Hypoxic perturbation of *nodal*-dependent axis specification in *Strongylocentrotus purpuratus*

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Sea urchin (*Strongylocentrotus purpuratus*) embryos cultured hypoxically fail to develop a dorsoventral axis owing to perturbed expression of the TGFβ-family signaling gene *nodal*. The purpose of this study was to characterize the developmental dynamics of this effect. We used quantitative PCR to assess *nodal* expression in *S. purpuratus* embryos at 9, 12, and 18 hours post-fertilization (hpf), under both normoxic and hypoxic conditions. It was found that hypoxia initially inhibited *nodal* expression at 9 hpf, but induced overexpression by 12 hpf, which was followed again by loss of expression by 18 hpf. Whole mount in-situ hybridization showed that the transient spike in *nodal* expression at 12 hpf is delocalized.

Localized expression of the *nodal* gene is required for dorsoventral axis specification in the sea urchin embryo[5,7]. When *nodal* expression is either blocked or fails to localize, a dorsoventral axis does not develop, producing larvae with an abnormal radialized morphology rather than the normal bilaterally symmetric larval form[5,7].

The *nodal* gene, which encodes a secreted TGFβ family signaling molecule, is transcriptionally activated between 6 and 9 hour post-fertilization in *S. purpuratus*[7,8]. Secreted Nodal protein binds to cell surface receptors and initiates a signal transduction process that leads to activation of the *nodal* gene in the cells receiving the signal, thus activating a positive feedback loop. This feedback is negatively controlled by *lefty*, another gene activated by Nodal signaling which is required for the localization of *nodal* to the prospective ventral side of the embryo in normal development[4].

Culturing embryos under hypoxic conditions blocks development of the dorsoventral axis[2]. In order to gain further insight into the cell physiology governing *nodal*-dependent axis specification we are studying the mechanism of action underlying this teratogenic effect. The goal of this study was to define the temporal and spatial changes in the pattern of *nodal* expression over a time course of 9, 12, and 18 hours post fertilization (hpf) in *S. purpuratus* embryos exposed to hypoxia (1 ppm oxygen or $pO_2 \simeq 14$ mmHg, as compared to normoxia of 10 ppm or $pO_2 \simeq 140$ mmHg). This time course interval corresponds to when the dorsoventral axis is normally specified via *nodal*.

Adult *S. purpuratus* were obtained from Point Loma Marine Invertebrate Lab (Coronal del Mar, CA). Gametes were obtained and eggs fertilized in filtered seawater (FSW) using standard procedures. Fertilized eggs were placed in 3 ml of either normoxic or hypoxic FSW. The hypoxic FSW was prepared by bubbling nitrogen gas into FSW until the oxygen content was 1 ppm, as assessed using an Ocean Optics fluorescence oxygen probe and sensor. The embryos in hypoxic seawater were put in a plexiglass chamber that was flushed with nitrogen gas for approximately 15 minutes, an interval that was previously determined to reduce oxygen levels within the chamber to ~1 ppm (hypoxia). The chamber was then sealed and placed in an incubator of 15° C. Control embryos were cultured in 3 ml of normoxic FSW and also incubated at 15° C. Embryos developed for 9, 12, or 18 hours. Embryos from the control conditions as well as hypoxic conditions were harvested by centrifugation. A small aliquot of each culture was allowed to continue developing under normoxic conditions to late gastrula stage (~48 hpf) for morphological assessment, to ensure that a radialized phenotype was obtained. The remaining embryos were stored at -80° C for later extraction of RNA.

Qiagen RNeasy Plus Mini Kit was used to extract RNA from each frozen sample and concentration was determined by Nanodrop spectrophotometry. Invitrogen Superscript III was used to synthesize cDNA from extracted RNA. Quantitative RT-PCR was performed using a Cepheid SmartCycler II using Quanta PerfeCTa SYBR Green Fastmix as described previously[1]. Fold-change in transcript abundance of *nodal* and *lefty* under hypoxic conditions was determined by normalizing the Ct values obtained for those amplicons to those obtained for *hprt*, which was assumed to be constant throughout hypoxic exposures. Whole Mount In-Situ Hybridization (WMISH) of hypoxia exposed embryos was carried out following the protocol of Ertl *et al.*[6], and embryos were imaged using a Zeiss 510 Meta confocal laser scanning microscope.

At 9 hpf *nodal* was found to be underexpressed 2-4 fold in hypoxic-cultured embryos compared to normoxic controls. At 12 hpf it was overexpressed 2-6 fold, and at 18 hpf it was again underexpressed 2-8 fold (Fig 1). These results were reproduced with three biological replicates.

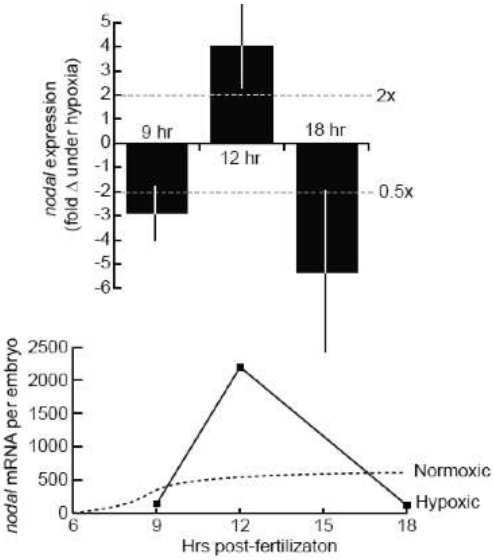

Figure 1. Quantitative effects of hypoxia on *nodal* expression at 9, 12, and 18 hpf. RNA extracted from untreated control embryos and embryos cultured under hypoxic conditions for the indicated times was used to make random-primed cDNA, which was then used as template for quantitative real-time PCR using primers specific to *nodal* and *hprt*, which was used for normalization[1]. The ddCt method was then used to obtain the fold difference in *nodal* expression between control and hypoxic-treated embryos. The upper graph shows the averages and standard deviations in the fold difference obtained in three different experiments (biological replicates). The lower graph plots the averaged data in relation to the normal developmental accumulation of *nodal* mRNA, obtained from previous measurements [8].

We also found that the expression of *lefty* in hypoxic-cultured embryos tracks that of *nodal*, in that *lefty* is underexpressed at 9 hpf and at 18 hpf, and dramatically overexpressed at 12 hpf (data not shown). This is not unexpected since *lefty* expression is activated by and requires Nodal signaling. This result shows that the dramatic up-regulation of *nodal* at 12 hpf in hypoxic-cultured embryos is not caused by loss of *lefty* expression.

Whole-mount in situ hybridization experiments showed that *nodal* expression was highly and globally expressed in 12 hr hypoxic-cultured embryos (not localized to one side of the embryo as in controls), but almost undetectable at 18 hpf (Fig 2). Since *nodal* localization is required for dorsoventral axis specification, this result explains the radialized development of hypoxic-cultured embryos.

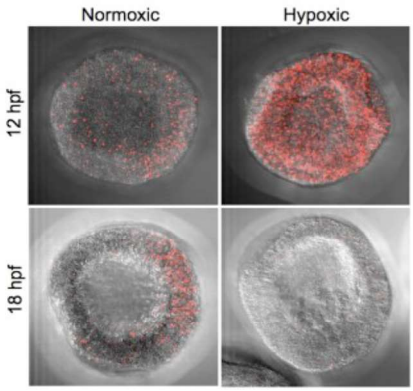

Figure 2. Spatial expression of *nodal* in embryos cultured under normoxic or hypoxic conditions. Embryos cultured normoxically or hypoxically for the indicated times were fixed then subjected to fluorescent WMISH using a riboprobes to *nodal*[6], then imaged confocal microscopy. Images depict projections of confocal z-series of each embryo; *nodal* WMISH signal is colored red. For each treatment and time point three out of three imaged embryos showed the same phenotype. These results show that the overexpression of *nodal* at 12 hpf in hypoxic-cultured embryos (see Fig 1) is caused at least in part by ectopic expansion of the expression domain.

We have shown previously that hypoxia causes a reduction in mitochondrial H_2O_2[3], which is required for the initial activation of *nodal*[1]. Hence, the initial suppression of *nodal* expression under hypoxia may be explained in part by reduced levels of mitochondrial H_2O_2. However, the mechanisms underlying the hypoxia-induced spike of delocalized *nodal* expression at 12 hpf, and its subsequent precipitous decline, remain unknown and are the subject of ongoing research.

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