SMAD1 and SMAD5 Expression Is Coordinately Regulated by FLI1 and GATA2 during Endothelial Development

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The bone morphogenetic protein (BMP)/SMAD signaling pathway is a critical regulator of angiogenic sprouting and is involved in vascular development in the embryo. SMAD1 and SMAD5, the core mediators of BMP signaling, are vital for this activity, yet little is known about their transcriptional regulation in endothelial cells. Here, we have integrated multispecies sequence conservation, tissue-specific chromatin, *in vitro* reporter assay, and *in vivo* transgenic data to identify and validate *Smad1* +63 and the *Smad5* promoter as tissue-specific *cis*-regulatory elements that are active in the developing endothelium. The activity of these elements in the endothelium was dependent on highly conserved ETS, GATA, and E-box motifs, and chromatin immunoprecipitation showed high levels of enrichment of FLI1, GATA2, and SCL at these sites in endothelial cell lines and E11 dorsal aortas *in vivo*. Knockdown of FLI1 and GATA2 but not SCL reduced the expression of SMAD1 and SMAD5 in endothelial cells *in vitro*. In contrast, CD31⁺ cKit[−] endothelial cells harvested from embryonic day 9 (E9) aorta-gonad-mesonephros (AGM) regions of GATA2 null embryos showed reduced *Smad1* but not *Smad5* transcript levels. This is suggestive of a degree of *in vivo* selection where, in the case of reduced SMAD1 levels, endothelial cells with more robust SMAD5 expression have a selective advantage.

Following the formation of the first blood vessels during vasculogenesis, embryo growth requires constant remodeling of the existing vascular network to form new blood vessels, a process termed angiogenesis (1). Angiogenesis proceeds through a series of steps, beginning with the selection of a tip cell and then the directed migration of the tip cells followed by stalk cells toward a vascular endothelial growth factor gradient, anastomoses of migrating sprouts, and, finally, perfusion and maturation of the newly formed blood vessel (2, 3).

The canonical bone morphogenetic protein (BMP) signaling pathway functions through BMP ligand binding membrane-bound receptors leading to the phosphorylation of the intracellular mediators SMAD1 and -5. Once phosphorylated, these receptor SMADs complex with SMAD4, leading to their translocation to the nucleus, where they work with other transcription factors (TFs) and chromatin remodelers to alter transcription (4–6). Several noncanonical pathway routes have been described, including receptor activation of the extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) pathways (7). BMP signaling has a well-defined role in endothelial cell biology, with previous work demonstrating that the canonical SMAD pathway regulates endothelial cell proliferation by interacting with the NOTCH pathway to upregulate angiogenic genes (8–12). Recent work using endothelium-specific Tie2-Cre mice crossed with mice modified to have floxed copies of the *Smad1* and *Smad5* genes demonstrated that the SMAD proteins work with the NOTCH genes to regulate tip/stalk cell selection in endothelial cells (13). Importantly, the phenotype was apparent only when at least three of the four *Smad1* and *Smad5* alleles were deleted, implying redundant roles for these genes (13, 14).

TFs acting at promoters and distal regulatory elements coordinate complex patterns of cell type-specific gene regulation important for multiple cellular processes and increasingly implicated in disease states. A diverse array of TF families has been implicated in the regulation of the development and function of the vascular system, including FOX, SOX, ETS, KLF, and GATA (15–17). More recently, the interplay between signal pathways and TFs has been shown to regulate several components of the NOTCH pathway to control its activation and function in the endothelium (18–20).

Despite their well-known function in multiple biological processes, little is known about the transcriptional regulation of

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† Deceased 3 October 2010 at the age of 27 years.

This article is dedicated to the memory of Thomas Bee.

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Smad1 and *Smad5*. We sought to address this by performing a comprehensive survey of gene regulatory elements of both genes in endothelial cells. Here, we show that *SMAD1* is regulated by an intronic enhancer, *Smad1*+63, and that *Smad5* is regulated by its promoter. Furthermore, we show that key endothelial ETS, GATA, and E-box TFs bind these *cis*-regulatory modules in developing embryos and that FLI1 and GATA2 regulate the expression of SMAD1 and SMAD5 in endothelial cells.

MATERIALS AND METHODS

Animal ethics. Ethics approval for animal work was granted by the Animal Care and Ethics Committee of the University of New South Wales.

BRE-lacZ embryos. BMP response element (BRE)-*lacZ* embryos were generated and processed as described previously (21). Samples were viewed on a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan), and images were taken with an Olympus Camedia C-3030 Zoom digital camera (Olympus, Melville, NY) and Adobe Photoshop (Adobe Systems Europe, Uxbridge, United Kingdom).

RNA isolation. After centrifugation, cell pellets were resuspended in RNeasy lysis buffer (Life Technologies) and stored at -20°C . RNA was isolated by using the mirVana microRNA (miRNA) isolation kit (Ambion) according to the manufacturer's protocol. RNA quality and quantity were assessed by using a 2100 bioanalyzer (Agilent Technologies), using RNA Pico chips.

Quantitative PCR (qPCR). Embryonic day 11 (E11) aorta-gonad-mesonephros (AGM) regions were dissected, and single-cell suspensions were obtained by collagenase treatment, as described previously (22). Staining was done with the following antibodies: CD41-Brilliant Violet 421 (Biolegend), clone MWReg30 CD34-fluorescein isothiocyanate (FITC) (BD Bioscience), clone RAM34 CD45-phycoerythrin (PE) (eBioscience), clone 30-F11 cKit-allophycocyanin (APC) (eBioscience), and clone 2B8. The sorts were performed on an Influx instrument. The following expression primers were used: forward (F) primer GTGTATGAACTCACAAAATGTGC and reverse (R) primer TAACATCCTGCCG GTGGTATTC for *Smad1*, F primer AACTTTCACCATGGCTTCCA and R primer CCAGAAGCTGAGCAAACTCC for *Smad5*, F primer GAGAC TGATACATACGCCTGCAGA and R primer TCACATGTCTCGATCCC AGTAGA for the $\beta 2$ microglobulin gene, and F primer ACCCACTCCT CCACCTTTGA and R primer CTGTTGCTGTAGCCAAATCGT for *Gapdh*.

Cloning and mutagenesis. Putative enhancer and promoter constructs were PCR amplified from human genomic DNA and cloned into pGL2 (Promega), pGLacZ, and hsp68LacZ vectors as indicated. Primers were designed by using Primer 3 (23). Genomic locations and primer sequences are listed in Table S1 in the supplemental material. Site-directed mutagenesis was performed by using the Agilent QuikChange kit according to the manufacturer's instructions. Mutagenesis products for the *Smad5* promoter were ordered as gene blocks from IDT. Mutagenesis primer sequences are listed in Table S2 in the supplemental material.

Cell culture. MS1 and SEND cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin (all from Life Technologies) at 37°C in 5% CO_2 .

Luciferase assays. Stable-transfection assays were performed as described previously (24). Briefly, 10^7 MS1 or SEND cells were electroporated at 220 V and 900 μF in microcuvettes along with 10 μg of the luciferase vector and 1 μg of the pGK-Puro resistance vector. Forty-eight hours after transfection, the cells were treated with puromycin (Life Technologies) at a final concentration of 0.1 $\mu\text{g}/\text{ml}$ for a period of 24 h before the medium was changed. Cells were grown for 2 to 3 weeks before being lysed and analyzed for luciferase activity.

Transgenics. F0 transgenic embryos were obtained from Cyagen or from the Transgenic Mouse Unit at the Weatherall Institute for Molecular Medicine (Oxford). Transgenic embryos were processed as described previously (25). Briefly, embryos were fixed in a solution containing 1%

formaldehyde, 0.2% glutaraldehyde, 2 mM MgCl_2 , 5 mM EGTA (pH 8.0), and 0.02% NP-40 in phosphate-buffered saline (PBS) for 1 h at 4°C and then stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) overnight at room temperature in the dark. Positive embryos were embedded in OCT compound and sectioned at a thickness of 10 μm on a Leica CM3050S cryostat. Images were taken by using an Olympus Camedia C-3030 Zoom digital camera and processed by using Adobe Photoshop.

Chromatin immunoprecipitation. For AGM chromatin immunoprecipitation (ChIP), the caudal aorta-mesenchyme (without the urogenital ridges) and fetal livers (FLs) of E11.5 embryos were dissected in PBS+10% fetal calf serum (FCS), treated with collagenase for 1 h at 37°C (dorsal aorta), disrupted by gentle pipetting, and resuspended in PBS. Dissections were staggered over a couple of weeks to collect tissues from a total of 60 embryos. At each sitting, cells were treated with 0.4% formaldehyde and lysed, and the retrieved nuclei were snap-frozen in a mixture of isopropanol-dry ice. Frozen nuclei were subsequently pooled and lysed. ChIP assays were performed as previously described (24). Antibodies used in the study were H3K9ac (catalog number 06-599; Millipore); GATA2 (catalog number sc-9008x), SCL (catalog number sc12984X), Ets1 (catalog number sc350x), Ets2 (catalog number sc351x), and Elf1 (catalog number sc631X) (all from Santa Cruz); and Runx1 (catalog number ab23980), Flt1 (catalog number ab15289), and FoxC2 (catalog number ab5060) (all from Abcam) antibodies,

and ChIP samples were quantified by using qPCR via Sybr green chemistry (Life Technologies) on a Stratagene Mx300p instrument (Agilent). Relative fold enrichment was calculated as the fold change over the enrichment seen in the IgG control and also over those for a negative-control region, the promoter of the *Afp* gene. Primers used for ChIP-qPCR were as follows: F primer GCACAGGAGGGAAGGA AATC and R primer CAGGCAATCTACCTCACACTC for *Smad1*+19, F primer ATGGTCTCACGCCACTCTTC and R primer CTGACAAG GTCAAAGCAGCA for *Smad1*+25, F primer CCGATCTCTTAAGCC TGACG and R primer CTCCCAACCTTTTGTTGAA for *Smad1*+63, F primer GTGGGTATTTCTGGCTGCTG and R primer TAGGCAAC AGGCATGATGTG for the *Smad5* promoter, F primer ACAAGTGAC CCCTGCTCTGT and R primer CCTGTTTAAGGGATGCCTGTT for the *Afp* promoter, and F primer GCTAGTTCCTGGGGAAGACC and R primer ATGTCCCAAAGCTGTGTCC for *Scl*+21.

siRNA knockdown. Small interfering RNA (siRNA) knockdown was performed by using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. The following siRNAs were ordered from Qiagen: Flt1-2 (AAGCATAATGTGACTATTGAA), Flt1-4 (CTGCT CTATATATGCAATATA), Gata2-1 (ATGGACAGGTTTACAGATTTA), Gata2-2 (CTCCAAGTGCATGCAAGAGAA), Scl-1 (CAGCTTCTGTTC AAATACTTA), and Scl-4 (ACCTGAATTCTGTTCTCATA). A total of 100,000 MS1 cells were plated into each well of a 6-well plate overnight, and transfections were performed the next day. Cells were grown for 72 h posttransfection and then used in assays. For gene expression experiments, RNA was extracted by using the RNeasy kit (Qiagen) according to the manufacturer's instructions. cDNA was made by using the SuperScript III kit (Life Technologies), and qPCR was performed by using Sybr green chemistry (Life Technologies). For Western blot assays, cells were lysed in a $2\times$ SDS lysis buffer and then scraped off the well. Protein electrophoresis was run on NuPAGE 10% Bis-Tris gels, and primary antibody incubations were performed overnight in 5% skim milk-Tris-buffered saline (TBS)+0.05% Tween. Antibodies used in Western blots were SMAD1 (catalog number 6944; Cell Signaling), SMAD5 (catalog number 9517; Cell Signaling), FLI1 (catalog number ab15289; Abcam), SCL (catalog number sd-12984; Santa Cruz), GATA2 (catalog number sc-9008; Santa Cruz), β -actin (catalog number sc-1616; Santa Cruz), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (catalog number ab9485; Abcam), and H3 (catalog number sc-8654; Santa Cruz) antibodies. Anti-

FIG 1 SMAD1 and SMAD5 are expressed in endothelial and subendothelial cells in E11 AGM regions. (A) Images showing BRE-*lacZ* expression in the endothelium of the dorsal aorta at E10.5. (B) qPCR using sorted endothelial cell (EC) ($CD34^{+}$ VEC^{+} $CD45^{-}$), hematopoietic stem cell (HSPC) ($CD34^{+}$ $cKit^{+}$ $CD41^{int}$ $CD31^{+}$), and mesenchymal cell (MC) ($CD34^{-}$ VEC^{-} $CD45^{-}$) populations from the E11 AGM region showing expression of *Smad1* and *Smad5* transcripts. Mean expression levels $\pm$ standard deviations from duplicate samples are shown as fold changes over *Hprt* expression levels. Expression levels in endothelial cells and HSPCs have been normalized to those in mesenchymal cells. (C) Confocal images of E11 embryo sections showing SMAD1 and SMAD5 protein expression in endothelial and subendothelial cells. BRE, BMP response element; DAPI, 4',6-diamidino-2-phenylindole.

horseradish peroxidase (anti-HRP) secondary antibodies were used to visualize the blots (Abcam), and images were taken on a ChemiDoc XRS system (Bio-Rad).

Confocal microscopy. Wild-type E10 embryos were fixed in 4% para-formaldehyde for 1 h, processed according to standard protocols, embedded in OCT compound, and sectioned on a cryostat. Primary antibody incubation mixtures were left overnight at 4°C, and secondary incubation was done at room temperature for 1 h. Slides were mounted with VectaShield (Vector Laboratories), imaged on a Zeiss 780 microscope, and processed by using Zen (Zeiss). Antibodies used for confocal microscopy were the same primary antibodies as those used for Western blotting as well as CD31 (catalog number 553370; BD) antibody, and secondary antibodies used were donkey anti-rabbit antibody–Alexa Fluor 555, donkey anti-goat antibody–Alexa Fluor 555, and donkey anti-rat antibody–Alexa Fluor 488 (all from Life Technologies).

Cell preparation and flow cytometry. *Gata2*^{+/+}, *Gata2*^{+/-}, and *Gata2*^{-/-} mouse embryos (26) were dissected as previously described (22), and single-cell suspensions were prepared by collagenase treatment (0.125% for 45 min at 37°C) and subsequently washed with a solution containing PBS, 10% heat-inactivated FCS, and 1% penicillin-streptomycin (PS). Cells were labeled with RαMCD31-PE-Cy7 (1:2,000; eBioscience) and RαMcKit-Brilliant Violet 421 (1:1,000; BD) for 30 min at 4°C, washed with PBS–10% FCS–1% PS, and sorted on a Sorp FACSARIA II instrument (BD). Viable cells were sorted directly into PBS supplemented with 50% FCS and 1% PS.

RESULTS

Identification of *Smad1* and *Smad5* cis-regulatory regions in endothelial cells in the embryo. To determine where canonical BMP/SMAD signaling was active in the developing embryo, we used the BMP response element (BRE)-*lacZ* reporter mouse line that expresses the *lacZ* reporter in response to Smad binding (21). The expression pattern observed showed reporter activity in the endothelium, including the dorsal aorta (Fig. 1A). To complement these data, qPCR was performed on sorted endothelial cell, hematopoietic stem and progenitor cell (HSPC), and mesenchymal cell fractions from the E11 aorta-gonad-mesonephros (AGM) region (Fig. 1B) (22). Confocal microscopy confirmed the presence of SMAD1 and SMAD5 protein expression in endothelial cells in E11 dorsal aortas (Fig. 1C). Together, these data demonstrate that canonical BMP/SMAD signaling is active in the developing endothelium of mouse embryos (13, 27).

Potential regulatory regions in the *Smad1* and *Smad5* loci were screened by aligning sequence conservation plots with chromatin accessibility data for human umbilical vein endothelial cells (HUVECs) from the ENCODE project (Fig. 2A). Four putative enhancer sites were identified in the *Smad1* locus and termed the +1, +19, +25, and +63 sites according to kilobase distance from the *Smad1* transcriptional start site (Fig. 2A). One putative site was

FIG 2 *Smad1+63* and the *Smad5* promoter are endothelial *cis*-regulatory elements. (A) Locus diagrams for the human *SMAD1* (left) and *SMAD5* (right) genes are shown with HUVEC DNase I, H3K9ac, and H3K27ac tracks from ENCODE and vertebrate sequence conservation tracks from the UCSC. Regions with high levels of sequence conservation are boxed, and the numbering is from the transcription start site. (B) qPCR analysis of H3K9ac ChIP material from MS1 and SEND endothelial cells showing high levels of enrichment at the corresponding regions in mouse. Data are shown relative to data for IgG and a negative-control region (*Afp* promoter) and are means $\pm$ standard deviations from duplicate samples. (C) Stable-transfection assays in MS1 and SEND endothelial cells using reporter constructs with *Smad1* and *Smad5* conserved regions. Data are shown as fold changes over values for empty vector controls and are means $\pm$ standard deviations. (D) The *Smad1+63* and *Smad5* promoter regions are active in endothelial cells *in vivo*. Representative images from β -galactosidase-stained Hsp68/*lacZ*/*Smad1+63* and *Smad5P*/*lacZ* E10 transgenic embryos show staining of endothelial cells in the dorsal aorta.

also identified in the *Smad5* promoter. No sites were observed in the intergenic regions between *Smad1* and *Smad5* and their respective flanking genes (data not shown). ChIP-qPCR performed with MS1 and SEND endothelial mouse cell lines showed strong enrichment of H3K9ac at the corresponding mouse regions, indicating that they are likely to be conserved (Fig. 2B).

Human DNA sequences for the four potential *Smad1* *cis*-regulatory regions and the *Smad5* promoter were cloned into luciferase vectors and tested in stable-transfection assays in endothelial cell lines (Fig. 2C). All regulatory regions showed some level of activity over the empty vector control, with the *Smad5* promoter and *Smad1+63* regions consistently showing the highest level of

Smad1+63

Smad5 Promoter

FIG 3 Activity of the *Smad1*+63 enhancer and *Smad5* promoter in endothelial cells is dependent on conserved ETS, GATA, and E-box motifs. Shown are data from stable-transfection assays in MS1 endothelial cells with wild-type and mutant enhancer and promoter constructs. Conservation plot diagrams are shown for each region, with conserved ETS sites highlighted in turquoise, GATA sites in red, and E-box sites in yellow. Conserved bases are black, and nonconserved bases are white. Diagrams to the right show the order of conserved binding sites for the respective regulatory region, and sites shaded in gray were mutated for that corresponding bar. Each stable-transfection experiment was performed in triplicate. The data shown are the averages of data from two separate experiments and are shown as means $\pm$ standard deviations relative to values for empty vector controls. Hs, *Homo sapiens*; Cf, *Canis familiaris*; Mm, *Mus musculus*; Md, *Monodelphis domestica*.

activity across both cell lines. The three most active *Smad1* elements, *Smad1*+19, *Smad1*+25, and *Smad1*+63, as measured by stable-transfection data for endothelial cells, and the *Smad5* promoter were cloned into *lacZ* vectors and tested in F0 transgenic embryo assays. The *Smad1*+63 (5/5) and *Smad5* promoter (6/6) transgenics showed staining in endothelial and mesenchymal cells consistent with *Smad1* and *Smad5* expression in the E11 AGM region (Fig. 2D). The *Smad1*+19 (3/3) and *Smad1*+25 (3/4) transgenics showed variable staining only in subendothelial mesenchymal cells in the dorsal aorta (data not shown).

Activity of the *Smad1*+63 and *Smad5* promoters is dependent on highly conserved ETS, GATA, and E-box motifs. Human, dog, mouse, and opossum *Smad1*+63 and *Smad5* promoter sequences were aligned (Fig. 3) and scanned for binding sites from TF families known to regulate endothelial cells. Multiple highly conserved ETS, GATA, and E-box sites were identified, with few binding sites for SOX, FOX, AP-1, or Klf. To test the importance of these binding sites for the activity of the regulatory regions, we mutated each site individually or in pairs and performed stable-transfection assays in MS1 cells. Mutation of the majority of these conserved sites resulted in significantly reduced luciferase activity (Fig. 3).

FLI1 and GATA2 are upstream regulators of SMAD1 and SMAD5 expression in endothelial cells. In order to determine which members of these TF families were bound at the *Smad1* and *Smad5* regulatory regions, we performed chromatin immunoprecipitation (ChIP) assays in MS1 and SEND endothelial cells for

members of each family (Fig. 4A). Several of the ETS factors showed some degree of enrichment, although FLI1 enrichment was robust at both regions in both cell lines. GATA2 enrichment at these regions was also consistent, although SCL enrichment was variable. FLI1, GATA2, and SCL form a recursively wired triad of TFs in the AGM regions and help maintain each other's expression (28). To evaluate whether these factors were bound at the *Smad1*+63 enhancer and *Smad5* promoter in the developing embryo, we performed ChIP assays using dorsal aortas harvested from day 11 embryos. The *Smad1*+63 enhancer and *Smad5* promoter had active chromatin marks and were bound by FLI1, GATA2, and SCL (Fig. 4B).

To test whether FLI1, GATA2, and SCL have nonredundant roles in controlling the expression of SMAD1 and SMAD5 in endothelial cells, we performed siRNA knockdown in MS1 cells using two separate siRNAs each. SMAD1 and SMAD5 transcript and protein levels fell in response to both FLI1 and GATA2 knockdown, although the former was more sensitive to FLI1 and the latter was more sensitive to GATA2 (Fig. 4C). There was no significant reduction in SMAD1 or SMAD5 transcript and protein expression levels with SCL knockdown (Fig. 4C).

GATA2^{-/-} embryos die at around E10.5 with severe anemia but are viable at E9.5 and, although pale, are of a size and a level of development comparable to those of their littermates with relatively normal endothelium (26). To evaluate the impact of GATA2 loss on the expression of SMAD1 and SMAD5 in endothelial cells *in vivo*, we sorted viable CD31⁺ cKit⁻ endothelial cells from E9

FIG 4 FLI1 and GATA2 positively regulate SMAD1 and SMAD5 expression in endothelial cells. (A) qPCR of ChIP material from MS1 and SEND endothelial cells for the indicated transcription factors. Data are shown relative to data for IgG and a negative-control region (*mAfp*) and are means $\pm$ standard deviations from duplicate samples. (B) The *Smad1+63* and *Smad5* promoter are active and bound by FLI1, GATA2, and SCL in E11 AGM regions. Data are shown relative to IgG values. (Ci) qPCR showing knockdown of FLI1, GATA2, and SCL in MS1 endothelial cells with two separate siRNAs and the scrambled control for each target gene with accompanying changes in *Smad1* and *Smad5* transcript levels relative to *Gapdh* levels. (ii) Western blots showing knockdown of FLI1, GATA2, and SCL in MS1 endothelial cells (top row), with corresponding changes in SMAD1 and SMAD5 protein expression along with loading controls. (D) qPCR for *Smad1* and *Smad5* transcripts relative to *Gapdh* transcripts in sorted endothelial cells (CD31⁺ cKit⁻) from E9 (18 to 26 somite pairs) AGM regions of *Gata2*^{-/-} ($n = 4$), *Gata2*^{+/-} ($n = 10$), and *Gata2*^{+/+} ($n = 6$) mouse embryos. Mean expression levels $\pm$ standard deviations from triplicate samples are shown. **, $P < 0.01$ by one-way ANOVA with a Tukey posttest. (E) Schematic diagram showing verified links between components of the BMP signaling pathway and the FLI1/GATA2/SCL triad in the AGM region.

(18 to 26 somite pairs) AGM regions of GATA2^{+/+}, GATA2^{+/-}, and GATA2^{-/-} embryos and quantified *Smad1*, *Smad5*, and *Gapdh* transcript levels. *Smad1* transcript levels were significantly reduced, but *Smad5* transcript levels were relatively unperturbed (Fig. 4D).

DISCUSSION

Disruption of both *Smad1* and *Smad5* together but not either one individually leads to abnormal vascular development. Here, we show that both genes are coordinately regulated by the same upstream regulators, FLI1 and GATA2, which bind at tissue-specific enhancer elements of these genes.

The total absence of GATA2 *in vivo* showed reduced SMAD1 but not SMAD5 transcript levels in embryonic endothelial cells. The greater dependence on a GATA2 input to maintain SMAD1 levels could be due to its activity on a SMAD1 distal enhancer rather than the gene promoter as in the case of SMAD5. Basal transcription of both SMAD1 and SMAD5 expression is clearly maintained in the absence of GATA2, in support of alternate upstream regulatory inputs. In contrast to the *in vivo* data, *in vitro* knockdown of GATA2 in endothelial cell lines resulted in nonredundant reductions in both SMAD1 and SMAD5 levels. Endothelial cells can tolerate reductions in SMAD1 and SMAD5 levels but not the total absence of both (13). Unlike imposed reductions of an upstream regulator in a homogenous cell line, there is probably some degree of *in vivo* selection of endothelial cells in GATA2^{-/-} embryos, where in the presence of reduced SMAD1, cells with more robust levels of SMAD5 are propagated.

We have previously shown that a transcriptional network module composed of FLI1/GATA2/SCL modulates BMP signaling and hematopoietic stem cell emergence in the embryo by regulating RUNX1 activity in the dorsal aorta (28, 29). This involves maintaining a fine balance between SMAD1/5, which positively regulates RUNX1, and SMAD6, which targets RUNX1 for degradation by the proteasome (Fig. 4E) (24, 30). FLI1, GATA2, and SCL, which we show here to bind SMAD1/5 regulatory elements, also positively regulate SMAD6 (29), emphasizing the high level of control exerted by these TFs not only in regulating BMP signaling but also in controlling RUNX1 levels in the hemogenic endothelium in the AGM region. It is noteworthy that SMAD1 and SMAD5, which have redundant roles in angiogenesis, are regulated by the same TFs. This could be an important compensatory mechanism to ensure that there is still *Smad1* or *Smad5* transcription if the expression of the other is lost.

The active regulatory elements all contained ETS, GATA, and E-box binding sites. ETS binding sites were previously found to be highly enriched in GATA2 binding peaks in endothelial cells (31). ETS factors such as FLI1 and ETV2 (ER71) have been positioned at the top of the cascade of TFs regulating the development of the vascular system from mesoderm tissues, along with the GATA binding factor GATA2 and E-box binding factor SCL/TAL1 (16). FLI1 is a known regulator of endothelial cells, and FLI1 knockout embryos exhibit vascular disruption (32, 33). Similarly, GATA2 regulates angiogenic sprouting *in vitro* (34). Although SCL/TAL1 knockdown in MS1 endothelial cells did not alter SMAD1 and SMAD5 protein levels to the same extent as FLI1 and GATA2, the strong enrichment of SCL at the endothelial regulatory elements in the developing aorta and its known role in endothelial development (35) suggest that it may still play a subtle role in modulating expression. These factors all act prior to the determination of ar-

terial or venous fate and are crucial for the formation of all endothelial cells. Binding sites from families involved later in vascular development, such as FOX (arterial) or SOX (venous), were largely absent (36, 37).

Given that BMP/SMAD signaling has been shown to govern angiogenic sprouting from both venous and arterial endothelial cells (13), it is possible to speculate that the ETS and GATA families are more general regulators of endothelial development and regulate the transcription of genes such as SMAD1/5, which are required for broad function of the vascular system. To our knowledge, this is the first study linking ETS and GATA factors to the regulation of BMP pathway activity in endothelial cells and provides an important framework to study the relationship in other contexts where both are crucial, including blood development (38, 39).

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We have no conflicts of interest to declare.

REFERENCES

- Carmeliet P, Jain RK. 2011. Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473:298–307. <http://dx.doi.org/10.1038/nature10144>.
- De Smet F, Segura I, De Bock K, Hohensinner PJ, Carmeliet P. 2009. Mechanisms of vessel branching: filopodia on endothelial tip cells lead the way. *Arterioscler Thromb Vasc Biol* 29:639–649. <http://dx.doi.org/10.1161/ATVBAHA.109.185165>.
- Herbert SP, Stainier DYR. 2011. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nat Rev Mol Cell Biol* 12:551–564. <http://dx.doi.org/10.1038/nrm3176>.
- Moustakas A, Heldin CH. 2009. The regulation of TGFbeta signal transduction. *Development* 136:3699–3714. <http://dx.doi.org/10.1242/dev.030338>.
- Schmieder B, Hill CS. 2007. TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol* 8:970–982. <http://dx.doi.org/10.1038/nrm2297>.
- Inman GJ, Nicolas FJ, Hill CS. 2002. Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity. *Mol Cell* 10:283–294. [http://dx.doi.org/10.1016/S1097-2765\(02\)00585-3](http://dx.doi.org/10.1016/S1097-2765(02)00585-3).
- Gaarenstroom T, Hill CS. 2014. TGF-beta signaling to chromatin: how Smads regulate transcription during self-renewal and differentiation. *Semin Cell Dev Biol* 32:107–118. <http://dx.doi.org/10.1016/j.semcdb.2014.01.009>.
- Larrivee B, Praht C, Gordon E, del Toro R, Mathivet T, Duarte A, Simons M, Eichmann A. 2012. ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway. *Dev Cell* 22:489–500. <http://dx.doi.org/10.1016/j.devcel.2012.02.005>.
- Pardali E, Goumans MJ, ten Dijke P. 2010. Signaling by members of the TGF-beta family in vascular morphogenesis and disease. *Trends Cell Biol* 20:556–567. <http://dx.doi.org/10.1016/j.tcb.2010.06.006>.
- Itoh F, Itoh S, Goumans MJ, Valdimarsdottir G, Iso T, Dotto GP, Hamamori Y, Kedes L, Kato M, ten Dijke P. 2004. Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. *EMBO J* 23:541–551. <http://dx.doi.org/10.1038/sj.emboj.7600065>.
- Valdimarsdottir G, Goumans MJ, Rosendahl A, Brugman M, Itoh S,

- Lebrin F, Sideras P, ten Dijke P. 2002. Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. *Circulation* 106: 2263–2270. <http://dx.doi.org/10.1161/01.CIR.0000033830.36431.46>.
12. Morikawa M, Koinuma D, Tsutsumi S, Vasilaki E, Kanki Y, Heldin CH, Aburatani H, Miyazono K. 2011. ChIP-seq reveals cell type-specific binding patterns of BMP-specific Smads and a novel binding motif. *Nucleic Acids Res* 39:8712–8727. <http://dx.doi.org/10.1093/nar/gkr572>.
13. Moya IM, Umans L, Maas E, Pereira PN, Beets K, Francis A, Sents W, Robertson EJ, Mummery CL, Huylebroeck D, Zwijsen A. 2012. Stalk cell phenotype depends on integration of Notch and Smad1/5 signaling cascades. *Dev Cell* 22:501–514. <http://dx.doi.org/10.1016/j.devcel.2012.01.007>.
14. Umans L, Cox L, Tjwa M, Bitto V, Vermeire L, Laperre K, Sipido K, Moons L, Huylebroeck D, Zwijsen A. 2007. Inactivation of Smad5 in endothelial cells and smooth muscle cells demonstrates that Smad5 is required for cardiac homeostasis. *Am J Pathol* 170:1460–1472. <http://dx.doi.org/10.2353/ajpath.2007.060839>.
15. De Val S, Black BL. 2009. Transcriptional control of endothelial cell development. *Dev Cell* 16:180–195. <http://dx.doi.org/10.1016/j.devcel.2009.01.014>.
16. Park C, Kim TM, Malik AB. 2013. Transcriptional regulation of endothelial cell and vascular development. *Circ Res* 112:1380–1400. <http://dx.doi.org/10.1161/CIRCRESAHA.113.301078>.
17. Meadows SM, Myers CT, Krieg PA. 2011. Regulation of endothelial cell development by ETS transcription factors. *Semin Cell Dev Biol* 22:976–984. <http://dx.doi.org/10.1016/j.semcdb.2011.09.009>.
18. Wu ZQ, Rowe RG, Lim KC, Lin Y, Willis A, Tang Y, Li XY, Nor JE, Maillard I, Weiss SJ. 2014. A Snail1/Notch1 signalling axis controls embryonic vascular development. *Nat Commun* 5:3998. <http://dx.doi.org/10.1038/ncomms4998>.
19. Wythe JD, Dang LT, Devine WP, Boudreau E, Artap ST, He D, Schachterle W, Stainier DY, Oettgen P, Black BL. 2013. ETS factors regulate Vegf-dependent arterial specification. *Dev Cell* 26:45–58. <http://dx.doi.org/10.1016/j.devcel.2013.06.007>.
20. Sacilotto N, Monteiro R, Fritzsche M, Becker PW, Sanchez-del-Campo L, Liu K, Pinheiro P, Ratnayaka I, Davies B, Goding CR. 2013. Analysis of Dll4 regulation reveals a combinatorial role for Sox and Notch in arterial development. *Proc Natl Acad Sci U S A* 110:11893–11898. <http://dx.doi.org/10.1073/pnas.1300805110>.
21. Blank U, Seto ML, Adams DC, Wojchowski DM, Karolak MJ, Oxburgh L. 2008. An in vivo reporter of BMP signaling in organogenesis reveals targets in the developing kidney. *BMC Dev Biol* 8:86. <http://dx.doi.org/10.1186/1471-213X-8-86>.
22. Fitch SR, Kimber GM, Wilson NK, Parker A, Mirshekar-Syahkal B, Gottgens B, Medvinsky A, Dzierzak E, Ottersbach K. 2012. Signaling from the sympathetic nervous system regulates hematopoietic stem cell emergence during embryogenesis. *Cell Stem Cell* 11:554–566. <http://dx.doi.org/10.1016/j.stem.2012.07.002>.
23. Koressaar T, Remm M. 2007. Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23:1289–1291. <http://dx.doi.org/10.1093/bioinformatics/btm091>.
24. Knezevic K, Bee T, Wilson NK, Janes ME, Kinston S, Polderdijk S, Kolb-Kokocinski A, Ottersbach K, Pencovich N, Groner Y, de Bruijn M, Gottgens B, Pimanda JE. 2011. A Runx1-Smad6 rheostat controls Runx1 activity during embryonic hematopoiesis. *Mol Cell Biol* 31:2817–2826. <http://dx.doi.org/10.1128/MCB.01305-10>.
25. Nottingham WT, Jarratt A, Burgess M, Speck CL, Cheng J-F, Prabhakar S, Rubin EM, Li P-S, Sloane-Stanley J, Kong-A-San J, de Bruijn MF. 2007. Runx1-mediated hematopoietic stem-cell emergence is controlled by a Gata/Ets/SCL-regulated enhancer. *Blood* 110:4188–4197. <http://dx.doi.org/10.1182/blood-2007-07-100883>.
26. Tsai FY, Keller G, Kuo FC, Weiss M, Chen J, Rosenblatt M, Alt FW, Orkin SH. 1994. An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* 371:221–226. <http://dx.doi.org/10.1038/371221a0>.
27. Dick A, Risau W, Drexler H. 1998. Expression of Smad1 and Smad2 during embryogenesis suggests a role in organ development. *Dev Dyn* 211:293–305. [http://dx.doi.org/10.1002/\(SICI\)1097-0177\(199804\)211:4<293::AID-AJAI>3.0.CO;2-C](http://dx.doi.org/10.1002/(SICI)1097-0177(199804)211:4<293::AID-AJAI>3.0.CO;2-C).
28. Pimanda JE, Ottersbach K, Knezevic K, Kinston S, Chan WY, Wilson NK, Landry JR, Wood AD, Kolb-Kokocinski A, Green AR, Tannahill D, Lacaud G, Kouskoff V, Gottgens B. 2007. Gata2, Flil, and Scl form a recursively wired gene-regulatory circuit during early hematopoietic development. *Proc Natl Acad Sci U S A* 104:17692–17697. <http://dx.doi.org/10.1073/pnas.0707045104>.
29. Pimanda JE, Donaldson IJ, de Bruijn MF, Kinston S, Knezevic K, Huckle L, Piltz S, Landry JR, Green AR, Tannahill D, Gottgens B. 2007. The SCL transcriptional network and BMP signaling pathway interact to regulate RUNX1 activity. *Proc Natl Acad Sci U S A* 104:840–845. <http://dx.doi.org/10.1073/pnas.0607196104>.
30. Narula J, Williams CJ, Tiwari A, Marks-Bluth J, Pimanda JE, Igoshin OA. 2013. Mathematical model of a gene regulatory network reconciles effects of genetic perturbations on hematopoietic stem cell emergence. *Dev Biol* 379:258–269. <http://dx.doi.org/10.1016/j.ydbio.2013.04.016>.
31. Linnemann AK, O'Geen H, Keles S, Farnham PJ, Bresnick EH. 2011. Genetic framework for GATA factor function in vascular biology. *Proc Natl Acad Sci U S A* 108:13641–13646. <http://dx.doi.org/10.1073/pnas.1108440108>.
32. Abedin MJ, Nguyen A, Jiang N, Perry CE, Shelton JM, Watson DK, Ferdous A. 2014. Flil1 acts downstream of ETV2 to govern cell survival and vascular homeostasis via positive autoregulation. *Circ Res* 114:1690–1699. <http://dx.doi.org/10.1161/CIRCRESAHA.1134303145>.
33. Spyropoulos DD, Pharr PN, Lavenburg KR, Jackers P, Papas TS, Ogawa M, Watson DK. 2000. Hemorrhage, impaired hematopoiesis, and lethality in mouse embryos carrying a targeted disruption of the Flil1 transcription factor. *Mol Cell Biol* 20:5643–5652. <http://dx.doi.org/10.1128/MCB.20.15.5643-5652.2000>.
34. Kanki Y, Kohro T, Jiang S, Tsutsumi S, Mimura I, Suehiro J, Wada Y, Ohta Y, Ihara S, Iwanari H, Naito M, Hamakubo T, Aburatani H, Kodama T, Minami T. 2011. Epigenetically coordinated GATA2 binding is necessary for endothelium-specific endomucin expression. *EMBO J* 30: 2582–2595. <http://dx.doi.org/10.1038/emboj.2011.173>.
35. Visvader JE, Fujiwara Y, Orkin SH. 1998. Unsuspected role for the T-cell leukemia protein SCL/tal-1 in vascular development. *Genes Dev* 12:473–479. <http://dx.doi.org/10.1101/gad.12.4.473>.
36. Cermenati S, Molero S, Cimbro S, Corti P, Del Gaudio L, Amodeo R, Dejana E, Koopman P, Cotelli F, Beltrame M. 2008. Sox18 and Sox7 play redundant roles in vascular development. *Blood* 111:2657–2666. <http://dx.doi.org/10.1182/blood-2007-07-100412>.
37. Seo S, Fujita H, Nakano A, Kang M, Duarte A, Kume T. 2006. The forkhead transcription factors, Foxc1 and Foxc2, are required for arterial specification and lymphatic sprouting during vascular development. *Dev Biol* 294:458–470. <http://dx.doi.org/10.1016/j.ydbio.2006.03.035>.
38. Zhang C, Lv J, He Q, Wang S, Gao Y, Meng A, Yang X, Liu F. 2014. Inhibition of endothelial ERK signalling by Smad1/5 is essential for haematopoietic stem cell emergence. *Nat Commun* 5:3431. <http://dx.doi.org/10.1038/ncomms4431>.
39. Lan Y, He W, Li Z, Wang Y, Wang J, Gao J, Wang W, Cheng T, Liu B, Yang X. 2014. Endothelial Smad4 restrains the transition to hematopoietic progenitors via suppression of ERK activation. *Blood* 123:2161–2171. <http://dx.doi.org/10.1182/blood-2013-09-526053>.