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Expression pattern of *bcar3*, a downstream target of Gata2, and its binding partner, *bcar1*, during *Xenopus* development

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ABSTRACT

Primitive hematopoiesis generates red blood cells that deliver oxygen to the developing embryo. Mesodermal cells commit to a primitive blood cell fate during gastrulation and, in order to do so the mesoderm must receive non-cell autonomous signals transmitted from other germ layers. In *Xenopus*, the transcription factor Gata2 functions in ectodermal cells to generate or transmit the non-cell autonomous signals. Here we have identified *Breast Cancer Antiestrogen Resistance 3* (*bcar3*) as a gene that is induced in ectodermal cells downstream of Gata2. Bcar3 and its binding partner Bcar1 function to transduce integrin signaling, leading to changes in cellular morphology, motility and adhesion. We show that *gata2*, *bcar3* and *bcar1* are co-expressed in ventral ectoderm from early gastrula to early tailbud stages. At later stages of development, *bcar3* and *bcar1* are co-expressed in the spinal cord, notochord, fin mesenchyme and pronephros but each shows additional unique sites of expression. These co-expression and unique expression patterns suggest that Bcar3 and Bcar1 may function together but also independently during *Xenopus* development.

1. Introduction

Hematopoiesis, or blood cell production, begins during early development and persists throughout adulthood. Hematopoiesis occurs in two phases. The first phase is primitive hematopoiesis, which occurs only during development, and generates mostly red blood cells that function to deliver oxygen. The second phase is definitive hematopoiesis, which begins during embryogenesis but persists in adults. Definitive hematopoiesis generates hematopoietic stem cells that differentiate into both red and white blood cell lineages. These blood cells function to deliver oxygen, form blood clots, and generate proper immune responses.

Primitive blood cells are derived from mesoderm that becomes specified with a hematopoietic fate by the end of gastrulation (Nakazawa et al., 2006; Palis et al., 1999; Smith and Turpen, 1985). It has been shown that the mesoderm needs to receive non-cell autonomous signals from other germ layers to commit to a blood cell fate. In mouse and chick, these signals come from endoderm, while in *Xenopus* they come from ectoderm (Baron, 2003; Belaoussoff et al., 1998; Maeno et al., 1994; Savary et al., 2005). We have shown that the transcription factor Gata2 is required in *Xenopus* ectoderm in order to generate or transmit these non-cell autonomous signals (Dalgin et al., 2007), but targets of Gata2 that mediate these signals have not been identified.

We have used microarray analysis to identify Gata2 target genes that are expressed in ectodermal cells of *Xenopus* embryos during gastrulation. We identified *Breast Cancer Antiestrogen Resistance 3* (*bcar3*, also known as *and-34*) as a gene that is induced in ectodermal cells downstream of Gata2. Bcar3 is an adaptor protein that interacts with Bcar1 (also known as p130 Crk-associated substrate (p130Cas)) to transduce signals downstream of activated integrins (Barrett et al., 2013; Cabodi et al., 2010). Bcar3 downregulates cadherin dependent cell–cell adhesion and promotes cell motility

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by controlling cytoskeletal remodeling in breast cancer cells (Makkinje et al., 2009; Near et al., 2009; Schrecengost et al., 2007; Sun et al., 2012; Wilson et al., 2013). Changes in cell–cell adhesion and cytoskeletal remodeling are integral components of cell movements that drive gastrulation, which brings the ectoderm and mesoderm into contact. This contact is required for the transmission of non-cell autonomous signals from ectoderm to mesoderm that enable mesoderm to commit to a blood cell fate (Kikkawa et al., 2001). For this reason, we chose to study *bcar3* as a potential key target of Gata2 that might be required in ectoderm to promote primitive hematopoiesis. We show here that *bcar3* is co-expressed with *gata2* in ventral ectoderm from early gastrula through neurula stages. We also expanded our analysis to examine patterns of expression of *bcar3*, and its binding partner *bcar1*, from early development through tailbud stages. We show that *bcar3* and *bcar1* are co-expressed in ectodermal cells during gastrula and neurula stages, consistent with their possible involvement downstream of Gata2 in primitive hematopoiesis, and that they show both unique and overlapping expression patterns in multiple other tissues in later embryos, suggesting involvement in other developmental processes as well.

2. Results

2.1. bcar3 is a downstream target of Gata2 in ventral ectoderm during gastrulation

We used microarray analysis to find Gata2 targets that might execute Gata2 functions in ectoderm during gastrulation (Mimoto et al., 2015). To perform the microarray, we injected Gata2 MOs or Gata2 RNA into both cells of two cell embryos, dissected ectoderm from early gastrula stage embryos (stage 10), and cultured the ectoderm until sibling embryos reached stage 12, which is near the end of gastrulation. As an additional method to verify targets, we analyzed gene expression in ectoderm isolated from embryos injected with RNA encoding Friend of Gata (Fog). We have shown that overexpression of Fog dominantly interferes with Gata function, causing disruption of erythropoiesis (Mimoto and Christian, 2012). Genes found to be downregulated in both Gata2 MO and Fog RNA injected embryos and found to be reciprocally upregulated in Gata2 RNA injected embryos, compared to uninjected controls, are expected to be positively transcribed downstream of Gata2. We identified *bcar3* as a putative positive target of Gata2. Specifically, microarray analysis revealed that expression of *bcar3* was reduced 1.8–2 fold in ectoderm from Gata2 morphants or embryos injected with Fog RNA, and was induced 2.4 fold in ectoderm from embryos overexpressing Gata2 (Fig. 1B). Bcar3 is a member of the Novel Src homology 2 (SH2)-containing Protein (NSP) family that has an SH2 domain at the amino terminus, a Serine/Proline-rich domain in the middle and a Guanine nucleotide exchange factor (GEF)-like domain at the carboxy terminus (Cai et al., 1999; van Agthoven et al., 1998). To validate *bcar3* as a Gata2 target gene in explanted ectoderm, we injected embryos with Gata2 MOs or Fog RNA, isolated ectoderm at stage 10, cultured it to stage 12 and then analyzed expression of *bcar3* using real time quantitative PCR (qPCR). As shown in Fig. 1C, expression of *bcar3* was nearly ablated in ectoderm isolated from embryos in which Gata2 expression or function was reduced.

We used three additional analyses to confirm that *bcar3* is a real in vivo target of Gata2 in ectodermal cells of intact *Xenopus* embryos during gastrulation. First, we injected control MOs or Gata2 MOs into both cells of two cell embryos, then dissected ventral ectoderm and ventral mesendoderm at stage 13, and performed qPCR to compare levels of *bcar3* in each sample as illustrated (Fig. 1D). Expression of *bcar3* was reduced 10 fold in ectoderm from Gata2 morphants compared to controls (Fig. 1D). Expression of *bcar3* was not detected in ventral mesendoderm at this stage (Fig. 1D). Second, we injected Fog RNA into one cell at the two cell stage, and examined expression of *bcar3* at stage 15 using whole mount in situ hybridization (WMISH). *bcar3* was expressed throughout the ventrolateral ectoderm, and in a dorsal midline stripe in control embryos (Fig. 1E). Expression of *bcar3* was reduced in ventrolateral ectoderm, in the half of the embryo in which Fog had been overexpressed relative to the uninjected half or to controls (Fig. 1E). Third, we examined whether the reduced expression of *bcar3* in Gata2 morphants was rescued by *gata2* RNA. We injected control MOs, Gata2 MOs alone, or Gata2 MOs together with Gata2 RNA that cannot be recognized by the Gata2 MO (Dalgin et al., 2007) into both cells of two cell embryos, dissected ectoderm at stage 10, cultured the ectoderm until sibling embryos reached stage 12, and then analyzed expression of *bcar3* using qPCR. Compared to control MO injected ectoderm, the expression level of *bcar3* was reduced by 2 fold in ectoderm from Gata2 morphants, and was upregulated 2.4 fold in ectoderm from embryos co-injected with Gata2 MOs and Gata2 RNA (Fig. 1F). Taken together, these data show that expression of *bcar3* in ectodermal cells of gastrula stage embryos requires Gata2.

2.2. Expression of bcar3 overlaps with that of gata2 in ectoderm during gastrula and neurula stages

Having shown that *bcar3* is a target gene downstream of Gata2 during gastrulation, we next wished to determine if expression of *bcar3* overlaps with that of *gata2* during gastrulation, and we expanded our examination to locate tissues and organs expressing *bcar3* throughout development. We used Northern analysis to examine temporal patterns of expression of *bcar3*, and WMISH to compare spatial patterns of expression of *gata2* and *bcar3*. Northern analysis showed that a single 4 kb *bcar3* transcript was present in eggs, and this persisted throughout development, while a second, more abundant transcript of approximately 2.1 kb was detected at later stages (Fig. 2A). This finding is consistent with the observation that there are multiple *bcar3* transcripts in human and mouse (Vervoort et al., 2007). WMISH analysis of stage 3 embryos confirmed the presence of maternal *bcar3* RNA (Fig. 2E). As previously shown (Kelley et al., 1994; Pieper et al., 2012; Walmsley et al., 1994; Zon et al., 1991), *gata2* was strongly expressed throughout the ectoderm during blastula and early gastrula stages (Fig. 2B–D), and expression became restricted to ventral and lateral ectoderm by late gastrula through neurula stages (Fig. 2M–Q). We found that *bcar3* was also strongly expressed throughout the ectoderm at blastula and early gastrula stages (Fig. 2F–H). At late gastrula stages, *bcar3* was strongly expressed in ventral ectoderm, in neural plate ectoderm and in the notochord (Fig. 2R and S). Strong expression of *bcar3* persisted in ventral and lateral ectoderm from stage 17–23 (Fig. 2T–V). Expression of *bcar3* was also detected in the notochord, and in specific stripes within the anterior neural folds during neurula stage (Fig. 2T) and in specific regions of the developing eye and brain at later stages (Fig. 2V, BB). At the tailbud stage, expression of *bcar3* was also detected in the cement gland, cranial ganglia, spinal cord, notochord, abdominal muscle anlagen, pronephros, fin mesenchyme and cloaca (Fig. 2BB–EE).

Our observation that expression of *bcar3* and *gata2* overlap in ectoderm during blastula and gastrula stages is consistent with the possibility that *bcar3* might function downstream of Gata2 to generate signals required for mesoderm to commit to a blood cell fate. In addition, *bcar3* is more widely expressed during later