Promotion of avian endothelial cell differentiation by GATA transcription factors

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ABSTRACT

In the avian embryo, endothelial cells originate from several sources, including the lateral plate and somite mesoderm. In this study, we show that Gata transcription factors are expressed in the lateral plate and in vasculogenic regions of the avian somite and are able to promote a vascular endothelial fate when ectopically expressed in somite precursors. A fusion of GATA4 to the transcriptional activator VP16 promoted endothelium formation, indicating that GATA transcription factors promote vasculogenesis via activation of downstream targets, while a fusion of GATA4 to the transcriptional repressor engrailed repressed expression of Vascular Endothelial Growth Factor Receptor 2, a marker of endothelial precursors. These findings indicate a role for GATA transcription factors in the differentiation of the endothelium.

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Introduction

Endothelial cells derive from multiple embryonic sources, including the lateral plate splanchnopleuric mesoderm and the somites (Ben-Yair and Kalcheim, 2008; Bertrand et al., 2010; Boisset et al., 2010; Dieterlen-Lievre and Jaffredo, 2009; Kissa and Herbomel, 2010; Pardanaud et al., 1996). The early splanchnopleuric mesoderm-derived endothelium is hemogenic: it can give rise to hematopoietic cells that are subsequently released into the circulation. In contrast, somite-derived endothelium appears to be restricted to an endothelial fate and does not give rise to hematopoietic tissue (Pardanaud et al., 1996). Some embryonic vessels, such as the aorta, are of dual origin, consisting of a lateral plate-derived hemogenic ventral side, and a somite-derived non-hemogenic dorsal side. After an initial period of hematopoiesis, the ventral wall of the aorta is replaced with somite-derived endothelium and ceases to be hemogenic (Pouget et al., 2006).

Multiple transcription factors have been implicated as playing roles in endothelial cell development (reviewed in De Val and Black, 2009), including Scl/Tal, MEF2C, and members of the Ets family

(Ferdous et al., 2009; Lee et al., 2008; Lin et al., 1998; Patterson et al., 2005; Visvader et al., 1998). However, aside from the recently characterized Ets family member Etv2 (Ferdous et al., 2009; Lee et al., 2008), most of these factors appear to play roles in either the later differentiation and morphogenesis of already-specified endothelial cells, or in regulating the hemogenic properties of endothelium, rather than a specific role in the initial formation of endothelial cells per se.

Members of the Gata family of transcription factors have been found to be required for hematopoiesis in mice (Tsai et al., 1994) or for formation of anterior hemangioblasts (a putative common precursor of endothelium and blood cells) in zebrafish (Peterkin et al., 2009), but a specific role for Gata factors in endothelial cell generation has not been described.

Several signaling pathways have been found to play roles during the early phases of endothelial cell differentiation including the VEGF (Coultas et al., 2005; Shalaby et al., 1997; Shalaby et al., 1995), hedgehog (Byrd et al., 2002), and BMP (Reese et al., 2004; Winnier et al., 1995) pathways. BMPs can promote endothelial cell formation in the chick (Bressan et al., 2009; Nimmagadda et al., 2005; Reese et al., 2004) and have also been implicated in blood vessel formation and in the formation of hemangioblasts in Xenopus and zebrafish (Chen et al., 2008; Liu et al., 2008; Walmsley et al., 2002). BMP signaling also appears to be important for the vasculogenic properties of other signaling pathways. In mice, BMPs have been found to mediate the vasculogenic activity of Sonic Hedgehog (Shh) (Astorga and Carlsson, 2007; Byrd et al., 2002), and BMPs have also been found to regulate Vascular Endothelial Growth Factor receptor 2 (VEGFR2; flk1) expression during early vascular development (Ben-Yair and Kalcheim, 2008; He and

Chen, 2005). However, the mechanisms by which BMP signaling mediates these endothelial-promoting effects have not been well characterized.

The current study reports that GATA transcription factors are expressed in vasculogenic regions of the embryo, can be induced by BMP signaling, can promote endothelial cell differentiation when ectopically expressed, and are required for normal expression of the endothelial cell marker VEGFR2. Taken together with previous data indicating that BMP signaling is required for induction of VEGFR2 and for the production of somite-derived endothelium (Ben-Yair and Kalcheim, 2008), these findings indicate a previously uncharacterized role for GATA factors during endothelial cell differentiation and suggest that they may play a role in mediating the vasculogenic properties of BMP signaling.

Materials and methods

Embryo culture and electroporation

Fertilized chick (*Gallus gallus*, white leghorn, Charles River Spafas) and Japanese quail (*Coturnix japonica*, Strickland Gamebird Farm) eggs were incubated at 38 °C to HH stage 3–5 (Hamburger and Hamilton, 1951) and then placed in modified New Culture as described (Sundin and Eichele, 1992). Electroporations were performed as described (James et al., 2006), and expression of Green fluorescent protein (GFP) in embryos was observed using a Leica dissecting microscope equipped with UV light source. Overall electroporation efficiency was between 50% and 75% of embryos. All plasmids were electroporated at a concentration of 0.6 mg/ml. If multiple plasmids were used, each plasmid was at a concentration of 0.6 mg/ml. Control electroporations used either pCAGGS-GFP or pCAGGs empty vector. DiI-labeled Acetylated Low Density Lipoprotein (acLDL, Molecular Probes) was injected for 5 seconds directly into the heart using pulled glass needles and embryos were incubated for 30 minutes at 37 °C before photographing and fixing as for immunostaining.

DNA constructs

pCAGGS-mGATA4 and pCAGGS-mGATA5 contain the full-length coding region of mouse GATA4 and mouse GATA5 respectively cloned into the pCAGGS vector (Niwa et al., 1991). pCAGGS GATA4-VP16 and pCAGGS GATA4-EnR encode for the mouse GATA4 DNA-binding domain (Zing finger domain) fused to either VP16 activator or Engrailed repressor sequences. All these pCAGGS constructs were obtained by subcloning inserts previously cloned into RCAS vectors, which will be described in detail elsewhere (Kempf et al., manuscript in preparation). pCAGGS-cGATA2 was constructed by isolating mRNA from 2-day-old chicken embryos using the RNeasy kit (Qiagen) and producing cDNA using Transcriptor reverse transcriptase (Roche), followed by PCR at 55 °C using Phusion polymerase (NEBiolabs) using a forward primer containing a XhoI restriction site 5′-ccg ctc gag agg ccc gag gcc tct a-3′ and a reverse primer containing an EcoRI restriction site 5′-ccg gat atc tct gcc acc ttt tgc tt-3′. PCR products were gel-purified, digested with EcoRI and XhoI and purified again using a Qiaprep kit (Qiagen) and cloned directly into pCIG, which is a pCAGGS vector containing an internal ribosome entry site upstream of GFP (gift from A. McMahon). pCAGGs H2B-GFP was a gift of C. Cepko.

Microarray

Approximately 50 explants of presomitic mesoderm from stage 7–8 chick embryos were cultured for 3 h either in serum-free chick embryo medium [SF-CEM; (DMEM-F12, 5 μg/ml human transferrin (Gibco), 100 μg/ml conalbumin (Sigma), 1× insulin-transferrin-selenium (Gibco), 1% Pen-Strep, 1% Glutamine)] or in SF-CEM supplemented with 10 ng/ml of human recombinant BMP-2 (R&D Systems). Cultures were harvested in Trizol, and RNA was prepared as in the manufacturer's instructions. Reverse Transcription and probe generation were carried out using the IVT Express kit (Affymetrix). Both control and BMP-treated probes were prepared in triplicate from three separate culture experiments. Probes were used to screen an Affymetrix Chick genome microarray, and data were analyzed with Affymetrix Gene Chip Operating software. A detailed description of the methods and results of the microarray experiments will be presented elsewhere.

Immunofluorescence and in situ hybridization

For immunofluorescence, embryos were fixed, processed and sectioned as previously described (James and Schultheiss, 2003; Schultheiss et al., 1997). Briefly, embryos were fixed in 4% paraformaldehyde for 30 minutes at room temperature, incubated in 5% sucrose/PBS briefly then 20% sucrose/PBS overnight before embedding in gelatin/15% sucrose/PBS for cryosectioning at 10 μm thickness and immunostaining. QH1 antibody ((Pardanaud et al., 1987) Developmental Studies Hybridoma Bank) ascites fluid was used at 1:100 dilution; rabbit anti-GFP antibody (Torrey Pines Biolabs) was used at 1:500 dilution; mouse anti-GFP antibody (Abcam) was used at 1:500 dilution; cleaved caspase3 antibody (Cell Signaling Technologies) was used at 1:100 dilution. Alexa-fluor 488 (Invitrogen) and Cy3 (Jackson Immunoresearch) conjugated secondary antibodies were used at 1:250–1:500 dilution. Slides were mounted and coverslipped with Vectashield (Vector Laboratories) and photographed using a Zeiss Axiophot or Nikon Eclipse E1000 microscope.

Whole mount in situ hybridization was carried out essentially as previously described (James and Schultheiss, 2005) with probes for chick Gata4 (Schultheiss et al., 1997), Gata5 (clone ChEST978f11 from the BBSRC Chick EST database, Ark Genomics (Boardman et al., 2002)), GATA2 (cloned by RT-PCR from embryonic chick cDNA using primers directed to the full-length GATA2 sequence) and VEGFR2 (Eichmann et al., 1993), a generous gift from C. Kalcheim. Following development of signal, some embryos were cryosectioned (20 μm), mounted in Gelvatol (Sigma) and photographed using DIC optics using a Zeiss Axiophot microscope.

Quantification

GFP co-localization with acLdl or QH1 was quantified by taking 20× merged photographs of 5 representative sections for each embryo and normalizing by the total number of GFP labeled cells and expressing this ratio as a percentage. Apoptosis was quantified by taking 20× photographs of 3 representative sections for each embryo and counting the number of cells exhibiting cleaved caspase3 staining, normalizing by the number of GFP labeled cells, and expressing this ratio as a percentage. Neuroectodermal regions were excluded from the analysis. Data were analyzed by Student's t-test (equal variance, 2 tails) and all error bars are one standard deviation above and below the mean.

Explant cultures, RCAS infection, and gene expression analysis

Presomitic mesoderm (PSM) and somite I–III were prepared from HH10 chicken embryos as described (Kempf et al., 2007; Zeng et al., 2002). For retroviral infection, freshly isolated explants were incubated with 10 μl of concentrated virus on ice for 2 h, before embedding in collagen gels. At day 5, individual explants were lysed and RNA was purified using a QIAGEN RNeasy mini kit, according to the manufacturer's instructions. Gene expression analyses were performed by real-time PCR using the 7900HT fast real-time PCR system (Applied Biosystems). GAPDH was used as an internal control. PCR conditions and primer sequences are available upon request.