Fli1 Acts at the Top of the Transcriptional Network Driving Blood and Endothelial Development

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Summary

Blood and endothelium arise in close association during development, possibly from a common precursor, the hemangioblast [1–4]. Genes essential for blood and endothelial development contain functional ETS binding sites, and binding and expression data implicate the transcription factor, friend leukaemia integration 1 (Fli1) [5–10]. However, loss-of-function phenotypes in mice, although suffering both blood and endothelial defects, have thus far precluded the conclusion that Fli1 is essential for these two lineages [11, 12]. By using Xenopus and zebrafish embryos, we show that loss of Fli1 function results in a substantial reduction or absence of hemangioblasts, revealing an absolute requirement. TUNEL assays show that the cells are eventually lost by apoptosis, but only after the regulatory circuit has been disrupted by loss of Fli1. In addition, a constitutively active form of Fli1 is sufficient to induce expression of key hemangioblast genes, such as Scl, Tal1, Lmo2, Gata2, Etsrp, and Flik1. Epistasis assays show that Fli1 expression is induced by Bmp signaling or Cloche, depending on the hemangioblast population, and in both cases Fli1 acts upstream of Gata2, Scl, Lmo2, and Etsrp. Taken together, these results place Fli1 at the top of the transcriptional regulatory hierarchy for hemangioblast specification in vertebrate embryos.

Results

Fli1 Is Required for Both Primitive and Definitive Hemangioblast Formation

In the early Xenopus embryo, we have described two populations of hemangioblasts that represent the precursors of the primitive and definitive blood and endothelial lineages [10]. The primitive hemangioblast population is located in the anterior of the neurula stage embryo (~16 hpi), just below the cement gland, whereas the definitive population is found in the dorsal lateral plate (DLP) of the tailbud stage embryo approximately 12 hr later. Fli1 is one of the earliest markers to be expressed in both populations [10].

To knock down Fli1 expression in Xenopus embryos, we made use of an antisense morpholino (MO) designed against the ATG region, and an optimal level of 40 ng per embryo was established by monitoring the downregulation of Scl expression in the anterior hemangioblast (Figure S1 available online). When targeted to the dorsal marginal zone (DMZ) at the 4/8 cell stage (the region giving rise to the primitive hemangioblast population [10], the expression of many genes later expressed in erythroid, myeloid, or endothelial cells was downregulated (Figures 1A–1L, 1S, 1T, 1V, and 1W, arrows). Thus, expression of Scl, Lmo2, Flik1, Mpo, Spib, and Runx1 were all severely reduced in Fli1 MO-injected embryos (morphants) monitored at stage 17. This effect was specific to the hemangioblasts: neither Runx1 expression in Rohon Beard cells of the neural plate nor Lmo2 expression in the somites was affected (Figures S4A–S4D). The effect on Gata2 expression, when Fli1 MO was injected, was monitored by in situ hybridization on sections, because Gata2 is expressed in all three germ layers at neurula stages [13]. Gata2 expression in the Fli1 morphants was reduced, albeit modestly, in the mesoderm of the anterior hemangioblast region (Figures 1I and 1L, arrows). The effect on Scl expression has been reported previously with the same MO [14], but here we show that all the hemangioblast genes tested were downregulated, consistent with a primary role for Fli1 at the top of the genetic hierarchy leading to formation of the embryonic hemangioblast.

Because the effect of the Fli1 MO on Gata2 expression in the anterior hemangioblast was a subtle one, we turned to Gata2 morphants to establish the hierarchy between Gata2 and Fli1. A combination of MOs directed against the ATG regions of Gata2a and B, the two pseudo-alleles of Xenopus laevis, have previously been shown to block globin expression [15], and we titrated the MOs monitoring this activity to optimize the dose (Figure S2). When we injected these Gata2 MOs into the DMZ at the 4-cell stage (the region containing the precursors of the anterior hemangioblast population), Scl and Lmo2 expression at stage 16/17 was significantly reduced whereas Fli1 expression remained unchanged (Figures 1M–1R). This, together with the reduction, albeit only slight, of Gata2 expression in Fli1 morphants, suggests that Fli1 may be above Gata2 in the regulatory hierarchy determining the development of the primitive hemangioblast population. The small effects of the Fli1 and Gata2 MOs on each other’s expression compared to their effects on Scl and Lmo2 expression are consistent with both Fli1 and Gata2 being upstream of Scl and Lmo2.

To further test the hierarchical relationship of Fli1 with Scl, Lmo2, and Gata2, we tried to rescue the Fli1 morphant phenotype by coinjection of mRNAs for Scl, Lmo2, and Gata2. As shown in Figures 1S–1X, the reduction of expression of SpiB and Runx1 was partially rescued by coinjection of the three mRNAs, in a dose-dependent manner (data not shown), consistent with Fli1 acting upstream of Scl, Lmo2, and Gata2 in the anterior hemangioblast population.

In order to look at the adult hemangioblast, we targeted the Fli1 MO to the ventral marginal zone (VMZ) at the 4/8 cell stage (the region giving rise to the DLP) [16]. We found that blood and endothelial gene expression in the adult DLP hemangioblast at tailbud stages was eliminated (Figures 2A–2L, black arrows). The expression of Scl, Gata2, Lmo2, Flik1, Msr, and Tie2 (data not shown) were all downregulated. Fli1 expression in

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the hemangioblast component of the DLP was also abolished (Figures 2A and 2B, cells outlined by red dotted line in Figure 2A). The remaining Fli1 expression in the DLP region in Fli1 morphants (Figure 2B, black dotted line) is in the pronephric duct (PND) precursors [10]. The reduced effect on the PND was confirmed with additional markers, Pax2 and Pax8, and expression of all these markers plus Runx1 and a T4Globin in the pVBI (also derived from the VMZ) was also decreased by this injection (Figures 2C–2L; Figure S3, red arrows). Overall, these results show that Fli1 is required for primitive and definitive hemangioblast formation in Xenopus embryos.

To confirm the hierarchy of transcription factors involved in establishing the definitive hemangioblast program in the DLP, we targeted the Gata2 MOs to the VMZ and monitored expression of Fli1, Scl, Lmo2, and Msr in the DLP soon after expression of these genes is first detectable. Whereas expression of Scl, Lmo2, and Msr was strongly downregulated in the Gata2 morphants (Figures 2O–2T, arrows), the expression of Fli1 remained unaffected (Figures 2M and 2N, arrows). These results suggest that Fli1 is at the top of the transcriptional hierarchy programming the definitive hemangioblast.

Incorrectly Programmed Hemangioblasts in Fli1 Morphants Undergo Apoptosis

We next asked whether the loss of expression of blood and endothelial markers in both embryonic and definitive hemangioblast populations was the result of respecification or loss of the cells. We monitored expression of MyoD, Nkx2.5, Blimp1, and BMP4, genes that are expressed in regions abutting the embryonic hemangioblast population, and found them unchanged in Fli1 morphants (Figures S4E–S4L), suggesting that respecification is not the cause of loss of hemangioblast gene expression. In contrast, TUNEL assays on Fli1 morphants at stage 17 (embryonic hemangioblasts) and stage 26 (adult hemangioblasts) revealed apoptosis in regions of the embryo where Scl expression was lost (Figures S5C, S5D, S5I, and S5J). Broader apoptosis in the DLP area at stage 26 may correspond to the partial loss of the PND (Figures S3E–S3H). Looking at times when Scl is first expressed (stage 14 for embryonic and stage 24 for adult hemangioblasts), we found loss of Scl expression in Fli1 morphants in the absence of apoptosis (Figures S5A, S5B, S5E, and S5H). Similar results were obtained for Fli1 expression (data not shown). These results suggest that loss of the hemangioblast program leads to apoptosis rather than being a consequence of it.

Activated Fli1 Transactivates Early Hemangioblast Genes through Scl, Etsrp, and Cloche

Our experiments so far in Xenopus have established the hierarchical relationship between the transcription factors Fli1, Gata2, Scl, and Lmo2. We were interested to investigate this relationship in zebrafish and also to define the position of
Fli1 in relation to the important regulators of blood and endothelium, Cloche and Etsrp, that are specific to the zebrafish. We began by looking at loss-of-function embryos with an MO against the 5’ untranslated region (UTR) immediately upstream of the Fli1 translation initiation codon. Although we were able to demonstrate activity of the MO by knocking down GFP expression from a transgene (data not shown), embryos injected with the MO were phenotypically normal and expression of blood and endothelial markers was unchanged (data not shown), raising the possibility of ETS factor redundancy [17]. Zebrafish have more ETS factors than other vertebrates (31 versus 24–29 for frog, mouse, and human), and, in particular, the Fli1 locus has been duplicated giving rise to the Fli1b gene [18]. We and others have used combinations of MOs but thus far have been unable to significantly disrupt both blood and endothelium (data not shown)[19, 20].

We therefore turned to gain-of-function experiments. To functionally test the ability of Fli1 to transactivate early hemangioblast genes, we injected full-length Fli1 RNA into zebrafish embryos but it had little effect on Scl, Lmo2, Gata2, or Flk1 expression (Figure 3, compare [B], [E], [H], [N] with [A], [D], [G], [J]). The lack of effect is possibly because, like other ETS factors, Fli1 needs activating signals [21], or alternatively partners [5]. We therefore also tested a constitutively active form of Fli1, in which the transactivation domain of the viral VP16 protein was fused to full-length Fli1 coding sequences. Fli1-VP16 RNA injection caused strong ectopic expression of Scl, Lmo2, Gata2, Flk1, and Etsrp as well as of Fli1 itself (Figure 3, compare arrowed regions in [C], [F], [I], [L], [O], [T] with [A], [D], [G], [J], [M], [S]). In contrast, there was no expansion of expression of the blood marker Gata1 (Figure 3, compare [R] with [P]). We therefore conclude that activated Fli1 protein can transactivate early hemangioblast genes but that the ectopic hemangioblast-like cells are unable to differentiate into red blood cells expressing Gata1. This might reflect an absence of the necessary blood differentiation signals in these ectopic regions of the embryo. Nevertheless, together with the observations that Fli1 expression is initially unaffected in Cloche embryos (Figures S6A and S6B) [9], or by the loss of Scl or Lmo2 [22, 23], these data are consistent with Fli1 acting near the top of the hemangioblast genetic hierarchy.

To further establish the hierarchy between these key regulators, we coinjected embryos with Fli1-VP16 RNA and either Scl or Etsrp MOs. As already discussed, injection of Fli1-VP16 RNA alone leads to increased expression of Flk1 (Figures 3O and 4B, arrows), whereas injection of Scl MO alone significantly
 activates at least one of its targets through topic expression in wild-type embryos (Figures 4G–4J, arrows). Thus, the activity of Fli1-Cloche must be dependent on Fli1 is upstream or parallel to cating that 1237 reduced Flk1 expression (Figure 4C, arrows), consistent with previous data from us and others [23, 24]. Injection of Fli1-VP16 RNA together with Scl MO failed to rescue the loss of Flk1 expression because of depletion of Scl protein, with the coinjected embryos showing the same phenotype as Scl MO injection alone (Figure 4D), suggesting that Scl is downstream of Fli1 in hemangioblast formation. Similarly, injection of Etsrp MO greatly reduced endothelial cell numbers as reported previously (Figure 4E) [18]. In the coinjected embryos, Fli1-VP16 RNA failed to rescue the Etsrp MO phenotype (Figure 4F), indicating that Etsrp is also downstream of Fli1. Thus, Fli1 transactivates at least one of its targets through Scl and Etsrp.

The Cloche gene has not yet been identified [25] and therefore we cannot determine whether Cloche expression is upregulated by Fli1-VP16. However, we can determine whether Fli1 activity is dependent on Cloche. We therefore injected Fli1-VP16 RNA into Clo−/− embryos to determine whether hemangioblast gene expression could be rescued. When Scl expression was monitored at 10 somites, no rescue was seen, even though the Fli1-VP16 RNA was able to induce ectopic expression in wild-type embryos (Figures 4G–4J, arrows). Thus, the activity of Fli1-VP16 we see in wild-type embryos must be dependent on Cloche. We therefore conclude that Fli1 is upstream or parallel to Cloche in the hemangioblast markers, Scl and Gata2, in the posterior lateral mesoderm (PLM) was substantially reduced (Figures S7A–S7F). We also checked Fli1 expression in the dino mutant, a well-characterized mutant resulting in enhanced Bmp signaling. Consistently, the expression of Fli1, together with Gata2 and Scl, was upregulated in the vicinity of the PLM (Figure S7G, arrows and data not shown). Cardiac precursor (Nkx2.5) and hindbrain (Krox20) marker expression acted as controls (Figure S7H).

The PLM in zebrafish gives rise to adult blood and endothelial cells and is therefore homologous to the DLP in frogs, in which Fli1 expression is also regulated by Bmp signaling [10]. Furthermore, because the PLM in fish also gives rise to embryonic erythroid but not myeloid cells, it could be viewed as homologous to the posterior VBI precursors in Xenopus rather than the anterior hemangioblast [27]. Therefore, in order to determine if the zebrafish population homologous to the anterior hemangioblast in Xenopus behaves similarly with respect to the Fli1 response to Bmp signaling, we monitored gene expression in the anterior lateral mesoderm (ALM) in heat-shocked embryos. We found that relatively normal numbers of Fli1-expressing cells were present at 6 somites (Figures S7I and S7J). We therefore conclude that the anterior hemangioblast populations in Xenopus and zebrafish that give rise to embryonic blood and endothelium express Fli1 in program. Taken together, these zebrafish data are consistent with Fli1 acting at the top of the hemangioblast genetic hierarchy through, or together with, other key regulators, such as Scl, Etsrp, and Cloche.

Differential Control by Bone Morphogenetic Proteins of Fli1 Expression in Adult and Embryonic Hemangioblasts

Inhibition of bone morphogenetic protein (Bmp) signaling in Xenopus embro- 
yos impairs the programming of both adult and embryonic hemangioblasts [10]. In the specific case of Fli1, though, although expression in the adult hemangio- 
blast is dependent on Bmp signaling, it appears to be independent of it in the embryonic hemangioblast [10]. Bmp signaling is also required for hemangio- 
blast induction in zebrafish but the re- 
sponse of Fli1 has not been recorded [26]. To determine the situation in zebra-
fish, we made use of a heat-shock-inducible dominant-negative Bmp recep- 
tor transgenic line [26]. When we heat 
shocked this line at 30% epiboly (i.e., before gastrulation), the expression of Fli1 as well as the other hemangioblast
a Bmp-independent manner in contrast to the posterior hemangioblasts that give rise to adult blood.

**Discussion**

Most of the genes known to be essential for the early blood and endothelial programmes contain ETS binding sites [5, 28]. Several members of the ETS family are present in the precursors to blood and endothelium in the embryo, and of these, Fli1 has emerged as a strong candidate based on its occupancy of these sites in putative hemangioblast-containing populations of cells [7]. However, the null phenotype in mice has been ambiguous [11, 12]. To clarify the early role of Fli1 in blood and endothelial development, we took advantage of two organisms that have been studied extensively for the early development of these cells. In particular, the locations and gene-expression profiles of the cell populations giving rise to these two tissues are well documented. We find that, in the absence of Fli1, neither population is programmed to the hemangioblast fate and that eventually the cells are lost by apoptosis, confirming Fli1 as a master regulator of blood and endothelial development.

In the building of genetic regulatory networks (GRNs), Davidson and his colleagues have suggested that three criteria need to be fulfilled to demonstrate regulatory connections between transcription factors [29]. First, the proposed target needs to respond appropriately when the proposed regulator’s expression or activity is perturbed; second, the expression profiles of the genes need to fit the proposed relationship; and finally, there needs to be evidence that the regulator binds to its target. Pimanda et al. have drawn a core GRN, or kernel, for the hemangioblast, based on criteria 2 and 3 [7]. Here we...
provide the perturbations necessary to test the validity of the proposed activities of Fli1 and to a lesser extent Gata2, i.e., criterion 1. We show that Fli1 fulfills all three criteria as a positive regulator of Gata2, Scl, Lmo2, and itself. Our data also show that Gata2 fulfills all three criteria for Scl and Lmo2, but we find no evidence that it regulates Fli1, in contrast to the network of Pimanda et al. [7]. These conclusions have been built into a new hemangioblast kernel (Figure 4K). Such a core regulatory network may be conserved even beyond vertebrates because overexpression of the Drosophila pointed gene (the ortholog of the ETS family of proteins in vertebrates) drastically increased the number of circulating hemocyes in larvae [30].

Perturbations of Scl and Lmo2 are consistent with the proposed locations of Fli1 and Gata2 in the hierarchy [22, 23]. Thus, loss of either has no effect on Fli1 or Gata2 expression, again in contrast to the network of Pimanda et al. [7]. Furthermore, Scl is required for Fli1/VP16 to drive Fli1 expression (Figures 4A–4D), and loss of downstream Runx1 and SpiB expression in Fli1 morphants is rescued by a combination of Scl, Lmo2, and Gata2 mRNAs (Figures 1S–1X). With respect to regulation of Fli1 expression, we show that Cloche is required in the anterior hemangioblast (Figure S6), whereas Bmp is required in the posterior hemangioblast (Figure S7; see also [10]). Furthermore, although Bmp is not required to drive Fli1 expression in the anterior hemangioblast, both Bmp and Fli1 are required to drive Scl. In principle, this could have indicated that Fli1 is upstream of Bmp. However, at least for Bmp4, the dominant Bmp in early Xenopus embryos, this does not seem to be the case (Figures S4K and S4L). Thus, a feasible scenario in the anterior hemangioblast is that Bmp drives Gata2 and Gata2 plus Fli1 drive Scl via the demonstrated ETS-Gata sites (Figure 4K) [5].

Careful temporal monitoring of hemangioblast marker expression and the onset of cell death in Fli1 morphants demonstrated that disruption of the hemangioblast program occurred before the cells underwent apoptosis. Thus, Fli1 is required for the induction of the hemangioblast program and also for cell survival, but whether Fli1 is acting directly on the cell-death pathway cannot be determined from the available data. It is noteworthy, though, that Fli1 has already been shown to display antiapoptotic activity in several cell types including avian erythroblasts, mouse fibroblasts, and lymphoid cells, possibly through the upregulation of Bcl2, an antiapoptotic protein [31]. So it is possible that the observed cell death is directly caused by the loss of Fli1 rather than being a consequence of the misprogramming of the cells.

In conclusion, the data presented here finally confirm a position for Fli1 at the top of the genetic cascade establishing the blood and endothelial programmes within cells of the mesoderm in the early embryo. We find some differences between the different populations with respect to the regulation of Fli1 itself, but in each case we find that Fli1 function is indispensable.

Accession Numbers

Tie2, Est clone, was deposited in the National Institute for Basic Biology (NIBB) (Japan) database with accession number ID XLo64122.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and seven figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/16/1234/DC1/.

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