PRIMER

Determining the timeline of germ layer formation in zebrafish in a primer for "Reduced Expression of the Nodal co-receptor Oep causes loss of mesendodermal competence in zebrafish"

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ABSTRACT

Background: The development of distinct **germ layers** is a vital part of vertebrate embryonic development. Nodal signaling is responsible for forming and maintaining mesendoderm. Mesendoderm is cell tissue found in zebrafish that will give rise to mesoderm and endoderm germ layers. In order for ectoderm to form, **competence** to Nodal needs to be lost. This primer investigates the developmental stage at which cells lose responsiveness to Nodal, and what is responsible for the loss of Nodal competence.
Results: Injection of Nodal into embryos at different developmental stages demonstrated that prospective ectoderm loses responsiveness to Nodal at 75% **epiboly**. Activin A, a ligand that signals through the same receptors as Nodal, but does not require co-receptor Oep, was injected to determine what is responsible for the loss of competence. Activin A was able to induce mesendodermal markers in the prospective ectoderm at 75% epiboly as well as earlier stages. Oep injection along with recombinant human Nodal (rhNodal) was able to extend the window of competence. This suggests that Oep is the missing factor.
Conclusion: The experiments show that the loss of competence in the prospective ectoderm is a relatively simple cell-autonomous mechanism. Due to the upstream receptors in the pathway, this mechanism ensures a global effect.

Germ layers: typically, early embryos consist of three cell layers that include the ectoderm, mesoderm, and endoderm. In addition to these three cell layers, zebrafish also have a cell layer called mesendoderm.

Competence: the ability for tissue to respond to surrounding tissues and signals

Epiboly: describes a major type of cell movement that occurs during gastrulation. During epiboly, all cells move around the yolk until all of the yolk is covered

Embryogenesis: the formation and development of an embryo

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INTRODUCTION

All vertebrates form three germ layers. These germ layers include the endoderm, mesoderm and ectoderm. The endoderm forms the digestive system and lining of visceral organs, the mesoderm develops into visceral organs such as the heart and lungs, and the ectoderm forms the epidermis and central nervous system (Figure 1). Germ layers are formed early in the developing embryo during the process of gastrulation. Being that the formation of germ layers if fundamental to all vertebrates to properly develop tissues and organs, understanding the developmental process is vital. Maldeveloped germ layers can lead to severe defects and death of the embryo.

Embryogenesis relies on cell signaling to activate gene expression needed for the development of germ layers. The Nodal signaling pathway is responsible for the transition between germ layers. In all vertebrates, Nodal induces the formation of mesoderm, endoderm, and mesendoderm (Shier 2003). If all components of Nodal signaling are present and functioning properly, mesoderm, endoderm and mesendoderm will form correctly. In cases where Nodal signaling is not functioning properly, the zebrafish embryo will have formed almost no mesoderm or endoderm and be composed mostly of ectodermal derivatives. Any remaining **somites** in the tail is due to Activin signaling which is still functioning in the zebrafish *oep* mutants. (Figure 2).

Somites: blocks of mesoderm that are located on either side of the neural tube in the developing vertebrate embryo. Will eventually form the vertebrae.

The family of Nodal proteins are a subset within the transforming growth factor beta (TGFβ) superfamily. In zebrafish, there are two types of Nodal proteins: Cyclops (Cyc) and Squint (Sqt). Nodals are secreted and then bind to Activin Like (Alk) receptors. Once bound, the alk receptors phosphorylate and recruit Smad2 and Smad4 to create the Smad2/4 complex. The Smad2/4 complex interacts with the transcription factors FoxHI and Mixer in order to regulate the transcription of Nodal genes (Shier 2003). Nodal acts as a morphogen, which are signals that act at a distance but also in a concentration-dependent manner. Therefore, Nodals can act both locally and distally to affect cell fate. (Barone et al. 2017, Shier 2003).

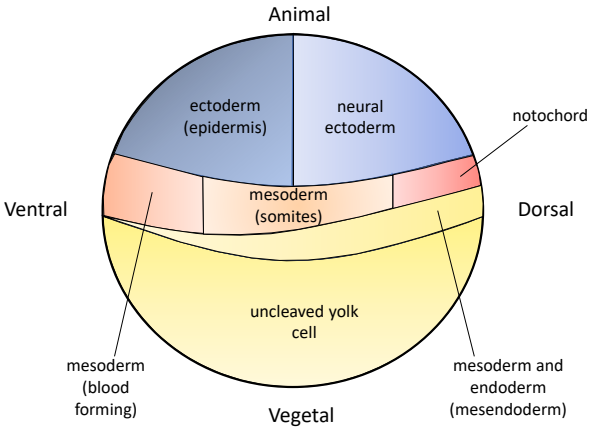

Figure 1: Fate map of a zebrafish embryo at early gastrula stage. Adapted from Principles of Development, 4th Edition, Oxford University Press.

Though it is known that Nodal is required for endoderm, mesoderm, and mesendoderm development, it is not known what causes the prospective ectoderm to lose its ability to respond to Nodal. Vopalensky, et al. (2018) revealed that the transition of mesendoderm to ectoderm results when Nodal is no longer able to signal the prospective ectoderm due to the reduced expression of Nodal co-receptor Oep during late gastrulation. This primer will review the experiments that led to this conclusion.

METHODS

Gathering embryos

Wild-type zebrafish were crossed to obtain embryos. The embryos were raised until they reached the desired stage (Figure 3) (Westerfield 2000).

Injection of rhNodal protein

Recombinant human Nodal (rhNodal) protein was injected into wildtype embryos at 50%

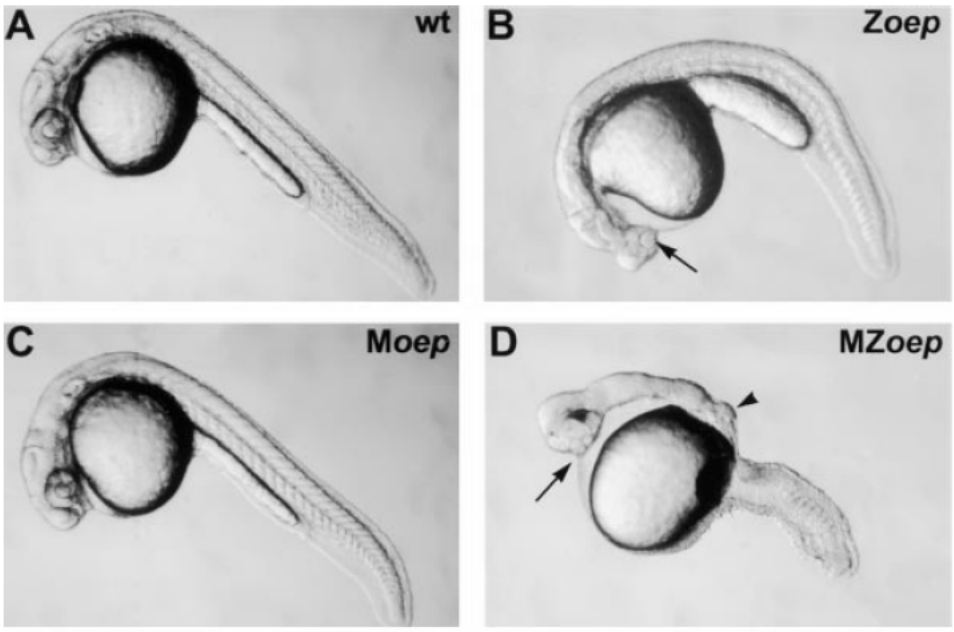

Figure 2: Maternal-zygotic effects of *oep*^{tz57} at 30 hpf. (A) Wild-type embryo (B-D) These mutant embryos have phenotypes of reduced somite development along the tail and abnormal shaped heads. (B) Zygotic mutant embryo (C) Zygotic mutant embryo (D) Maternal and zygotic embryo. Adapted from Gritsman et al., 1999.

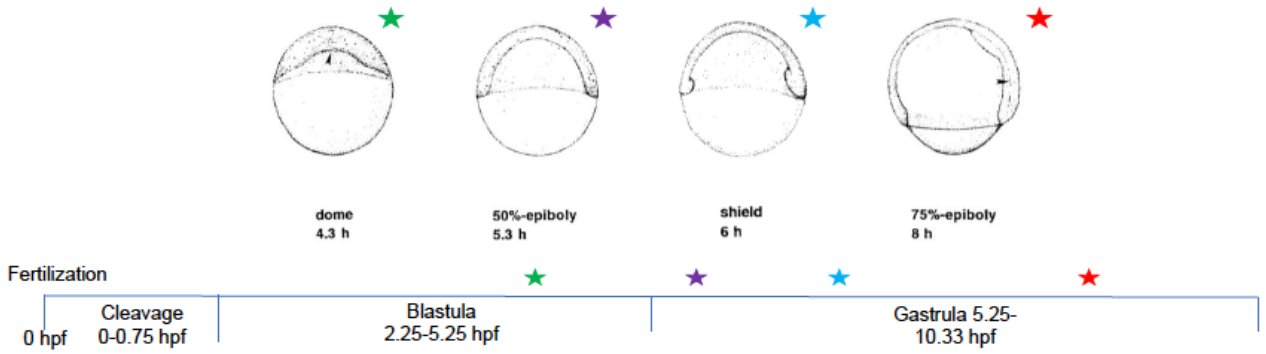

Figure 3: Embryo stages used for protein injections. Stars are indicative of embryo stage along hours post fertilization timeline. Embryo images adapted from Kimmel et al., 1995.

Ectopic: located in an abnormal place or position

epiboly (5.3 hpf) to determine if cells were competent to respond to Nodal signaling. The response is tested by analyzing the embryos 1.5 hours later for the **ectopic** expression of mesendodermal markers *no tail* (*ntl*), *sebox*, and *bhikhari* (*bhik*).

To test whether the loss of Oep in the prospective ectoderm causes the inability to respond to Nodal, *oep* mRNA was injected at the 1-cell stage of WT embryos in order to increase Oep protein levels during gastrulation stages. rhNodal was then injected intracellularly to *oep* mRNA injected embryos at 50% epiboly, shield, or 75% epiboly stages. Embryos used as controls were injected with *egfp* rather than *oep* mRNA.

Intercellularly: located or occurring between cells

Injection of Activin A protein

Activin A was injected **intercellularly** at 50% epiboly, shield, and 75% epiboly. Since Activin A is able to activate the Nodal signaling pathway without Oep, it was used to determine whether or not Oep is the limiting factor of prospective ectoderm competence to Nodal.

Injection of oep mRNA

To test whether the loss of Oep in the prospective ectoderm results in Nodal's inability to induce the tissue, Oep mRNA was injected at the 1-cell stage of WT embryos in order to increase *oep* mRNA levels during gastrulation stages. Nodal was then injected intracellularly into Oep mRNA injected embryos at 50% epiboly, shield, or 75% epiboly stages. Embryos used as controls were injected with *egfp* rather than Oep mRNA, also at the 1-cell stage.

Fixation

After protein injections or transcription inhibition, the embryos were fixed in order to preserve the structure and molecules such as proteins and RNAs.

Whole mount in situ hybridization (WISH)

WISH was used to label mRNAs. First, a probe labeled with the small moleculedigoxigenin is made that is complementary to a specific mRNA sequence. During WISH, holes are made in the cell membrane, which allows the probe to enter the cell and bind to its complementary mRNA. Alkaline phosphatase-conjugated antibody to digoxigenin is added which enters the cells and binds to the probe. A colorless compound containing phosphates is added and enters the cell. The phosphatase that bound to the antibody removes the phosphates, resulting in the appearance of purple dye (Figure 4).

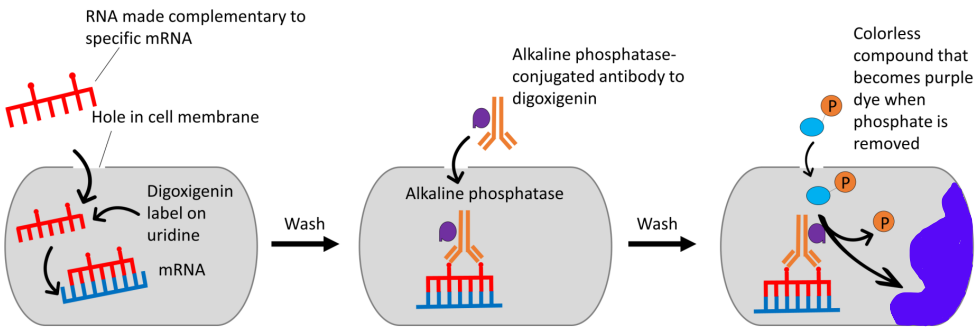

Figure 4: The process of whole-mount in situ hybridization (WISH). Adapted from Developmental Biology, 6th Edition..

RESULTS

Prospective ectoderm loses responsiveness to Nodal between shield and 75% epiboly

In their first experiment, the authors repeated studies done earlier to demonstrate that they could replicate results from other laboratories (Dubrelle et al., 2015). rhNodal was injected intercellularly into WT embryos at 50% epiboly. As expected from previous studies, WISH studies found that the mRNA for the mesodermal marker *ntl* and endodermal marker *sebox* were ectopically expressed in the prospective ectoderm. In contrast, ectodermal markers, *sox2* and *sox3*, were reduced (Figure 5). This suggests the prospective ectoderm cells were turned into mesendoderm by the addition of rhNodal.

To determine the time window of responsiveness to Nodal in the prospective ectoderm, rhNodal was also injected into WT embryos at later stages (shield and 75% epiboly). After 1.5 hours, the injected embryos were fixed using paraformaldehyde and the mesodermal marker *ntl* was analyzed using WISH. *ntl* was expressed in injected embryos up until late shield stage, but not in embryos injected at 75% epiboly (Figure 6). This suggests that prospective ectoderm loses its ability to respond to Nodal and is determined to become ectodermal tissue at 75% epiboly.

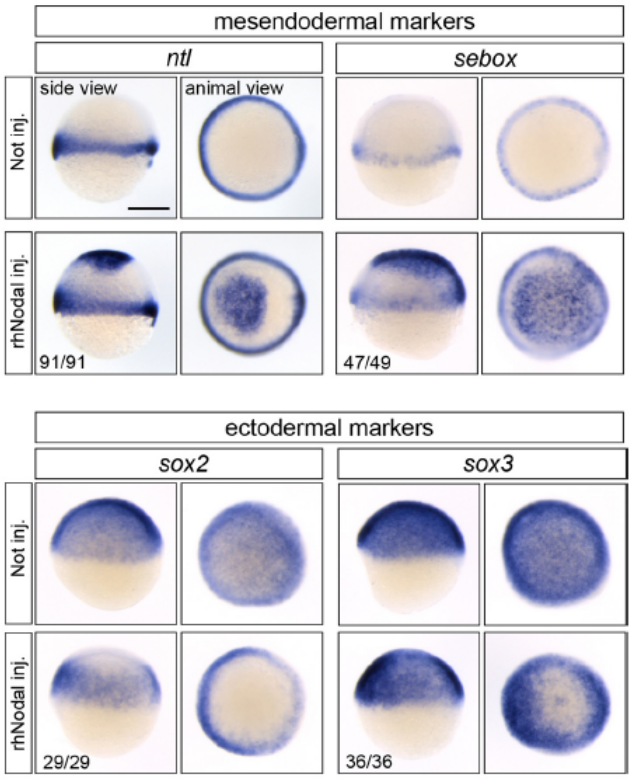

Figure 5: Expression levels of mesendodermal and ectoderm markers in embryos injected with rhNodal at 50% epiboly. (A) Mesodermal marker *ntl* and endodermal marker *sebox* are ectopically expressed in rhNodal injected embryos. (B) The expression of ectodermal markers *sox2* and *sox3* is reduced in rhNodal injected embryos. Adapted from Vopalensky et al., 2018.

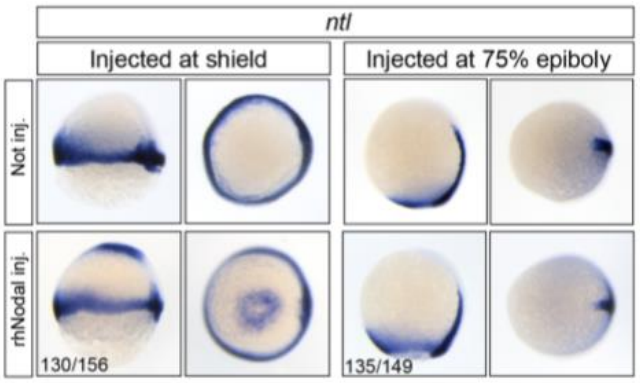

Figure 6: *ntl* expression in embryos injected with rhNodal at shield stage or 75% epiboly . Emrbyos injected with rhNodal at shield stage show ectopic expression of *ntl* while embryos injected at 75% epiboly show no ectopic expression of *ntl.* Adapted from Vopalensky et al., 2018.

Nodal co-receptor Oep needed for the prospective ectoderm to be responsive to Nodal

The second experiment was a repeat of the first, except the protein Activin A was injected rather than rhNodal. Activin A, a Nodal-related ligand, was used since it does not require *oep* to activate the Nodal pathway. By using Activin A, they wanted to test whether oep was the missing factor in the tissue that will become the ectoderm. Activin A was injected intercellularly into WT embryos at 50% epiboly, shield stage, or 75% epiboly. The embryos were then analyzed for *ntl* (mesoderm), *bhik* (mesoderm) and *gsc* (mesendoderm) expression.

Activin A injected embryos expressed ectopic induction of all three genes, *ntl*, *bhik* and *gsc* in the prospective ectoderm when analyzed 1.5 hours after injection. Expression of these genes occurred in embryos injected at all three developmental stages (Figure 7). This suggests that reduced levels of *oep* in the prospective ectoderm is responsible for the loss of Nodal responsiveness in this tissue.

To better determine if the loss of Oep in the ectoderm results in its inability to respond to Nodal, embryos were injected with *oep* mRNA at the one-cell stage. These embryos were then also injected with rhNodal at later developmental stages (50% epiboly, shield stage, or 75% epiboly). It was found that embryos injected with *oep* mRNA at all three stages ectopically expressed *bhik* and *gsc* up to 75% epiboly (Figure 8). This experiment reveals that extending the time of Oep expression results in a longer competence window for Nodal.

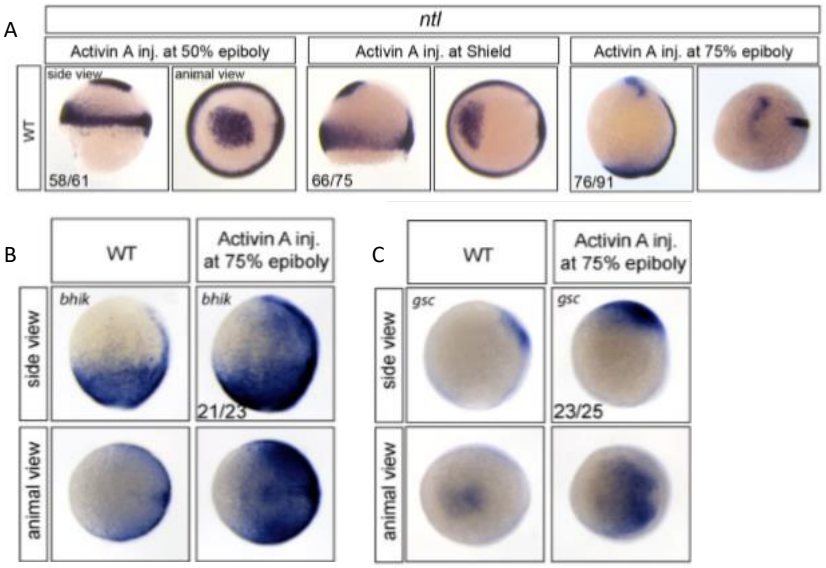

Figure 7: Expression of mesendodermal markers after Injection of Activin A into embryos between 50% epiboly, shield stage, and 75% epiboly. (A) Injection of Activin A at 50%, shield stage, and 75% epiboly resulted in the ectopic expression of mesodermal marker *ntl.* (B) Injection of Activin A up to the stage of 75% epiboly resulted in the ectopic expression of mesodermal marker *bhik.* (C) Injection of Activin A up to the stage of 75% epiboly resulted in ectopic expression of mesendodermal marker *gsc*. Adapted from Vopalensky et al., 2018.

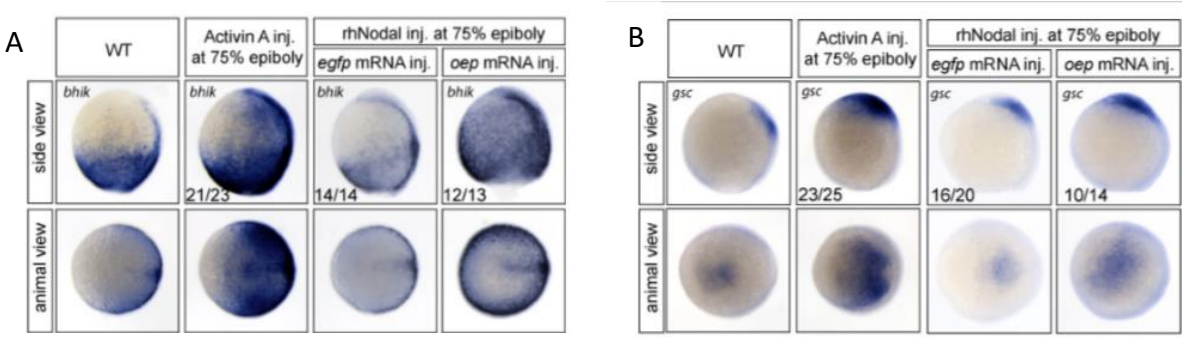

Figure 8: Injection of rhNodal and oep mRNA extends the window of mesendodermal competence to 75% epiboly for mesodermal markers *bhik* and *gsc.* (A) *bhik* expression ectopically expressed at 75% epiboly. (B) *gsc* expression ectopically expressed at 75% epiboly. Adapted from Vopalensky et al., 2018.

DISCUSSION

Differentiate: make or become different in the process of growth or development

The formation of a correct body plan during development is vital to the survival of vertebrate organisms. During the developmental stage of gastrulation, the body plan is established. Cells begin to move and **differentiate** throughout the embryo to form the germ layers and its derivatives, specifically mesendoderm. Nodal acts to induce mesendoderm both spatially and temporally.

The study of Vopalensky et al. revealed that the decrease in one component of the Nodal signaling pathway results in the inability of cells to respond to Nodal. The inability for the embryos to become ectoderm shows that competence was lost. By injecting embryos with Nodal protein, it was found that the responsiveness to Nodal on the prospective ectoderm is lost between shield stage and 75% epiboly in developing zebrafish embryos. Injection of Activin A led to ectopic expression of all mesendodermal markers, including in embryos as old as 75% epiboly. Since Activin A signals through the same receptors as Nodal, but does not require co-receptor Oep, this suggests that Oep is the missing factor. This finding complements an earlier study by Ho and Kimmel (1993). In their experiments, **hypoblast** cells were transplanted into the animal pole of older embryos. One-third of the transplanted hypoblast cells from shield stage (6 hpf) embryos that were transplanted into embryos at 50% epiboly kept their hypoblast fate. The other one-third expressed an epiblast fate. Though Ho and Kimmel did not include the fate of the last one-third, it could be inferred that the fate of these embryos was mixed. In other words, these cells could have become a mixture of endoderm, mesoderm, or ectoderm. This reveals that at shield stage, cells are not yet fully committed to any fate. Comparatively, 75% epiboly (8 hpf) hypoblast cells maintained their hypoblast fate when transplanted into the animal of 50% epiboly embryos, showing that by 75% epiboly, cells are committed to their fate.

Hypoblast: a layer formed during the onset of gastrulation. Generates mesodermal and endodermal derivatives

Taken together, the follow-up studies of Vopalensky et al. 2018 on germ layer competence show that by mid-gastrulation, specifically 75% epiboly, hypoblast cell types of the germ layers of developing embryos are committed to their fate. Up until 75% epiboly, cells are able to change their fate in response to their environment. Once the stage of 75% epiboly is reached, differentiation prevents the cells from changing their fate. Loss of Nodal co-receptor Oep on the prospective ectoderm was shown to be an important factor in the differentiation process. This seemingly simple process of regulating germ layer formation by expressing reduced levels of upstream receptors represents a cell-autonomous mechanism.

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