Stony Brook University

The roles of *sox2* and canonical Wnt signaling pathway in fate specification of tailbud bipotential stem cells

A Thesis Presented

by

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to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science

in

Biochemistry and Cell Biology

Stony Brook University

December 2015

Stony Brook University

The Graduate School

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Abstract of the Thesis

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During vertebrate development, formation of the head -to-tail body axis is shaped by a group of unspecified cells at the caudal region of the embryo. The undifferentiated tail bud stem cells are capable of adopting mesodermal or neural cell fates as the embryo develops, but the mechanism of this patterning process remains largely unknown. Our previous studies confirm that Wnt signaling is required in tail bud stem cells to repress the neural transcription factor *sox2* and induce mesodermal fate. However, it is unclear if *sox2* repression at the cell-autonomous level is the critical factor required for Wnt mediated mesoderm induction. Using zebrafish transgenic lines, we reveal that downregulation of *sox2* is necessary for mesodermal progenitors to exit the tail bud. Surprisingly, we also find that ectopic *sox2* can induce neural tissue in cells normally destined to give rise to somites in a Wnt signaling-dependent manner. Constitutive expression of Wnt represses *sox2* expression and promotes adaption of mesodermal fate, whereas overexpression of *sox2* downregulates Wnt pathway activity, inducing neural fate adaption. Moreover, simultaneous high level of Wnt signaling and high level of *sox2* promotes the long term bipotential state of tailbud stem cells. Together our results reveal the requirement of precise regulation of differential expression levels of *sox2* and Wnt pathway in proper tissue patterning during embryo development.

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List of Abbreviations

BCIP/NBT - 5-bromo-4-chloro-3-indolyl-phosphate/ nitro blue tetrazolium

BMP - Bone morphogenetic protein

CNS - Central nervous system

DAPI - 4',6-diamidino-2-phenylindole

EM - Embryo media

EpiSCs - Epiblast stem cells

ESCs - Embryonic stem cells

FGF - Fibroblast growth factor

GFP - Green fluorescent protein

Hpf - Hours post-fertilization

HS - Heat shock

Hsp70 - Heat-shock protein 70

Lefty1 - Left-right determination factor 1

MEP – Mass embryo production

Mf20 - Myosin Heavy Chain Antibody

Msgn1 - Mesogenin 1

Ngn1 - Neurogenin 1

Oct - Octamer-binding transcription factor

PFA – Paraformaldehyde

RD - Rhodamine dextran

Sox2 - SRY (sex determining region Y)-box 2

Tbx6 - T-box 6

TCF - T-cell factor

TLB wild-type - Tupfel long-fin cross to Brian's wild-type

Wnt - Wingless-type MMTV integration site family

WT – Wild-type

Acknowledgments

I would like to thank my principle investigator, Dr. Benjamin Martin, and all members of the Martin lab. For Dr. Martin, thank you for allowing me to join your lab and for your patience in teaching and guiding me. I have learned a lot from you, not only in lab work but in writing and presenting skills. You are a great advisor and I am lucky to have the opportunity to learn from you. For Richard Row, Brian Kinney, Hana Goto, Lily Sarrafha and Eric Paulissen, thank you for teaching me all lab techniques, giving me advices on my thesis and my future plan. Most importantly, thank all of you for accompanying me throughout my Master study.

I would like to thank Dr. Sirotkin for being my second reader, providing several suggestions for my project at the lab meeting. Also, I would like to thank Dr. Dean for guiding me throughout my Master's career, advising and helping me in academics, lab work and PhD application. Last but not least, I would like to thank Neal Bhattacharji for taking care of our zebrafish.

I. Introduction

During vertebrate development, the posterior body of an embryo is derived from a structure called the tailbud. This anatomical protrusion forms at the posterior end of the embryo after gastrulation and gives rise to distinct germ layers (Beck, 2014). The tailbud contains a group of bipotential stem cells that give rise to the presomitic mesoderm, axial mesoderm and posterior spinal cord. These tailbud stem cells are divided into several domains and undergo specification depending on the patterns of gene expression (Beck, 2014). During the past decades, several signaling pathways including Wnt, BMP, FGF and Nodal pathways have been identified as regulators of tailbud formation and posterior body development (Thorpe et al.,2005, Szeto and Kimelman, 2004), but the mechanism behind this process remains unknown. Our lab is dedicated to understanding the signaling pathways that control the fate decision of uncommitted cells within the tailbud, especially focusing on the adoption of mesodermal fate and somitogenesis.

Canonical Wnt signaling pathway (hereby known as Wnt) plays several roles in vertebrate development, including posterior mesoderm formation. In mouse, Wnt/β-catenin signaling is thought to promote the formation of mesoderm and tailbud. Mouse embryos with null allele of *wnt-3a* show absence of posterior somite, disrupted notochord and the lack of tailbud (Takada et al., 1993). Wnt-3a-mutant cells do not migrate normally through primitive streak to adopt paraxial mesodermal fates, instead they stay within the streak and form ectopic neural cells (Yoshikawa et al., 1997). Research in zebrafish has also revealed the crucial role of Wnt pathway in formation of posterior body. Embryos with downregulation of both *wnt-3a* and *wnt-8* via morpholinos fail to form tails (Thorpe et al., 2005). In addition, the zebrafish contains a niche of embryonic mesodermal progenitors within the tailbud which is supported by a Brachyury-Wnt autoregulatory loop (Martin and Kimelman, 2008 and 2010). This autoregulatory loop creates an environment with high levels of Wnt signaling and low levels of retinoic acid, promoting somitogenesis as well as posterior body development (Martin and Kimelman, 2008 and 2010). The canonical Wnt pathway not only helps to maintain progenitor niche, but also regulates the fate decision of uncommitted stem cells within the tailbud. Continuous expression of Wnt signaling is required and sufficient for bipotential tailbud stem cells to adopt mesodermal fate and give rise to somites. Wnt pathway first induces bipotential stem cells in tailbud to form mesodermal progenitors; after progenitors migrate out of the tailbud, Wnt signaling works in mesodermal progenitors to promote somite formation. Downregulation of canonical Wnt

signaling results in adoption of neural fate or formation of posterior vascular endothelium among the mesodermal progenitors during second fate decision (Martin and Kimelman, 2012). Intriguingly, loss of Wnt leads to upregulation of a neural progenitor marker *sox2*, and the expression pattern is identical with the region of where fate decision takes place (Martin and Kimelman, 2012). These results not only suggest the potential interaction between canonical Wnt pathway and transcription factor *sox2,* but also reveals that the fate decision of tailbud stem cells is a continuous process - bipotential stem cells face several fate decision points during development in order to be fully and correctly specified into specific structures.

SRY (Sex Determining Region Y)-box 2 (*sox2*) belongs to the SRY-related HMG-box (SOX) family of transcription factors. *Sox2* is known to regulate embryonic development at different stages, such as maintaining neural stem cells and pluripotency of embryonic stem cells (Graham et al., 2003). SoxB1 transcription factors are believed to play a key role in specification of neuroectoderm. *Sox2/3/19a/19b* loss of function study suggests that SoxB1 genes coordinate cell patterning via regulating several developmental-dependent genes (Okuda et al., 2010). *Sox2*-mutant mouse embryos show early embryonic lethality with the phenotype that lacks epiblast and abnormal trophectoderm formation, suggesting that *sox2* functions in maintaining stem cell pluripotency and promoting early cell fate decisions collaborating with *oct4* (Avilion et al., 2002). Moreover, *sox2* can regulate the fate of axial stem cells in a *tbx6*-dependent manner. Activation of *tbx6* represses *sox2 cis*-regulatory module N1, and therefore reduces *sox2* expression level and promotes formation of paraxial mesoderm. Loss of *tbx6* results in upregulation of *sox2*, leading to cells originally with mesodermal fate to adopt neural fate and form ectopic neural tubes. Moreover, misexpression of *sox2* in primitive paraxial mesoderm of wild-type embryos induces the development of ectopic neural tube, which is similar to *tbx6*-loss-of-function embryos (Takemoto et al., 2011).

Interaction between SOX proteins and Wnt signaling has been revealed to affect several developmental processes as well as cancer progression. In most cases SOX proteins cause repression of downstream Wnt target genes, while a few of them can activate the expression of Wnt-target genes (Kormish et al., 2010). For example, *sox17* inhibits β-catenin/TCF signaling in SW480 cells through inducing degradation of both β-catenin and TCF. However, *sox4* stabilizes β-catenin and promotes proliferation of colon cancer, indicating that SOX transcription factors can regulate the activity of canonical Wnt signaling by acting as antagonists and agonists (Sinner

et al., 2007). Interestingly, in mouse and chick, some reports suggest that canonical Wnt signaling seems to stimulate *sox2* expression by activating *cis*-regulatory modules N1, thus promoting the formation of neural tissue (Takemoto et al., 2006, 2011). However, other studies reveal that high expression level of *wnt-3a* in mouse epiblast represses endogenous *sox2* and induces mesodermal structure without affecting neural formation (Jurberg et al., 2014). Moreover, in zebrafish inhibition of Wnt signaling leads to upregulation of *sox2* (Martin and Kimelman, 2012). Our preliminary data also implicates that overexpression of *sox2* in zebrafish embryos seems to have little influence on the formation of normal spinal cord. Such contrasting results suggest that *sox2* plays a variable role during fates specification of bipotential stem cells, depending on the combinational expression levels with Wnt signaling.

Using several transgenic zebrafish lines, our main purpose is to understand the possible role of *sox2* in fate decision of tailbud stem cells, mapping the potential interaction between *sox2* and canonical Wnt signaling. Zebrafish are the best vertebrate model system to use for this project due to the transparency of their embryos. The optical clarity of zebrafish allows the application of techniques such microinjection and cell labeling, making them a very useful tool. In addition, the fast developmental speed of zebrafish makes it easier to conduct lineage tracing compared to mouse and Xenopus. We speculate that during development, continuous expression of Wnt signaling and downregulation of *sox2* are both required for tailbud bipotential stem cells to leave the tailbud and adopt mesodermal fate, ultimately giving rise to somites. We found that overexpression of *sox2* after gastrulation results in formation of ectopic neural tissue at the positions where muscle tissue should normally be, and these ectopic neurons seem to be derived from uncommitted progenitors within somites. The ratio between *sox2* and Wnt determines the cell fate acquisition during tailbud stem cells development. High expression level of Wnt and low expression level of *sox2* lead to somite formation whereas low level of Wnt and high level of *sox2* give rise to neurons. Simultaneous high level of Wnt signaling and high level of *sox2* promotes the long term bipotential state of tailbud stem cells. Our hypothesis has revealed the coordination of Wnt signaling and *sox2* in allocation of cell fates during development as well as the possibility to alter the fate of mesodermal progenitors even after they leave the tailbud.

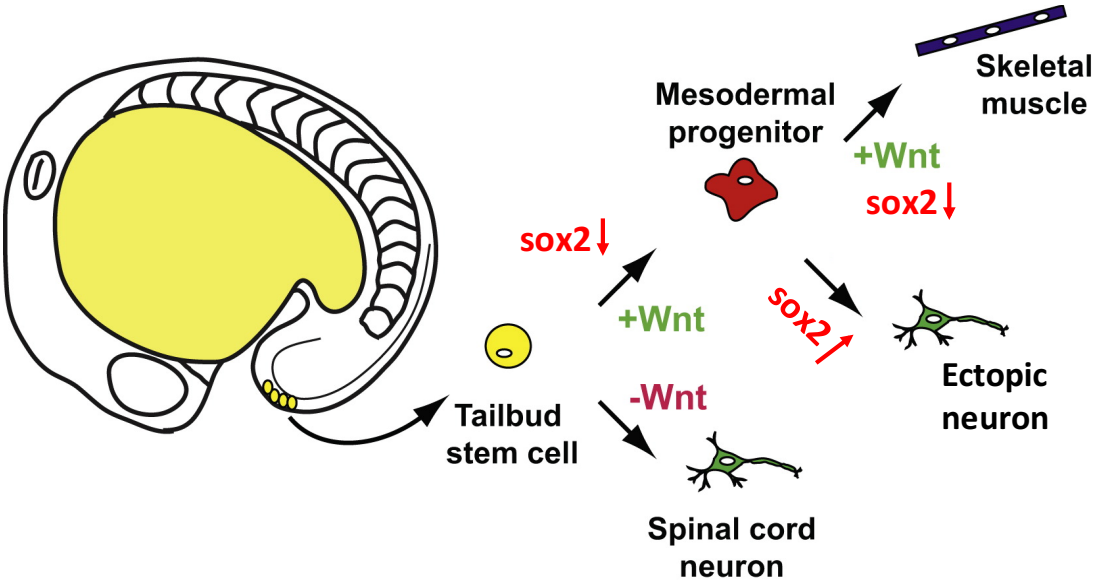

Figure 1. A model of how *sox2* and Wnt signaling function in fate decisions of tailbud stem cells. Continuous high expression level of Wnt signaling represses *sox2*, promoting tailbud stem cells to form mesodermal progenitor and somites. Overexpression of *sox2* inhibits tailbud bipotential stem cells from leaving the tailbud and induces mesodermal progenitor to adopt neural fate even after they join the somites.

II. Materials and Methods

1. Whole-Embryo Heat Shock

Embryos collected from different crosses were grown at 28°C and heat-shocked in pre-warmed (41°C) embryo media (EM) for 30 minutes at a given developmental stage. After heat shock, embryos were placed under the desired temperature (18°C, 23°C or 28°C) for further development. Embryos were fixed in 4% Paraformaldehyde (PFA) at 4°C overnight after heat shock.

2. *In situ* Hybridization

Whole-mount *in situ* hybridization was performed as described by Griffin et al (1995). Probes for *sox3*, *ngn1* and *GFP* were applied at 1:200 dilutions and anti-digoxigenin Fab fragment was used at a 1:5000 dilution. The probes were developed in Alkaline Phosphatase Buffer with BCIP/NBT, varying amounts of time depending on the probe and developmental stage. Staining was stopped by one 5-minute wash in 100% methanol, then replaced with fresh 100% methanol and stored at -20 for at least 30 minutes. Embryos were gradually transferred from methanol to 70% glycerol and mounted on the slides for imaging.

3. Antibody Staining

Immunohistochemistry was conducted similarly as previously described (Martin and Kimelman, 2012). Embryos were fixed using 4% PFA at 4°C overnight. Mf20 antibody (Developmental Studies Hybridoma Bank) was used at a 1:50 dilution as a muscle marker, and DAPI was used at 1:200 dilution of 1mg/ml stock to detect nuclei. Anti-mouse Alexa-568 conjugated secondary antibody (Invitrogen) was used at a 1:500 dilution to detect the primary antibody. Embryos were mounted on the slides via 70% glycerol for imaging using spinning disk confocal microscopy.

4. Double Fluorescence *in situ* Hybridization

Double fluorescence *in situ* hybridization experiment was performed essentially as described previously (Lauter et al., 2011). *Sox2*-fluorescein probe and *GFP*- digoxigenin probe were used at 1:100 dilutions. Anti-FL-POD Fab fragments (1:500 dilution) and anti-DIG-POD Fab

fragments (1:1000 dilution) were used to detect *sox2* and *GFP* probes, respectively. DAPI was used at a 1:200 dilution for 5 minutes as nuclear marker.

5. Cell Transplantation and Microinjection

The DNA or RNA injections were conducted using wild-type embryos at 1-cell stage. Transplantation was performed by removing cells from sphere-stage donors (either wild-type or transgenic lines, 2% rhodamine dextran-injected) and transplanting them to the dome-stage or 30%-epiboly-stage wild-type hosts using an eppendorf cell-tram vario. The transplanted host embryos were grown to later stage for further observation.

6. Generation of *HS:sox2* Transgenic Line and *HS:gal4sox2* Transgenic Line

For *HS:sox2* transgenic line, zebrafish *sox2* coding sequence was inserted into a *Tol2*-flanked *hsp70:p2a-NLS Kikume* plasmid vector using the ligation-independent cloning (LIC) method. The annealed plasmid was transformed into DH5α cells and the insertion was confirmed by sequencing. For *HS:gal4sox2* transgenic line, Gal4 activation domain (constructed by David Turner) was fused to the C-terminus of zebrafish *sox2* coding sequence and inserted into a *Tol2 hsp70:p2a-NLS Kikume* plasmid vector. The resulting plasmid (*Tol2 hsp70:sox2-Kikume* or *Tol2 hsp70:gal4sox2-Kikume*) was injected into wild-type embryos obtained from Mass Embryo Production (MEP) system (AquaticHabitats) to create a stable transgenic line as previously described (Kawakami, 2004). The injected embryos were grown in EM for 6 days after injection then placed in the system to establish a stable line. F0 generation transgenic fish were outcrossed with wild-type and screened by heat-shocking F1 embryos at bud stage and screening for green fluorescence 3 hours post heat-shock. Strong positive adults were outcrossed with wild-type again to establish the F1 generation.

7. Transgenic Zebrafish Lines and Wild-Type

In this thesis, four transgenic lines including *HS:sox2*, *HS:TCF△C*, *HuC-GFP*, *HS:β-catenin* and *siam-GPF* were used. Heat shock-inducible Wnt inhibitor transgenic line *HS:TCF△C* and heat shock-inducible Wnt activator transgenic line *HS:β-catenin* were used to investigate the relationship between *sox2* and Wnt signaling pathway during development of tailbud stem cells. *HuC-GFP* transgenic line was used as a neuron reporter to confirm the position and morphology

of *sox2*-induced ectopic neurons. Wnt reporter transgenic line *siam-GPF* was used to identify the expression patterns of canonical Wnt signaling in wild-type embryos and *HS:sox2* embryos. Embryos from Tupfel Long-fin cross to Brian's (TLB) wild-type fish were used for injection, transplantation and outcrossing with transgenic lines.

III. Results

1. Overexpression of *sox2* induces the formation of ectopic neural tissue

In order to test our hypothesis that downregulation of *sox2* is necessary for tailbud stem cells to exit the tailbud and adopt somitic cell fate, a heat shock (HS) inducible *sox2* transgenic zebrafish line created by previous lab member Steve Tsotras was used in whole embryo or transplant experiments. Several heat shock-inducible transgenic line were used in this project. A target gene inserted downstream of hsp70 promoter can be conditionally activated simply by raising the temperature, which is especially beneficial in studying gene functions at specific time-points of development. By transplanting cells from *HS:sox2* embryos to wild-type embryos, we found that cells overexpressing *sox2* via multiple heat-shocks mostly stay within the tailbud (figure 2B-C). Compared to cells transplanted from wild-type embryos, *sox2*-upregulated cells cannot properly give rise to somites, while the formation of spinal cord seems to be less affected (figure 2). This result supports the hypothesis that *sox2* overexpressing tailbud stem cells cannot properly undergo the mesodermal progenitor program and remain in the tailbud longer. In addition to transplantation experiments, we are also interested in how modulation of *sox2* can affect other progenitor domains and signaling pathways. Embryos collected from outcross of *HS:sox2* to wild-type were heat-shocked at bud stage and fixed at 24 hpf (hours post fertilization) for *in situ* hybridization (Figure 3). As indicated by two different neuronal markers *sox3* and *ngn1*, overexpression of s*ox2* results in formation of ectopic neural cells along the ventro-lateral regions of the embryo (Figure 3B and E). Two lines of ectopic neural tissue are distinct from normal spinal cord and seem to be located at the lateral edge of somites (Figure 3C and F). Notice that compared to the wild-type, embryos from *HS:sox2* transgenic line have relatively shorter body and enlarged tail, suggesting that cells do not normally migrate out of the tailbud, causing disruption of the posterior body formation.

To further confirm that these *sox2*-induced ectopic cells are neural tissue, neuron reporter transgenic line *Huc-GFP* was crossed to *HS:sox2* transgenic fish. HuC-GFP is a neuron-specific protein that expresses in most regions of the nervous system (Kim et al., 1996). In *Huc-GFP* transgenic line, 5'-upstream fragments of HuC promoter is able to drive GFP expression (Park et al., 2000), therefore if the ectopic cells are actually neurons, they should exhibit green fluorescence. As shown in Figure 4, embryos with overexpression of *sox2* present two distinct

lines of ectopic neural tissue apart from the spinal cord, and the pattern is consistent with the results from *in situ* hybridization. Embryos positive for both *HS:sox2* and *HuC:GFP* also exhibit a phenotype with shorter body and larger tailbud, but the pattern of spinal cord seems to be similar to that of wild-type embryos. This suggests that *sox2* overexpression does not have a significant effect on the development of nervous system but affects the elongation of posterior body and somites formation.

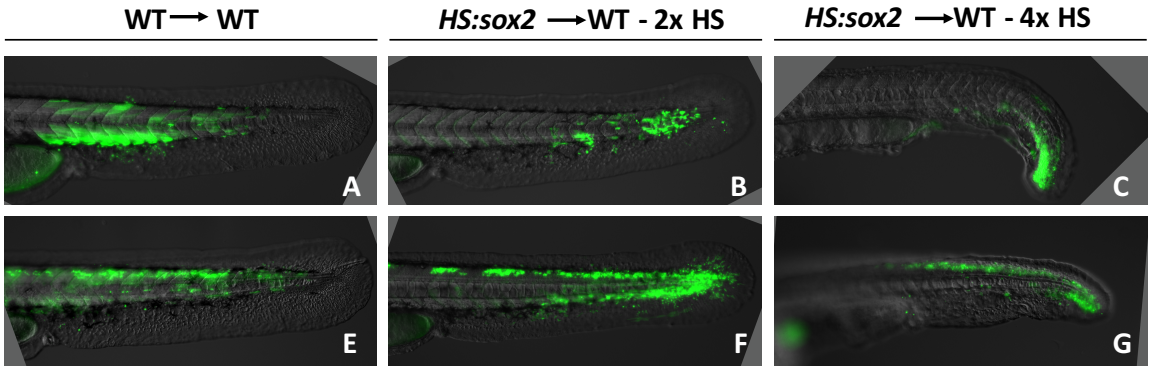

Figure 2. Overexpression of *sox2* via multiple heat-shocks prevents tailbud bipotential stem cells from leaving the tailbud and differentiating into somites. Transplantation was conducted by removing cells from sphere-stage *HS:sox2* (B-C, F-G) or wild-type (A, E) donors and transplanting them to shield-stage wild-type hosts. 2-time heat-shocks (B, F) or 4-time heat-shocks (C, G) were conducted at dome/30% epiboly-stage (1st heat shock, before transplantation), bud-stage (2nd heat shock), three hours after the 2nd heat shock (3rd heat shock) and three hours after the 3rd heat shock (4th heat shock). Transplanted cells that give rise to either mesoderm (A-C) or neurons (E-G) are shown (data from Richard Row).

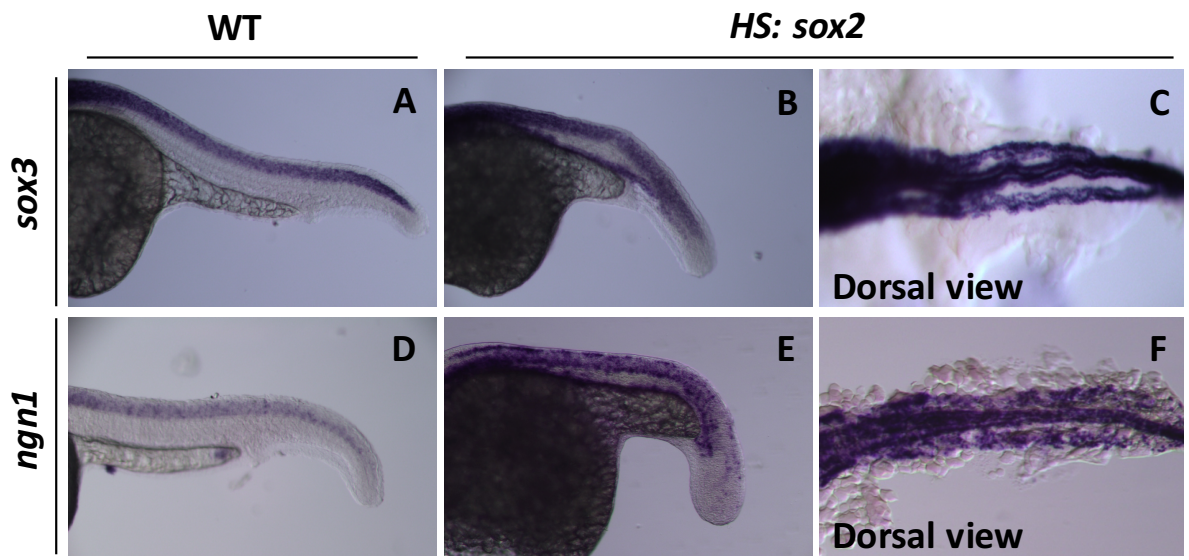

Figure 3. Overexpression of *sox2* induces ectopic neural tissue formation. Embryos from wild-type (A and D) and *HS:sox2* transgenic line (B-C, E-F) were heat shocked at bud stage and fixed at 24 hpf. Two neuronal markers *sox3* (A-C) and *ngn1* (D-F) were applied to embryos for *in situ* hybridization.

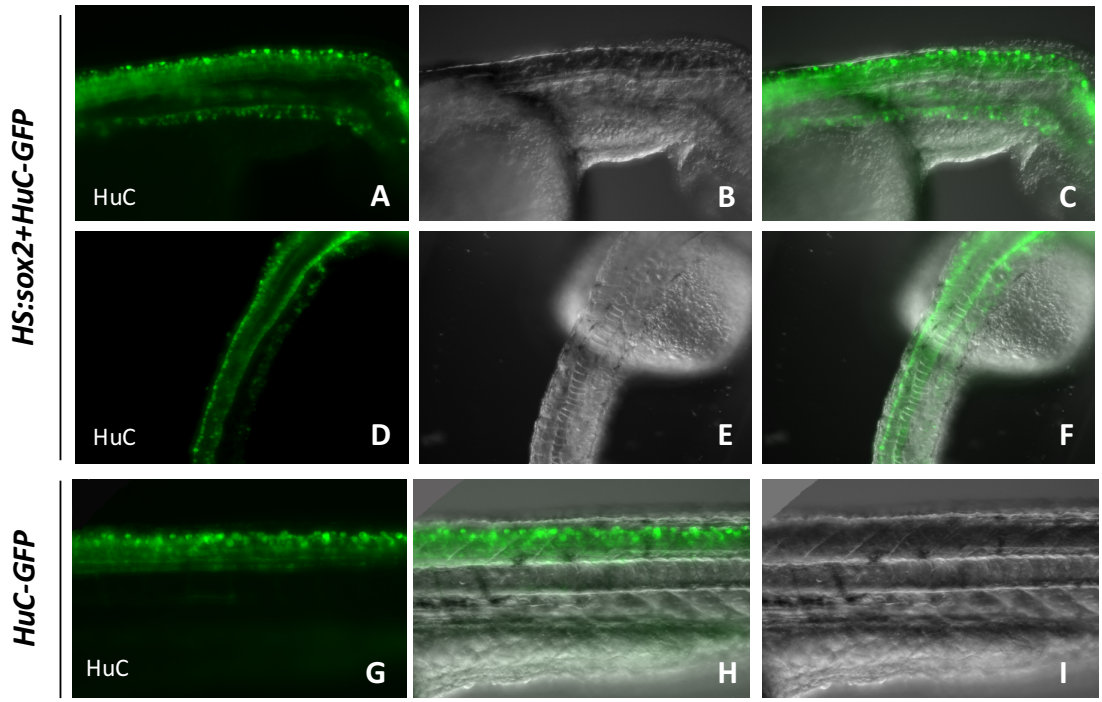

Figure 4. Ectopic cells induced by *sox2* overexpression are confirmed to be neurons. *HuC-GFP* transgenic line was used as neuron reporter. Embryos from *HS:sox2*/*HuC-GFP* cross (A-F) were heat shocked at bud stage, live imaging was applied at 30 hpf. *HuC-GFP*-only embryos act as control (G-I).

After confirming that *sox2*-induced ectopic cells are indeed neurons, we wanted to further examine whether these ectopic neurons morphologically resemble normal neural cells. Using spinning disk confocal microscopy to image *HS:sox2*/*HuC-GFP* embryos, we were able to observe the distinct compartments of *sox2*-induced ectopic neurons, including cell bodies and axons. The ectopic neurons are morphologically similar to neurons from normal spinal cord, suggesting that they might be functional as normal neural tissue (figure 5).

In addition to whole-embryo heat shock, transplantation was conducted to determine if *sox2* can cell-autonomously induce neurons in cell that have already joined the paraxial mesoderm. Cells removed from sphere-stage *HS:sox2*/*HuC-GFP* embryos (donors) were transplanted to dome-stage wild-type embryos (hosts) and heat shocked when hosts reached bud stage. 2% rhodamine dextran (RD) was injected into donors to mark the transplanted cells. As shown in Figure 6, some of transplanted cells were induced into ectopic neurons within somite tissue,

which were marked by the expression of HuC-GFP. The *sox2*-induced ectopic neurons are morphologically normal, with axons extending from cell bodies (figure 6I).

Together these results show that *sox2* plays a role in tailbud stem cells fate decisions by regulating their ability to leave the tailbud and inducing neural fate in cells after they leave the tailbud. Continuous repression of *sox2* is required for normal mesodermal development.

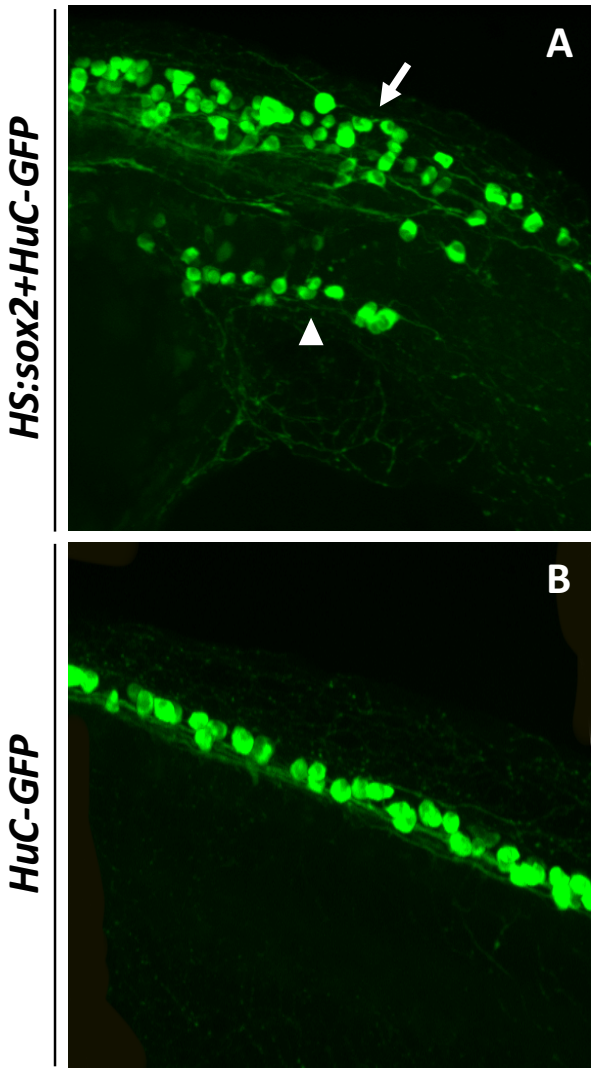

Figure 5. *Sox2*-induced ectopic neural cells are morphologically normal. *HS:sox2*/*HuC-GFP* embryos (A) were heat shocked at bud stage then fixed at 30 hpf for imaging. *Sox2*-induced ectopic neurons are indicated by arrowhead, whereas spinal cord is marked by arrow (A). Neural cells from the spinal cord of *HuC-GFP*-only embryos are presented as control (B).

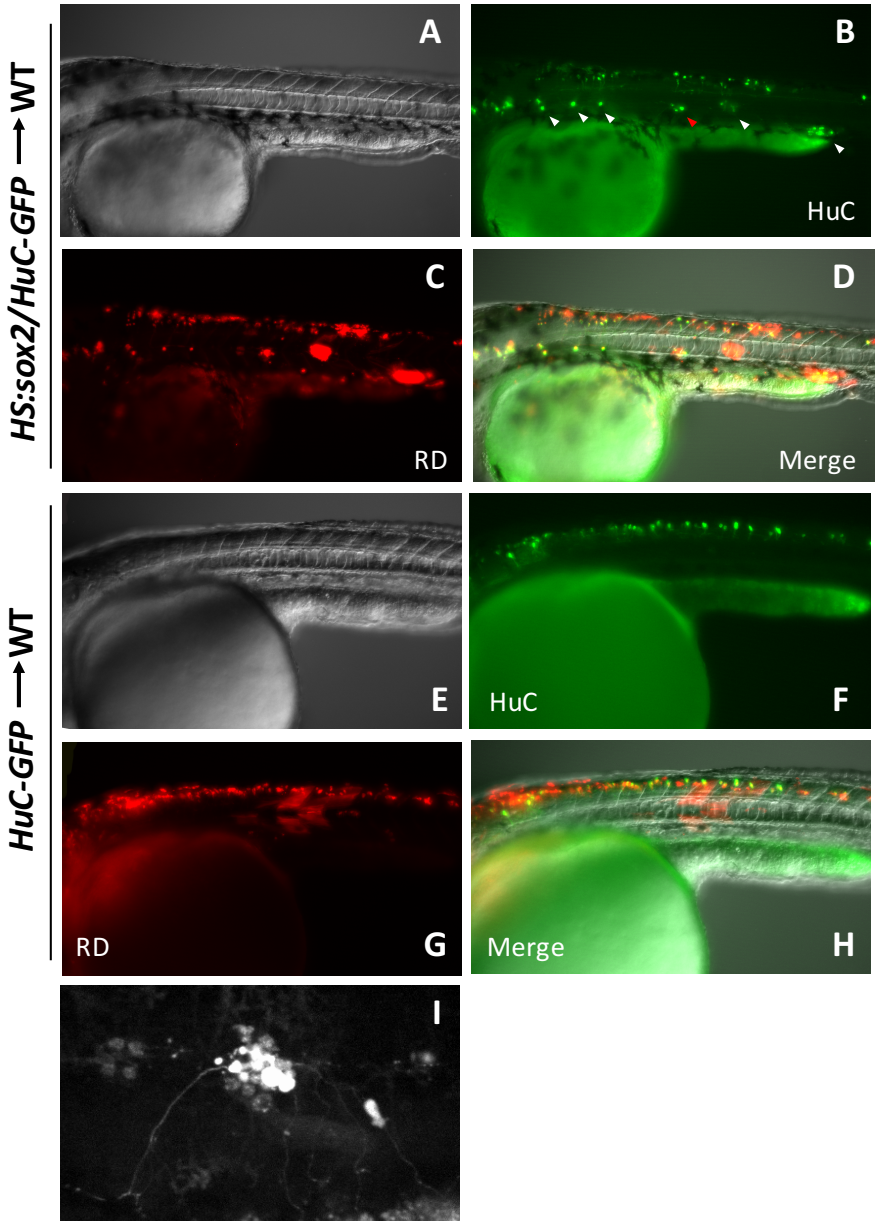

Figure 6. Overexpression of *sox2* induces ectopic neuron formation within somites. Transplantation was conducted by removing cells from sphere-stage *HS:sox2*/*HuC-GFP* embryos (A-D) or *HuC-GFP* (E-H, act as control) embryos and transplanting them to dome-stage wild-type hosts. 2% rhodamine dextran (RD) was injected into donors at one-cell stage to mark the transplanted cells (C). *Sox2*-induced ectopic neurons are indicated by arrowhead (B), neurons indicated by red arrowhead were examined under confocal microscopy for morphological identification (I). A merged picture (D) reveals that ectopic neurons are located within somites.

2. *Sox2*-induced ectopic neurons are derived from uncommitted cells within somites

In both transplantation and whole-embryo heat shock experiments, we found that overexpression of *sox2* does not affect the formation of nervous system. We assume that the ectopic neural tissue shown in figure 2 and 3 are derived from unspecified progenitors within somites. In order to test our hypothesis, antibody staining was applied to *HS:sox2*/*HuC-GFP* embryos to identify the positions of *sox2*-induced ectopic neural cells (figure 7). Apart from the few overlaps between neural marker HuC and muscle marker mf20, we found that the ectopic neurons are mostly located at the lateral edge of somite tissues (figure 7D). Since the lateral regions of somites contain undifferentiated cells (dermomyotome) (Keynes and Stern, 1988), the locations of *sox2*-stimulated ectopic neural tissue support our hypothesis that upregulation of *sox2* can interrupt paraxial mesoderm patterning via altering the fate of mesodermal progenitors within somites.

To further support our speculation, we investigated the patterns of ectopic neurons when paraxial mesoderm is first forming. Two heat shocks were applied to *HS:sox2*/*HuC-GFP* embryos. The first heat shock was done at shield stage, which is the stage that mesodermal patterning starts to take place (Stickney et al., 2000). The second heat shock was done at bud stage to maintain the high expression level of *sox2* through out the early stages of somite development (figure 8). If the ectopic neurons are indeed derived from uncommitted cells within somites, the early heat-shock should cause a significant increase in ectopic neural tissue. As opposed to two distinct lines of ectopic cells demonstrated in figure 3 and 4, overexpression of *sox2* during early somitogenesis results in distribution of ectopic neurons throughout the region where somites would normally form (figure 8A-F). This pattern is confirmed by the results from *in situ* hybridization experiments (figure 9). Apart from the change of ectopic neurons pattern, double heat shocked *HS:sox2*/*HuC-GFP* embryos exhibit a more severe phenotype compared to single heat shocked embryos, with a significantly shorter body and large tailbud (figure 8B and C). The enlarged tailbud illustrates the inability of tailbud stem cells to leave the tailbud and join presomitic mesoderm when they have expression level of *sox2*.

In summary, our results indicate that stable repression of *sox2* is required for the second fate decision of the tailbud stem cells to differentiate into mature muscle tissue after they leave the tailbud. Upregulation of *sox2* induces unspecified progenitors within somites to adopt neural fate and give rise to ectopic neural tissue.

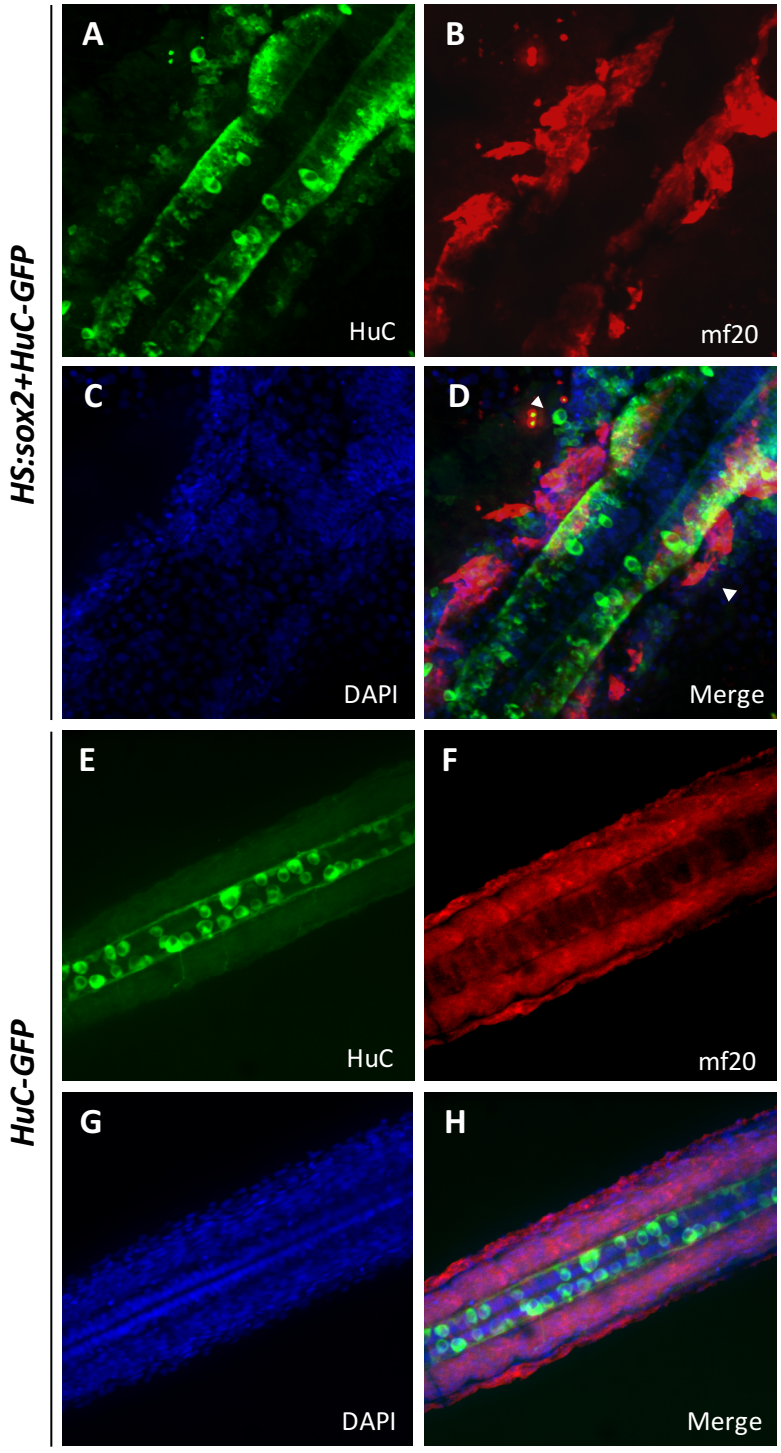

Figure 7. The positions of *sox2*-induced ectopic neural cells. Embryos collected from *HS:sox2*/*HuC-GFP* cross (A-D) were heat shocked at bud stage. Antibody staining of muscle protein (B, F) was applied. DAPI was used to stain nuclei. Arrowheads (D) indicate the positions of ectopic neurons. *HuC-GFP*-only embryos act as control (E-H).

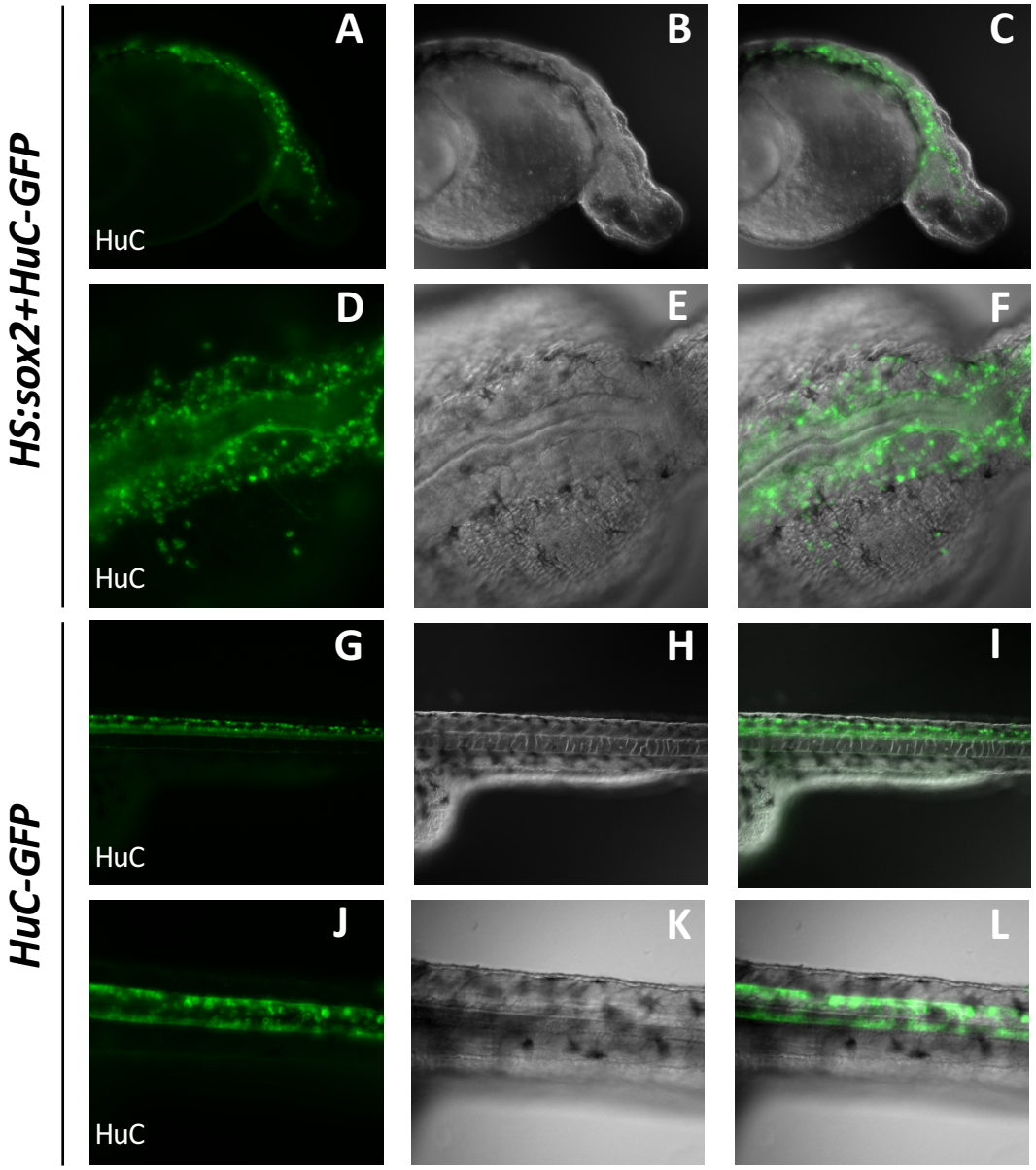

Figure 8. Overexpression of *sox2* at early stage of somitogenesis alters the patterns of *sox2*-induced ectopic neural cells. Embryos from *HS:sox2*/*HuC-GFP* cross (A-F) were heat shocked at shield stage and bud stage, live imaging was applied between 30-36 hpf. *HuC-GFP*-only embryos act as control (G-L).

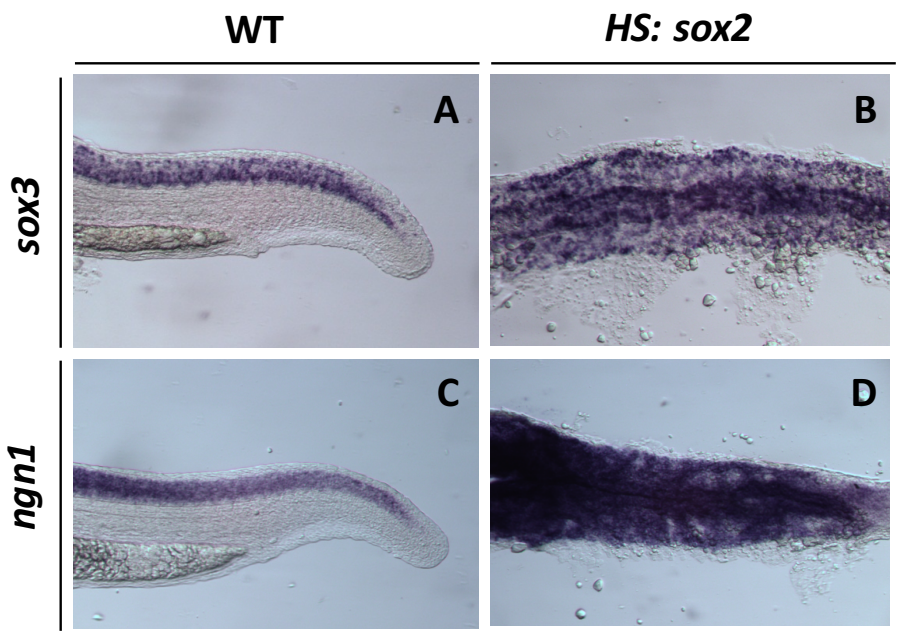

Figure 9. The pattern of *sox2*-induced ectopic neurons is confirmed by *in situ* hybridization. Embryos from wild-type (A and C) and *HS:sox2* transgenic line (B and D) were heat shocked at shield stage and bud stage, *in situ* hybridization was applied at 24 hpf. Ectopic neurons were labeled via two neuronal markers *sox3* (A-B) and *ngn1* (C-D).

3. Interaction between *sox2* and canonical Wnt signaling pathway

SOX family transcription factors can act as antagonists or agonists via regulating the activity of downstream Wnt factors, while Wnt pathway can also control some *Sox* genes expression (Kormish et al., 2010). In zebrafish, downregulation of Wnt signaling increases the expression level of *sox2* within the tailbud (Martin and Kimelman, 2012). Based on the hypothesis that *sox2* represses Wnt signaling, we took advantage of Wnt reporter transgenic line *siam-GFP*, in which the expression of GFP is driven by Wnt response element upstream of the promoter of Xenopus direct β -catenin target gene *siamois*, to investigate the expression level of Wnt signaling in *sox2*-overexpressing embryos (figure 10). As predicted, overexpression of *sox2* results in a reduction of Wnt pathway activity in embryos heat-shocked at bud stage (figure 10A-F). The decrease can be clearly observed in tailbud region as well as in body trunk. However, *sox2* seems to not be able to reduce Wnt pathway activity at the very tip of the tailbud, creating an environment with high Wnt and high *sox2*. This is presumably the reason why transplanted *sox2*-overexpressed

cells don't leave the tailbud. In addition, overexpressing *sox2* at the 12-somite stage does not exhibit obvious difference in expression level of Wnt signaling between wild-type embryos and *HS:sox2/siam-GFP* embryos (figure 10G-L), suggesting that *sox2* alone is not sufficient to repress expression of Wnt signaling during late developmental stages.

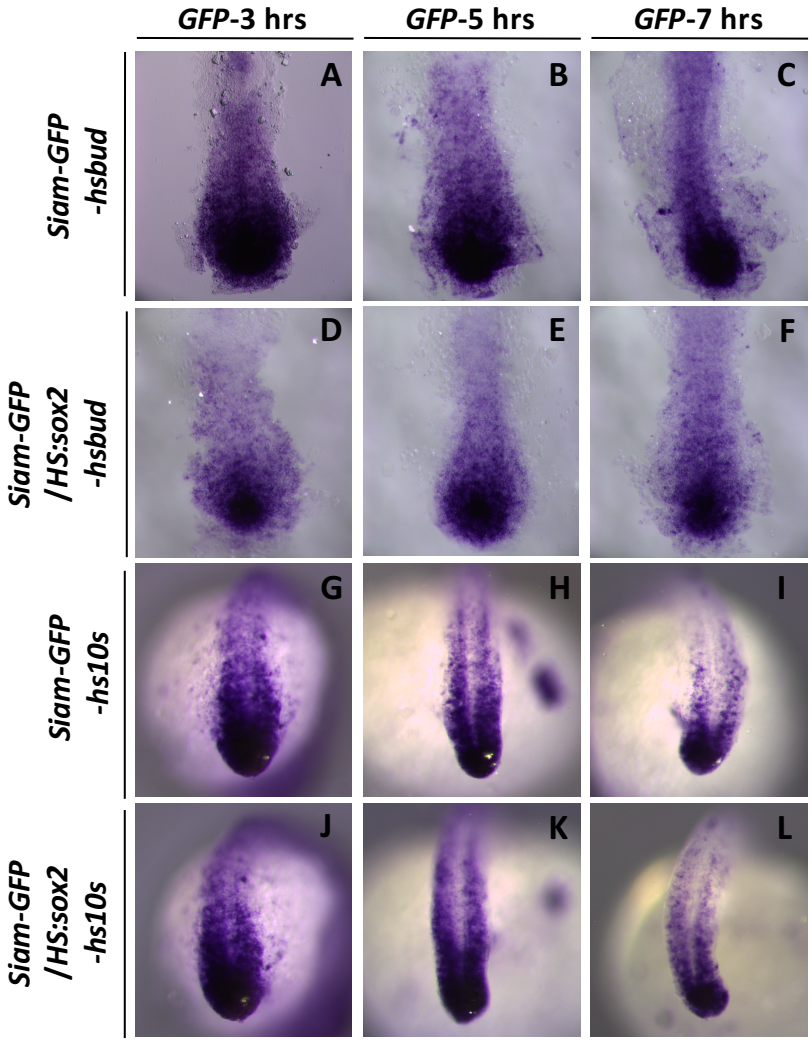

Figure 10. Overexpressing *sox2* at early developmental stage represses activity of Wnt signaling. Embryos from *HS:sox2/siam-GFP* cross were heat shocked at bud stage or 12-smoite stage. Embryos were sorted into four different groups (wild-type, *siam-GFP*-only, *HS:sox-only* and *HS:sox2/siam-GFP*) based on fluorescence and fixed at 3 (A, D, G, J), 5 (B, E, H, K) and 7 (C, F, I, L) hours post heat shock. *In situ* hybridization was applied to embryos from the *HS:sox2/siam-GFP* group (A-F) and the *siam-GFP*-only group (G-L) using a *GFP* RNA probe to mark the expression patterns of Wnt signaling pathway activity.

Since the expression level of *sox2* and Wnt pathway seem to be partially dependent on *sox2*-Wnt interaction and is crucial for cell fate decision during development, we aimed to identify the effects of differential expression level between Wnt pathway *sox2* in fate specification of bipotential tailbud stem cells. We speculate that via simultaneous upregulation of *sox2* and downregulation of the Wnt pathway, *sox2*-induced ectopic neural tissue should be expanded due to the elimination of *sox2*-repressive Wnt signaling pathway. *HS:sox2* and heat shock inducible Wnt inhibitor transgenic line *HS:TCF△C* were used to test our hypothesis. *HS:TCF△C* transgenic line is a heat shock-inducible dominate repressor of Wnt signaling which can produce C-terminally truncated TCF after heat shock. As shown in figure 11, embryos with both overexpression of *sox2* and inhibition of Wnt signaling (figure 11D-E, I-J) exhibit a significant expansion of ectopic neurons in either bud stage heat shock or 12 somites heat shock compared to *sox2*-overexpression-only embryos (figure 11B and G). The expression patterns of neuronal marker *sox3* extend to the very end of the tail and present all over somites (figure 11E and J). This patterns reveal that downregulation of Wnt signaling enables *sox2* to induce neural fate at the very tip of the tailbud (figure 11E), which is normally blocked by high Wnt pathway activity due to the inability of *sox2* to repress Wnt signaling (figure 10). Notice that *HS:sox2*-only embryos which were heat-shocked at 12-somite stage induce less ectopic neural tissue formation compared to bud-stage-heat-shocked embryos (figure 11G). This may be related to the inability to repress Wnt pathway expression via overexpressing *sox2* at late developmental stages (figure 10J-L). Notably, inhibition of Wnt signaling alone does not cause an expansion of neural tissue in cells that have already joined the paraxial mesoderm, indicating a synergistic effect when both *sox2* is up-regulated and Wnt signaling is down-regulated.

In addition to Wnt loss-of-function, heat shock-inducible Wnt gain-of-function transgenic line *HS:β-catenin* was crossed to *HS:sox2* transgenic fish to further confirm the roles of *sox2* and Wnt during the induction of ectopic neurons (figure 12). Compared to *sox2*-overexpressing embryos, embryos overexpressing both *sox2* and *β-catenin* exhibit a reduction in *sox2*-induced ectopic neurons when heat-shocked at bud stage (figure 12C-D, G-H). For embryos heat-shocked at 12-somite stage, *sox2/β-catenin*-overexpressing embryos present a decrease in the quantity of *sox3*-marked ectopic neurons but an increase in the amount of ectopic neurons marked by the other neuronal marker ngn1, compared to embryos that only overexpressed *sox2* (figure 12K-L, O-P). Nevertheless, embryos with *sox2*/*β-catenin* overexpression still display an elimination in

total amount of ectopic neurons relative to *sox2*-overexpression group. This is possibly due to the temporal expression patterns of *sox3* and *ngn1* during neuron development. *Sox3* is one of the earliest-expressed transcriptional factors in neurons and is responsible for maintenance of undifferentiated stem cell state; whereas *ngn1* is know to stimulate the differentiation of neural progenitors, marking the neurons that undergo differentiation (Dee et al., 2008, Ma et al., 1996, Korzh et al., 1998). In *sox2/β-catenin*-overexpressing embryos, the expression level of Wnt signaling is higher than *sox2*-overexpressing embryos, thus promoting cell differentiation. As a result, more ectopic neurons are marked by *ngn1* relative to *sox3* in *sox2/β-catenin*-overexpressing embryos, causing an expansion of ngn1 marker compared to embryos that only overexpress *sox2*.

Together, our results indicate that *sox2*-Wnt interaction is likely to be repressive, and the formation of *sox2*-indcued ectopic neurons can be expanded or eliminated depending on differential expression level between *sox2* and Wnt pathway.

In summary, our study reveals a potential regulatory mechanism in cell fate decision of tailbud biopotential stem cells. Continuous downregulation of *sox2* and expression of Wnt is required for tailbud stem cells to adopt mesodermal fate and correctly give rise to somites after leaving tailbud. High *sox2* expression and repression of Wnt pathway favor the adoption of neural fate, leading to the formation of ectopic neurons within somites. High level of *sox2* and high level of Wnt pathway keep tailbud bipotential stem cells staying in the tailbud in a lineage primed state. Precise regulation of differential expressing levels of *sox2* and Wnt pathway is crucial for proper tissue patterning during embryo development.

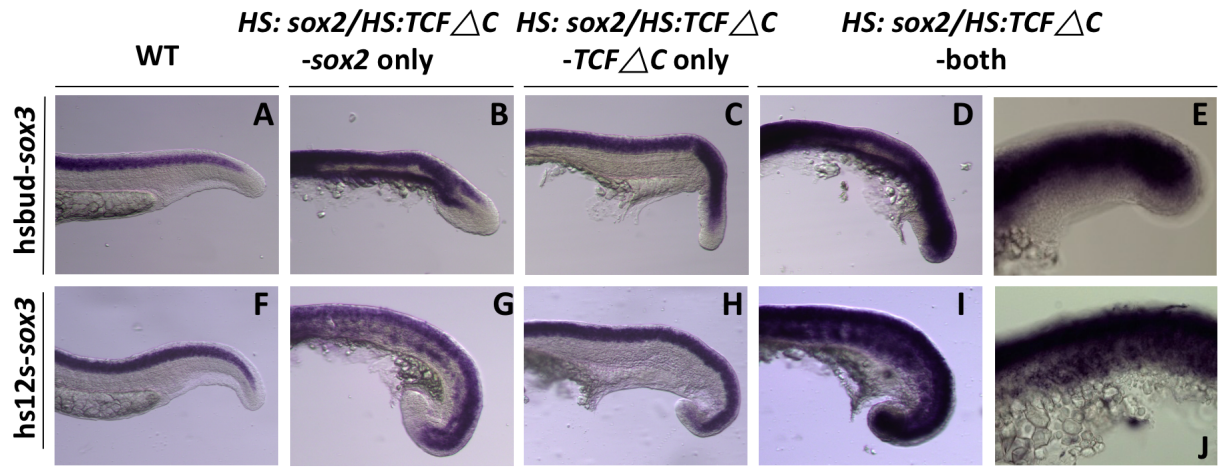

Figure 11. Simultaneous overexpression of *sox2* and downregulation of Wnt signaling leads to an expansion of *sox2*-stimulated ectopic neurons. Embryos collected from *HS:sox2*/*HS:TCF△C* cross were heat shocked at bud stage (A-E) or 12-somite stage (F-J). Embryos were sorted into four groups: *HS:sox2*-only (B and G), *HS:TCF△C*-only (C and H), *HS:sox2*/*HS:TCF△C*-both (D-E, I-J) and wild-type (A and F) based on fluorescence. Embryos from all four groups were fixed at 24 hpf. *In situ* hybridization was applied to detect the formation of ectopic neurons via neuronal marker *sox3*.

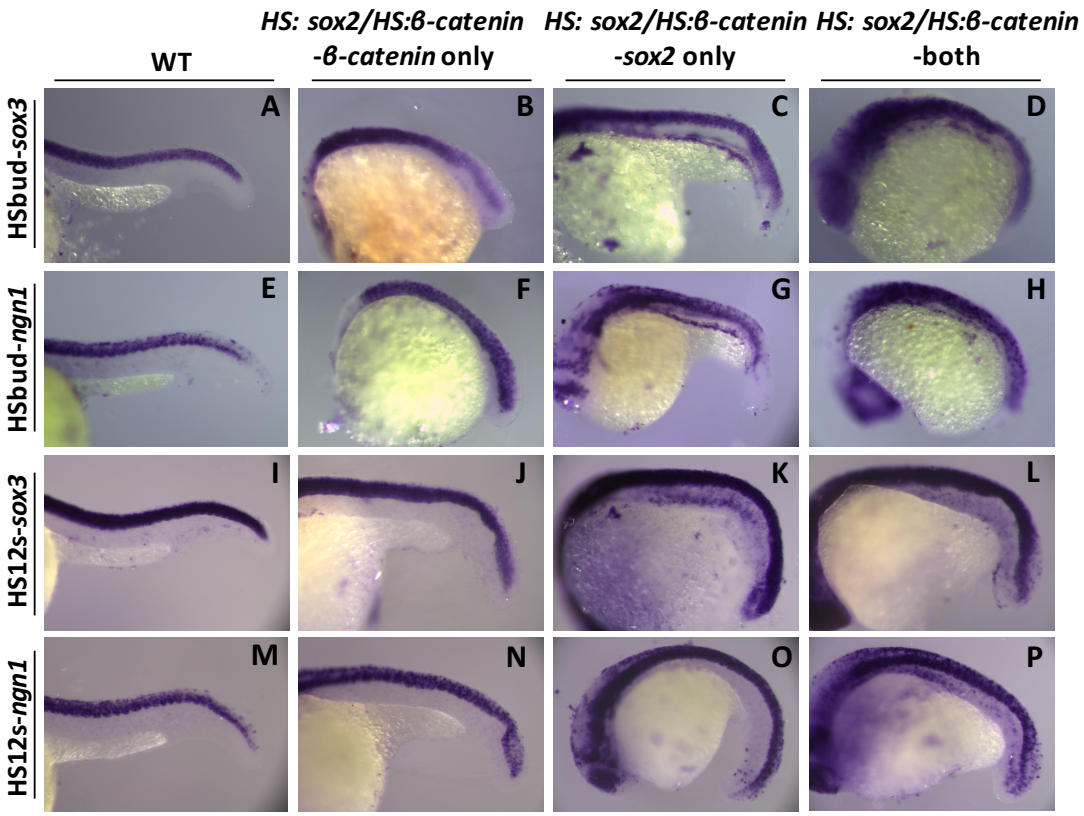

Figure 12. Simultaneous overexpression of *sox2* and Wnt signaling leads to a reduction of *sox2*-stimulated ectopic neurons. Embryos collected from *HS:sox2*/ *HS:β-catenin* cross were heat shocked at bud stage (A-H) or 12-somite stage (I-P) and sorted into four different groups: wild-type (A, E, I, M), *HS:β-catenin*-only (B, F, J, N), *HS:sox2*-only (C, G, K, D) and *HS:sox2*/*HS:β-catenin*-both (D, H, L, P) based on fluorescence. Embryos were fixed at 24 hpf and analyzed by *in situ* hybridization. Two different neuronal markers *sox3* (E-H, M-P) and *ngn1* (A-B, I-L) were utilized to detect the level of ectopic neurons.

IV. Discussion

Various functions of the transcription factor *sox2* have been determined in different model organisms. *Sox2* is a key player in stabilizing the pluripotency of embryonic stem cells via cooperation with *oct3/4* to stimulate *oct-sox2* enhancers, which in turn activate the expression of various pluripotent stem cell-specific genes (Masui et al., 2007). Small elevation of *sox2* in mouse ESCs downregulates several *oct-sox2* target genes such as *nanog* and *lefty1*, promoting the differentiation of ESCs into a wide range of differentiated cell types (Kopp et al., 2008). In mouse and chick, *sox2* is expressed in proliferating neural precursors and multipotent neural stem cells within CNS, maintaining the potency and proliferative capacity of stem cells (Graham et al., 2003, Avilion et al.,2002). *Sox2* is also implicated in regulation of neural and mesodermal patterning in mice. Ectopic expression of *sox2* in the paraxial mesodermal compartment results in formation of ectopic neural tube, suggesting its role in specification of neural fate (Takemoto el at., 2011). Our data reveal the role of *sox2* in zebrafish as a determinator during fate specification. Constitutive expression of *sox2* prevents tailbud bipotential stem cells from differentiating into mesodermal tissue. Overexpressing *sox2* promotes mesodermal progenitors within somites to adopt a neural fate, inducing formation of ectopic neurons. Our results are consistent with models in mouse and chick in which *sox2* plays a role in maintaining pluripotent state of stem cells as well as functioning in neural development.

Interestingly, the induction of ectopic neurons by *sox2* can only occur after progenitor cells leave the tailbud. Overexpressing *sox2* before gastrulation or during initiation stage of gastrulation maintains the undifferentiated state of tailbud bipotential stem cells and keeps them from leaving the tailbud, without inducing the formation of ectopic neurons within the tailbud. One possible explanation is that the neural inducing function of *sox2* is determined by the differential expression level of Wnt signaling. Within the tailbud, the Wnt pathway is highly expressed, promoting specification of mesodermal progenitors and inhibiting adoption of neural fate. Overexpressing *sox2* in this Wnt-abundant region creates an environment with both high Wnt signaling and high *sox2* level, holding bipotential stem cells within the tailbud as undifferentiated progenitors. This is similar to the concept of “lineage-priming” of stem cells differentiation, which proposes that undifferentiated stem cells express a subset of genes that are related to different lineages which they can commit. Differentiation of stem cells into a given lineage is achieved by increasing the expression level of marker genes related to this specific

signaling pathway and reducing the expression of genes associated with other lineages (Delorme et al., 2009, Zipori D., 2004). Evidences for this lineage-priming theory are provided by lineage-committed of hemopoietic stem cells, in which undifferentiated hemopoietic stem cell co-activate more than one lineage-affiliated gene expressing programs prior to exclusive commitment and differentiation to a single hemopoietic lineage at single cell level (Hu et al., 1997). After stem cells are specified into mesodermal progenitors and migrate to the locations of somites, where the expression level of Wnt signaling is relatively low, *sox2* is able to perform its role in promoting neural development thereby inducing formation of ectopic neurons.

Our hypothesis is supported by our results that overexpression of *sox2* decreases overall expressing level of Wnt pathway while the high-expressed Wnt signaling at the end of the tail remains unaffected (figure 10D-F). Activation of *sox2* expression via heat shock at 12-somite stage fails to repress the expression level of Wnt (figure 10J-L), which is possibly responsible for the reduction of ectopic neurons formation relative to bud-stage heat shock (figure 11B and G). The expansion of *sox2*-induced ectopic neurons under the environment with low level of Wnt signaling and high level of *sox2* (figure11D and I) and the elimination of ectopic neurons in embryos with both high Wnt and *sox2* expressing levels (figure 12D and L) further strengthen our interpretation. The ability of Wnt pathway to control specification of epiblast stem cells (EpiSCs) via differential expression also reveals the potential of Wnt signaling to regulate fate decision of stem cells in a dose-dependent manner (Tsakiridis et al., 2014). Low expression level of Wnt signaling retains undifferentiated EpiSC cultures in a pluripotent, partially neural-like fraction. Slightly higher Wnt levels reverses EpiSCs from neural-like state to uncommitted state, while further elevation of Wnt signaling promotes specification of uncommitted cells exclusively into mesodermal and neuromesodermal progenitors. However, in addition to this Wnt-dependent model, the neural-promoting function of *sox2* may also be regulated by different epigenetic states of stem cells and/or interactions between *sox2* with different sets of co-factors during development.

The interaction of *sox2* and Wnt pathway in stem cell fate specification has been controversial. The negative regulatory role of *sox2* in Wnt signaling during development is reported by previous studies (Mansukhani et al., 2005, Kormish et al., 2010), however the role of Wnt pathway in *sox2*-promoted neural development remains unclear. Our results support a model that

differential expressing levels of *sox2* and Wnt signaling pathway determine fate decision of tailbud stem cells cooperatively. Constitutive expression of Wnt represses *sox2* expression and promotes adaption of mesodermal fate, whereas overexpression of *sox2* downregulates Wnt pathway expression, inducing neural fate adaption. Our hypothesis is similar to one of the reports in mouse model, in which high expressing level of *wnt3a* downregulates *sox2* expression in the epiblast and inhibits bipotent neural/mesodermal progenitors to take neural fate (Jurberg et al., 2014). However, this repressive interaction between *sox2* and Wnt during stem cell fate specification seems to be opposite to another report in mouse study, which states that expression of Wnt signaling in *tbx6*-mutated embryos activates *sox2* expression via *cis*-regulatory module N1 thus promoting neural development (Takemoto et al., 2006, 2011). A possible explanation for these discrepancies is that the activation of *sox2* is affected by differential expression level of Wnt signaling. High level of Wnt pathway activates mesodermal genes such as *tbx6* and *msgn1* (Wittler et al., 2007, Szeto and Kimelman, 2004), which in turn inhibit *sox2* expression. The repression of *sox2* by Wnt-targeted genes may eliminate and overwhelm the possible positive effect of Wnt signaling in *sox2* activation. Upregulation of *sox2* under low Wnt signaling activity is possibly regulated by other Wnt-independent pathways, promoting neural fate adaption.

The ability of *sox2* to switch the fate of progenitors from forming mesoderm to neural tissue indicates a reversible mechanism of stem cell fate decisions. Interactions between *sox2* with Wnt signaling and possibly other pathways seem to guide the direction of specification of undifferentiated progenitors, determining the potency as well as fate adoptions during development. Together our results suggest a requirement of precise regulation between levels of *sox2* and Wnt signaling in specification of tailbud bipotential stem cells. Based on levels of Wnt pathway activity, elevation of *sox2* can either maintain the undifferentiated state of stem cells (in cases of high Wnt activity) or induce neural fate (in cases of low Wnt activity). Future research into the details of how these factors are repressed and activated within the tailbud, including the identification of other potential regulators of sox2, can help us get a clearer insight of tailbud stem cell fate determination during body formation.

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