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GATA2 regulates Wnt signaling to promote primitive red blood cell fate

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ABSTRACT

Primitive erythropoiesis is regulated in a non cell-autonomous fashion across evolution from frogs to mammals. In *Xenopus laevis*, signals from the overlying ectoderm are required to induce the mesoderm to adopt an erythroid fate. Previous studies in our lab identified the transcription factor GATA2 as a key regulator of this ectodermal signal. To identify GATA2 target genes in the ectoderm required for red blood cell formation in the mesoderm, we used microarray analysis to compare gene expression in ectoderm from GATA2 depleted and wild type embryos. Our analysis identified components of the non-canonical and canonical Wnt pathways as being reciprocally up- and down-regulated downstream of GATA2 in both mesoderm and ectoderm. We show that up-regulation of canonical Wnt signaling during gastrulation blocks commitment to a hematopoietic fate while down-regulation of non-canonical Wnt signaling impairs erythroid differentiation. Our results are consistent with a model in which GATA2 contributes to inhibition of canonical Wnt signaling, thereby permitting progenitors to exit the cell cycle and commit to a hematopoietic fate. Subsequently, activation of non-canonical Wnt signaling plays a later role in enabling these progenitors to differentiate as mature red blood cells.

1. Introduction

During vertebrate hematopoiesis, stem cells differentiate along one of the various blood lineages (Drevon and Jaffredo, 2014). The first blood progenitors arise from mesodermal cells that are specified with a hematopoietic fate during gastrulation. These cells will differentiate predominantly as red blood cells (RBCs) several days later in a process termed primitive hematopoiesis. A second phase of blood development, definitive hematopoiesis, generates hematopoietic stem cells (HSCs) that give rise to progenitors of the erythroid, lymphoid and myeloid lineages.

Signals from the microenvironment, or niche, that determine the balance between quiescence, proliferation and differentiation of HSCs during adult hematopoiesis are similarly required for primitive hematopoiesis. Tissue recombination studies have shown that non-hematopoietic cells must send a signal to the nascent mesoderm during gastrulation in order for it to form blood (Belaoussoff et al., 1998; Maeno et al., 1994). In *Xenopus*, ectodermal cells provide this signal. When ectoderm is physically separated from the hematopoietic ventral mesoderm in *Xenopus* embryos, RBCs fail to form despite the normal differentiation of other mesodermal cell types (Kikkawa et al., 2001).

Although the precise molecular nature of the signal (s) transmitted by ectodermal cells during primitive blood development is unknown, it is known that bone morphogenetic proteins (BMPs) are needed to generate this signal. BMPs are required for ventral patterning of all germ layers, and RBCs fail to develop when BMP signal reception is blocked in either mesodermal or ectodermal cells (Kumano et al., 1999; Walters et al., 2001). It has been suggested that BMPs secreted from the ectoderm provide the signal that enables mesoderm to form blood (Kumano et al., 1999; Maeno, 2003; Tran et al., 2010), However, activation of the intracellular BMP signaling cascade is not sufficient to rescue erythropoiesis in isolated ventral mesoderm (Dalgin et al., 2007).

Wnt signaling is also required for primitive blood development. Wnts signal via two general pathways, termed canonical and non-canonical (Chien et al., 2009). Canonical Wnt signaling is associated with progenitor cell proliferation whereas non-canonical Wnt signaling is often necessary for exit from pluripotency and progenitor cell specification. In *Xenopus,* Wnt4 signals from mesodermal cells during gastrulation to activate the canonical Wnt cascade in the overlying ectoderm, and possibly also the

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mesoderm, and this is required for primitive blood formation (Tran et al., 2010).

The transcription factor GATA2 functions cell-autonomously in blood progenitors during erythropoiesis (Tsai et al., 1994), but is also required in ectodermal cells of *Xenopus* embryos for primitive blood formation (Dalgin et al., 2007). Epistasis analysis in whole embryos and explants support a model in which BMPs signal to ectodermal cells to activate transcription of *GATA2*, and GATA2 subsequently induces expression of a secondary signaling molecule(s) that is essential for mesoderm to form blood (Dalgin et al., 2007). The current studies explore the nature of the signals that are induced by GATA2.

2. Materials and methods

2.1. Embryo culture and manipulation

Embryos were obtained, microinjected, and cultured as described (Mimoto and Christian, 2012). Embryos were staged according to Nieuwkoop and Faber (1967). Ectoderm or mesoderm explants were dissected with watchmakers' forceps and cultured independently or as recombinants as described previously (Goldman et al., 2006). Ectoderm removal assays were performed by removing the vitelline coat with watchmakers' forceps, dissecting away the dorsal half of the embryo and then peeling off the ectoderm using tungsten needles or an eyebrow knife. Stage 13 embryos were incubated in 0.005% Trypsin for 1–5 min prior to ectoderm removal and then transferred to 0.02% trypsin inhibitor for 20 min prior to culture. The vitelline coat was removed from late gastrula stage embryos using watchmakers' forceps, the ventral half was removed using tungsten needles and then separated into ectodermal and mesendodermal fragments using an eyebrow knife. Transient activation of the canonical Wnt pathway was achieved by culture of whole embryos in 0.25 M LiCl in 0.5X Modified Barth's Saline (MBS) for 20 min followed by 4–5 washes in 0.5X MBS. Double label in situ hybridization assays were performed as described in Harland (1991) except that the vitelline coat was not removed prior to fixation and probes were detected using BM purple as a substrate.

2.2. Microarray analysis

To generate samples for the microarray analysis, FOG RNA (250 pg), GATA2 RNA (250 pg) or GATA2 anti-sense morpholino oligonucleotides (MOs) (20 ng) (Dalgin et al., 2007) was injected into each cell near the animal pole of embryos at the two-cell stage. Embryos were allowed to develop to stage 10, at which point ectoderm was removed using watchmakers' forceps and cultured to stage 12 in 0.5X NAM. Approximately 40 ectodermal explants from a single injection condition were pooled and RNA samples were generated for microarray analysis according to standard protocols provided by the manufacturer (Affymetrix). Briefly, RNA was extracted using Trizol (Invitrogen) and purified with an RNA clean-up kit (QIAGEN). RNA samples were sent to the Oregon Health & Science University Affymetrix Microarray Core facility for further processing and hybridization to the microarrays. Three biological replicates collected during three separate days of experiments were used for each of the four microarray conditions. Array probe levels were summarized using the Robust Multiarray Analysis RMA method (Irizarry et al., 2003a, 2003b), implemented in the Affymetrix package under BioConductor in the R programming language. A second level of probeset normalization was performed using the Global Rank-Invariant Set Normalization (GRSN) method (Pelz et al., 2008). Hierarchical clustering based on a Pearson's correlation coefficient was performed as an unsupervised method to identify outliers. Based on this analysis, Uninjected #3 was identified as a significant outlier and was removed from subsequent analysis. A batch adjustment was performed using an internal method based on Distance Weighted Discrimination (DWD) (Benito et al., 2004), which was developed by our statistician, Carl Pelz. This analysis was used to correct for a batch effect from the different biological replicates, likely due to the fact that *Xenopus laevis* is not isogenic, creating a significant degree of background variability between the biological replicates that was unrelated to our scientific question. To facilitate analysis of a smaller sample size, the eBayes method (implemented through (Smyth, 2004) was used to identify up and down-regulated genes. Genes that showed changes +/− 1.2-fold were considered potentially significant.

2.3. Analysis of RNA

For Northern blotting, total RNA was isolated and analysis was performed as described previously (Christian et al., 1991). Bands were quantified using the NIH ImageJ software. For quantitative reverse transcription PCR (qPCR) analysis, total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions, from which cDNA was generated using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Life Sciences, Inc.) with a poly d(T) primer. qPCR was performed using a SYBR Green-based assay (QIAGEN) and a 7900 HT Sequence Detector (ABI). Each sample was analyzed in triplicate and normalized to the housekeeping gene, ornithine decarboxylase (ODC). Forward (F) and reverse (R) primers used for PCR are listed in Table S1. Tm used was between 58 and 60 °C.

2.3.1. Morpholinos and cDNA constructs

GATA2 (Dalgin et al., 2007) morpholino antisense oligonucleotides (MOs) were purchased from Gene Tools, LLC (Philomath, OR), along with a standard control MO. All cDNAs were subcloned into pCS2+ for RNA transcription.

3. Results and discussion

3.1. Ectodermal signals required for blood differentiation in the mesoderm are transmitted by the end of gastrulation

In order to identify GATA2 targets in the ectoderm that might be required for blood development, we first wanted to determine the developmental window during which ectoderm is required for primitive erythropoiesis. To do this, the ventral half of embryos, which gives rise to the majority of RBCs in the VBI (Ciau-Uitz et al., 2010; Maeno et al., 2012), was explanted and ectoderm was either retained or removed at successive stages of development, from mid-gastrulation (stage 11) through the end of gastrulation (stage 13). The ventral explants were cultured until wild type siblings reached the tailbud stage (stage 34) and then assayed by whole mount in situ hybridization (WMISH) for expression of *globin*, a marker of differentiated RBCs (assay illustrated in Fig. 1A). Expression of *globin* was detected in nearly all explants cultured in the presence of ectoderm (Fig. 1B, top row, Fig. 1C). By contrast, expression of *globin* was absent in ventral explants in which ectoderm was removed at stages 11 and 12 (Fig. 1B, middle and bottom rows, Fig. 1D). When ectoderm was removed at stage 13, expression of *globin* was detected in 50–70% of ventral explants in three independent experiments (Fig. 1B, middle and bottom rows, Fig. 1D). Whereas *globin* expressing mesoderm was located near the superficial surface of explants cultured in the presence of ectoderm (Fig. 1B, top row), it was present at a more internal location in explants from which the ectoderm was removed at stage