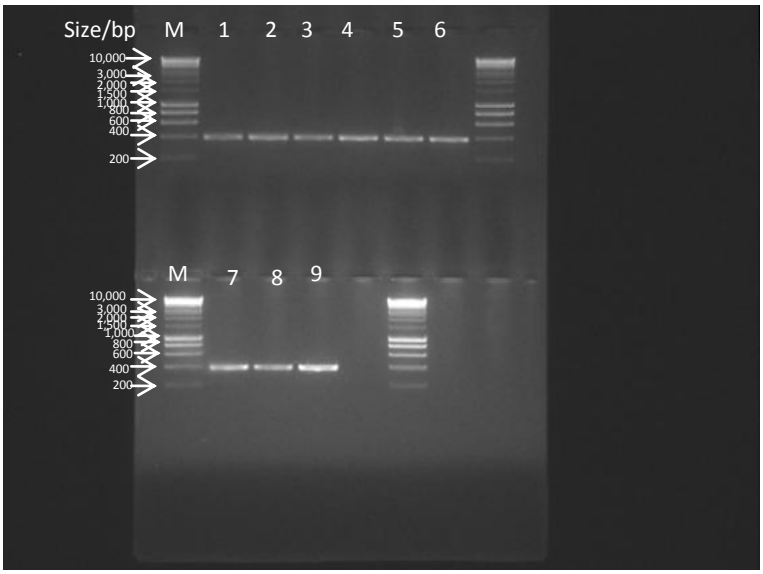

Figure 22. PCR amplification of the *beta-actin* segment

In lanes 1-9 are *beta-actin* segments amplified from cDNAs of nine samples (1-3, three replicates of embryos injected with 100 pg human *NANOG*; 4-6, three replicates of embryos injected with 100 pg mouse *Nanog*; 7-9, three replicates of uninjected control embryos). M, Marker Hyperladder I. There is a clear single band of about 400 bp in each lane.

Quantitative RT-PCR

Quantitative RT-PCR is an effective way of quantifying gene expression (Freeman et al., 1999). The work described in this session is aimed to quantify expression of five genes *chordin*, *squint, goosecoid*, *wnt8a,* and *bmp2b* in *NANOG*-injected embryos, *Nanog*-injected embryos and uninjected control embryos. Standard curves are generated by plotting log 10 of the initial quantities of these five genes and *beta-actin* against their cycle numbers (Ct) at which amplification detected by fluorescence from their samples reaches the threshold in a series of diluted cDNA mix (undiluted, 1 in4, 1 in 16, 1 in 64, 1 in 256 and 1 in1024) of uninjected replicates (Results Appendix). Log 10 of quantities of *chordin*, *squint*, *goosecoid*, *wnt8a*, *bmp2b* and *beta-actin* in *NANOG/Nanog*-injected and uninjected replicates are then inferred from standard curves with their cycle numbers used to reach the threshold of amplification (Results Appendix). Therefore expression of *chordin*, *squint*, *goosecoid*, *wnt8a*, and *bmp2b* in *NANOG/Nanog*-injected replicates and uninjected control replicates can be quantitatively measured by normalizing their quantities with quantities of *beta-actin*. Figure 23 illustrates quantities of *chordin*, *squint*, *goosecoid, wnt8a,* and *bmp2b* relative to quantities of *bactin*.

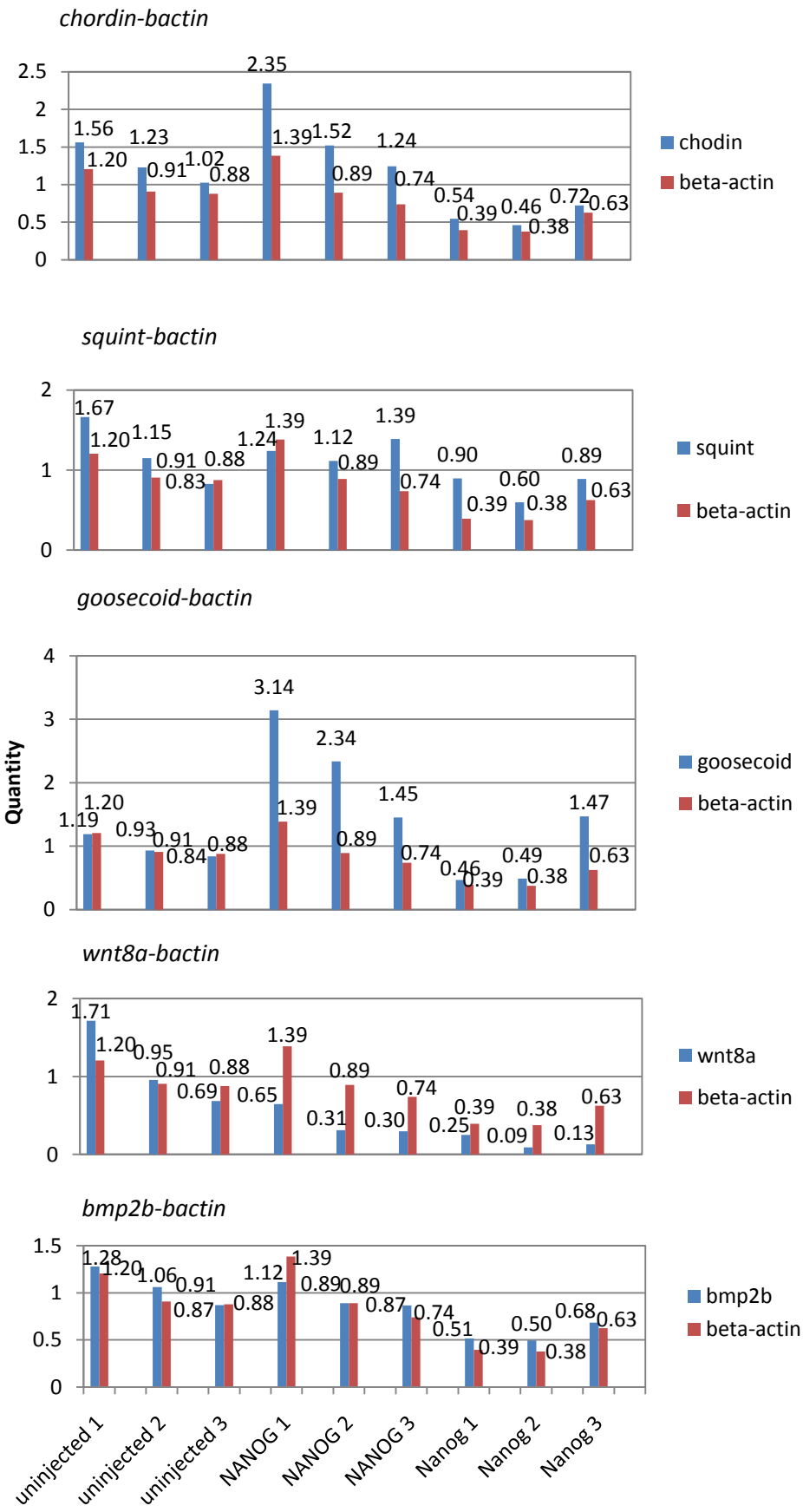

Figure 23. Quantities of *chd*, *sqt*, *gsc*, *wnt8a* and *bmp2b* relative to *bactin*

Figure 23. Quantities of *chd*, *sqt*, *gsc*, *wnt8a* and *bmp2b* relative to *beta-actin*. Three replicates of uninjected embryos are labelled uninjected 1, uninjected 2 and uninjected 3 in the horizontal axis. In a similar way, three replicates of embryos injected with human *NANOG* are labelled as *NANOG* 1-3. Three replicates of embryos injected with mouse *Nanog* are labelled as *Nanog* 1-3.

T-tests

T-tests were performed to test whether injection of mammalian *NANOG/Nanog* mRNA causes significant changes in gene expression of *chordin*, *squint*, *goosecoid*, *wnt8a* and *bmp2b* (p values are shown in table 4). The results show that overexpression of *NANOG/Nanog* significantly upregulates expression of the dorsal gene *goosecoid*, and significantly downregulates expression of the ventral gene *wnt8a* (Figure 24; Table 4). This is consistent with the dorsalized phenotypes of *NANOG/Nanog*-injected embryos.

Table 4. p values of rejecting significant difference in five genes' expression between *NANOG/Nanog*-injected and uninjected embryos

Comparison in gene expression between *NANOG/Nanog*-injected and uninjected embryos	p value
chordin	0.128799
squint	0.177210
goosecoid	0.009049
wnt8a	0.070615
bmp2b	0.666329

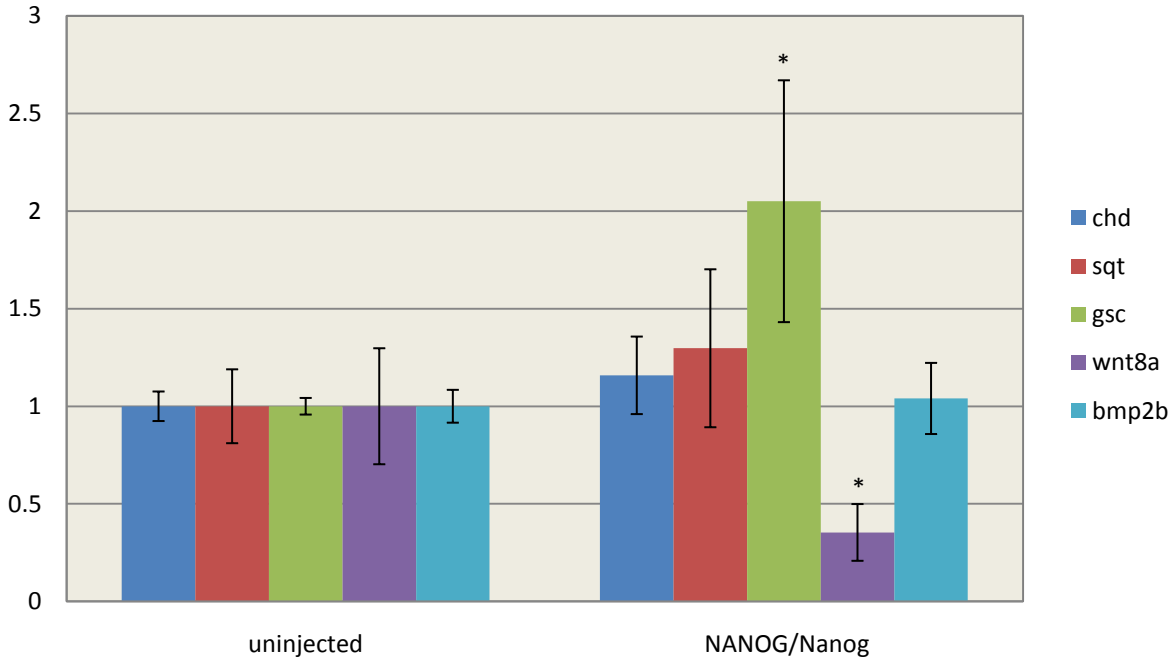

Figure 24. Bar chart showing the changes in expression of five dorsal and ventral genes after injection with *NANOG/Nanog* mRNA. Expression of *goosecoid* is significantly upregulated, while *wnt8a* expression is significantly downregulated. Upregulation of *goosecoid* expression and downregulation of *wnt8a* expression are labelled with * in the chart. The change would be rejected with p value smaller than 0.1 for them. Expression of *chordin*, *squint* and *bmp2b* are not significantly altered in injected embryos.