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Five Transcription Factors and FGF Pathway Inhibition Efficiently Induce Erythroid Differentiation in the Epiblast

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SUMMARY

Primitive erythropoiesis follows a stereotypic developmental program of mesoderm ventralization and internalization, hemangioblast formation and migration, and erythroid lineage specification. Induction of erythropoiesis is inefficient in either ES/iPS cells in vitro or nonhemangioblast cell populations in vivo. Using the chick model, we report that epiblast cells can be directly and efficiently differentiated into the erythroid lineage by expressing five hematopoietic transcription regulators (SCL+LMO2+GATA2+LDB1+E2A) and inhibiting the FGF pathway. We show that these five genes are expressed with temporal specificity during normal erythropoiesis. Initiation of *SCL* and *LMO2* expression requires FGF activity, whereas erythroid differentiation is enhanced by FGF inhibition. The lag between hematopoiesis and erythropoiesis is attributed to sequential coregulator expression and hemangioblast migration. Globin gene transcription can be ectopically and prematurely induced by manipulating the availability of these factors and the FGF pathway activity. We propose that similar approaches can be taken for efficient erythroid differentiation in vitro.

INTRODUCTION

During primitive erythropoiesis in amniotes, hemangioblast cells are generated from the pluripotent epiblast through gastrulation at the posterior primitive streak (ventral mesoderm precursors) and give rise to predominantly primitive erythrocytes. In both chick and mouse models, a delay of approximately half a day has been reported between the onset of early hematopoietic markers in newly ingressed extraembryonic mesoderm cells and that of globin genes (McGrath and Palis, 2008; Minko et al., 2003; Nakazawa et al., 2006; Silver and Palis, 1997). A comparable delay is seen in *Xenopus* (Ciau-Uitz et al., 2010; Walmsley et al., 2008), zebrafish (Brownlie et al., 2003; Ciau-Uitz et al., 2010; Patterson et al., 2007), and erythroid differentiation from embryonic stem cells/induced pluripotent stem cells in vitro (Chang et al., 2006; Lu et al., 2008). The molecular nature of this delay is unclear but has been hypothesized to reflect a requirement for combinatorial and sequentially expressed transcription factors, for extraembryonic endoderm contact, or for the embryo to reach a conducive developmental stage (Galloway et al., 2008; Gering et al., 2003; McGrath and Palis, 2008). Data from our lab and other labs suggested that this delay may also reflect a requirement for hemangioblasts to downregulate fibroblast growth factor (FGF) activities before the onset of erythropoiesis (Kumano and Smith, 2000; Nakazawa et al., 2006; Walmsley et al., 2008). In this work, we used the chick model and showed that it was possible to bypass this requirement and achieve ectopic and accelerated erythroid induction in postingression mesoderm and pluripotent epiblast cell populations.

RESULTS AND DISCUSSION

Primitive Erythropoiesis Follows a Hemangioblast-Intrinsic Program

During chicken primitive erythropoiesis, *SCL* and *LMO2* are first detected in nascent extraembryonic mesoderm at Hamburger and Hamilton stage 4 (HH4), the full streak stage (Figure 1A) (Minko et al., 2003; Nakazawa et al., 2006). By HH5, migrating hemangioblasts (Weng et al., 2007) express these genes robustly as revealed by RNA in situ hybridization analysis (Figures 1B and 1C). Erythroid lineage differentiation, marked by embryonic *rho* globin (*HGB1* gene) expression, is initiated only after HH7 (zero to one somite) (Figures 1D and 1E). As development proceeds, primitive erythropoiesis intensifies (Figure 1F) and generates differentiated primitive red blood cells before initiation of cardiac activity at HH11 and of circulation at HH12. To understand molecular mechanisms controlling the progression from hematopoiesis (HH4) to erythropoiesis (HH7), we first investigated whether avian primitive erythropoiesis follows a hemangioblast intrinsic or extrinsic developmental program by graft analysis. Hemangioblasts from HH6 embryos (before erythropoiesis) were grafted isotopically to HH6 host, and host embryos were incubated postgraft to HH8 (Figure 1G). All graft hemangioblasts (circles) (15/15 grafts) initiated *rho* expression as strongly as in the host (arrowheads). HH6 hemangioblasts grafted to HH4 host, grown to HH6+ postgraft (Figure 1H), were also *rho* positive (circle and inset) (11/11 grafts) despite the host still being *rho* negative, and the differentiation potential of HH6 hemangioblast graft in HH4 host was not dependent on graft location (Figures

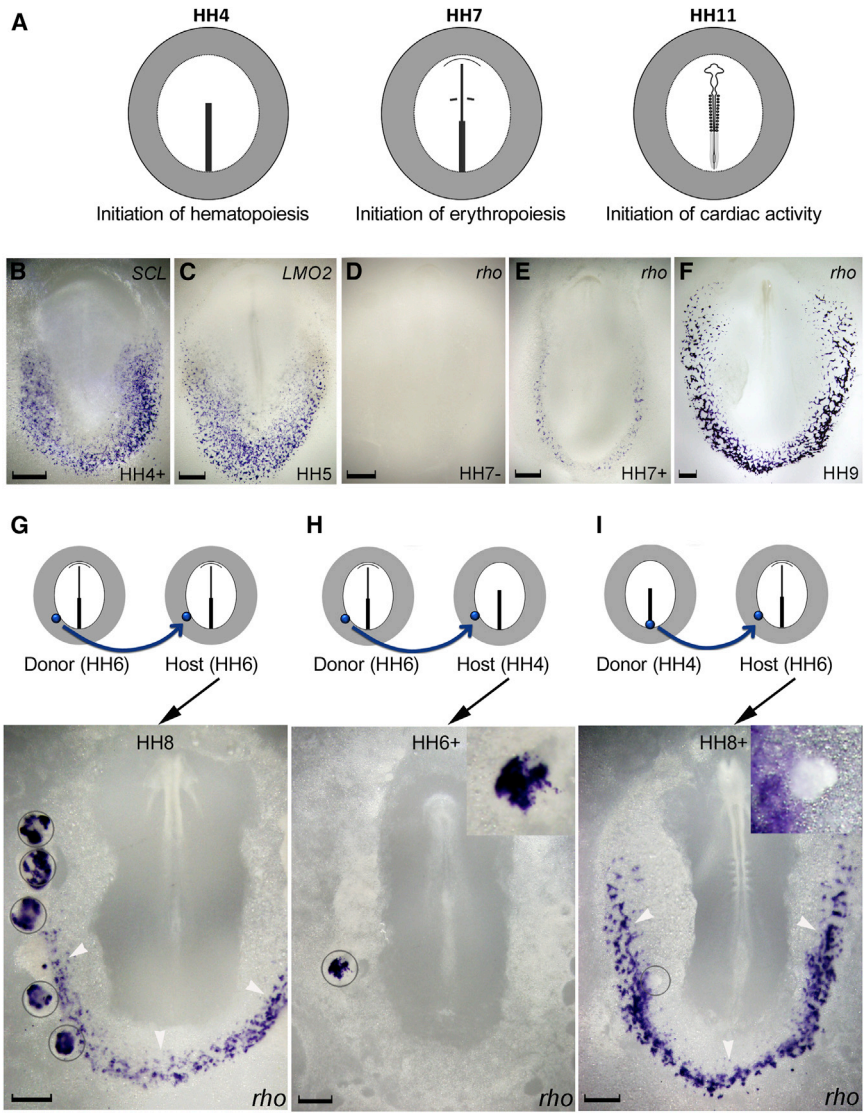

Figure 1. Developmental Delay from Hematopoiesis to Erythropoiesis and Hemangioblast-Intrinsic Program of Primitive Erythropoiesis during Early Chicken Development

(A) Schematic diagram of developmental landmarks. Early hematopoietic markers begin their expression at HH4. Erythroid lineage differentiation, marked by globin gene expression, starts at HH7. Cardiac activity is initiated at HH11 and circulation is established at HH12–13.

(B) *SCL* is expressed robustly at HH4+ in migrating hemangioblast cells.

(C) *LMO2* expression is strong at HH5 in migrating hemangioblasts.

(D) *rho* globin is not expressed at HH7−.

(E) *rho* globin is weakly expressed at HH7+.

(F) *rho* globin is strongly expressed at HH9. Expression analyses of *SCL*, *LMO2*, and *rho* (B–F) were performed using the whole-mount RNA in situ hybridization method.

(G) HH6 donor hemangioblasts were grafted isotopically to HH6 host embryo. The host was further cultured to HH8 and stained for *rho* mRNA expression by in situ hybridization. *rho* expression was detected robustly in both graft (circles) and host (white arrowheads) hemangioblasts.

(H) HH6 donor hemangioblasts were grafted isotopically to HH4 host embryo. The host was further cultured to HH6+ and stained for *rho*. *rho* expression was detected only in graft (circle and inset) tissues. The host hemangioblasts were still negative for *rho* at this stage.

(I) HH4 donor hemangioblast precursors in posterior primitive streak were grafted to HH6 host embryo. The host was further cultured to HH8− and stained for *rho*. *rho* was detected only in host hemangioblasts (white arrowheads). The graft tissue (circle and inset) remained negative because graft cells followed their own timing of erythropoiesis. Scale bars, 500 μm.

S2A–S2C available online). In contrast, when HH4 posterior streak cells (hemangioblast precursors) were grafted to HH6 host, cultured postgraft to HH8+ (Figure 1I), erythropoiesis in host embryos was marked by strong *rho* expression (white arrowheads), whereas graft cells remained *rho* negative (circle and inset) (zero out of nine grafts). Avian primitive erythropoiesis therefore follows a hemangioblast-intrinsic developmental program.

SCL and LMO2 Initiate Erythropoiesis in a Time-Dependent, but Not Stage-Dependent, Manner

To test whether this program could be initiated or accelerated by exogenously introduced SCL and LMO2, two key early hematopoietic regulators, we cloned these two genes into pCAGGS expression vector (Figure 2A, top). When the SCL+LMO2-expressing construct was electroporated into mesoderm precursors at HH4 and host embryos were cultured postelectroporation to HH6+ (Figure 2B), neither host hemangioblasts nor electroporated mesoderm cells (Figure 2B, brown) expressed *rho* (zero out of six embryos). When similarly electroporated embryos were incubated to HH8–10 (Figure 2C), exogenous SCL+LMO2 induced ectopic *rho* in lateral somite and medial lateral plate territories normally negative for globins (boxed area, section in inset; magnified view in Figures S1A and S1B) (80/91 embryos). The induction was not optimal because only a

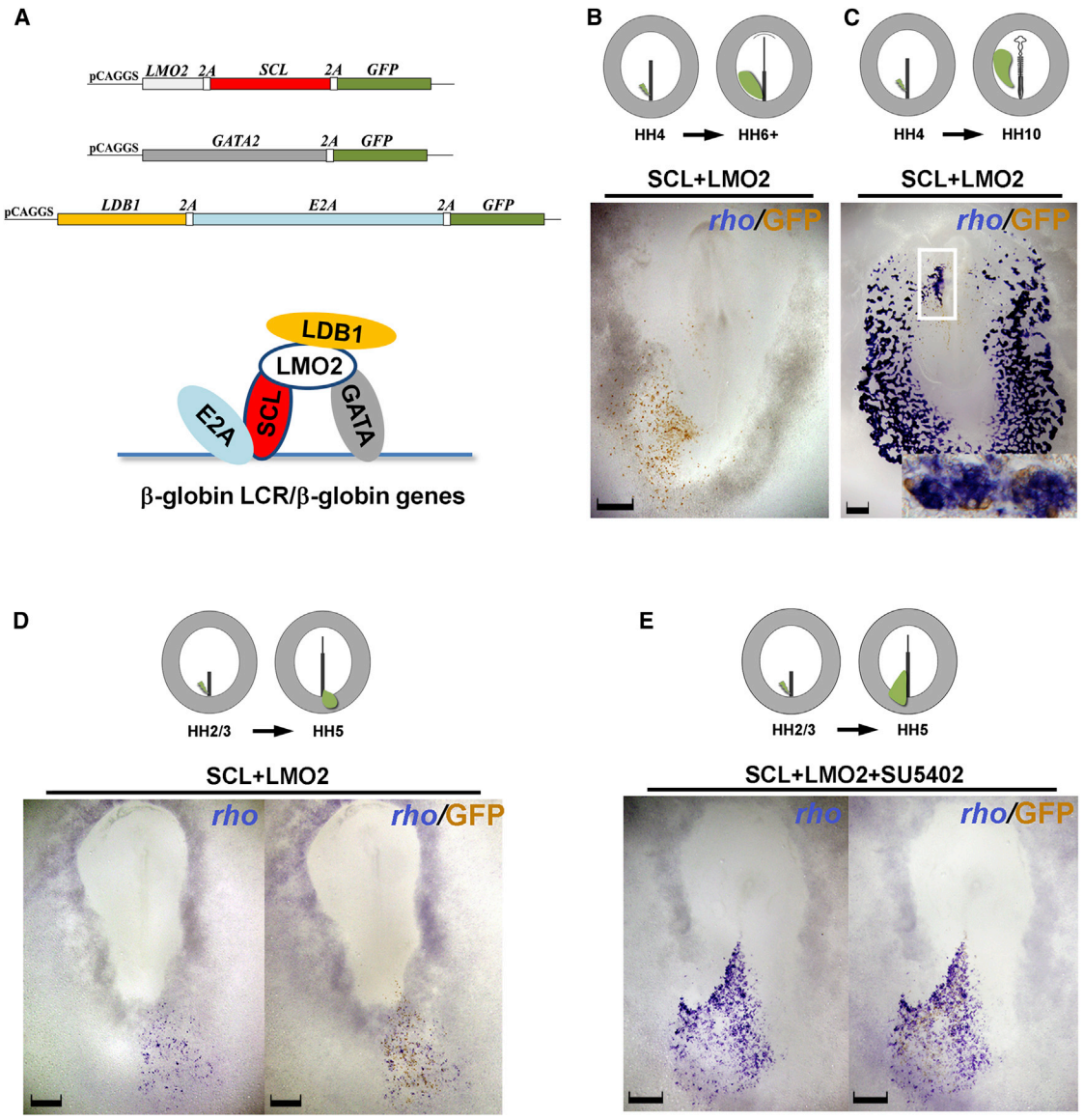

Figure 2. Induced Erythroid Lineage Differentiation in the Mesoderm by SCL+LMO2 and FGF Pathway Inhibition

(A) Schematic diagrams of expression constructs and of transcriptional regulatory complex for erythroid differentiation containing SCL, LMO2, LDB1, E2A, and GATA. Details are found in Experimental Procedures.

(B) SCL+LMO2 expression construct was electroporated into the posterior streak at HH4. Electroporated embryos were grown to HH6+ and stained for *rho* mRNA and GFP protein. Neither the endogenous hemangioblasts nor electroporated cells were positive for *rho*.

(C) SCL+LMO2 expression construct was electroporated into the posterior streak at HH4. Electroporated embryos were grown to HH10 and stained for *rho* mRNA and GFP protein. Endogenous erythropoiesis was robust (strong *rho* expression in extraembryonic mesoderm). SCL+LMO2 induced ectopic *rho* expression in cells located in lateral somites and medial lateral plate (white rectangle with section shown in inset; magnified views in Figures S1A and S1B).

(D) SCL+LMO2 expression construct was electroporated into the posterior streak at HH2/3, about 6 hr prior to endogenous *SCL* and *LMO2* expression. Electroporated embryos were grown to HH5 and stained for *rho* mRNA and GFP protein (bottom left: *rho* only; bottom right: *rho*+GFP). Electroporated cells initiated *rho* expression at HH5 because they had been under SCL+LMO2 control for approximately 12 hr.

(E) SCL+LMO2 expression construct was electroporated into the posterior primitive streak at HH2/3 and electroporated embryos were grown to the equivalent of HH5 in the presence of SU5402 and stained for *rho* mRNA and GFP protein expression (bottom left: *rho;* bottom right: *rho*+GFP). Combination of SCL+LMO2 and SU5402 induced strong *rho* expression in the mesoderm. The induction was widespread and not limited to the endogenous hemangioblast territory. Scale bars, 500 μm.

fraction of SCL+LMO2-expressing cells in each embryo were *rho* positive. When electroporation was performed at HH2/3 (about 6 hr before the endogenous *SCL* and *LMO2* expression) and embryos grown postelectroporation to HH5 (Figure 2D), SCL+LMO2-expressing cells were able to initiate *rho* expression, albeit still inefficiently, in a population of otherwise *rho*-negative extraembryonic mesoderm cells (bottom left: *rho*; bottom right: *rho*+GFP) (10/38 embryos). These results suggested that SCL and LMO2 are able to initiate, although not optimally, erythroid differentiation in a time-dependent, but not stage-dependent, manner.

FGF Inhibition Enhances Erythroid-Inducing Potentials of SCL and LMO2 in the Mesoderm

To achieve optimal induction, we analyzed the role of FGF pathway in early erythropoiesis. We have previously reported that FGF activity enhances endothelial and inhibits primitive blood differentiation (Nakazawa et al., 2006). Treatment of embryos from HH4+/5 to HH10 with SU5402, a potent FGF inhibitor, led to a medial expansion of globin-positive territory (Figures S1C and S1D, arrows) (control: zero out of five embryos; SU5402: six out of six embryos; Nakazawa et al., 2006) without hindering overall development (Figures S1C, S1C′, S1D, and S1D′). SU5402 treatment from HH2/3 caused growth arrest and failed to induce *rho* (Figures S1E and S1E′) (0/14 embryos). In combination with exogenous SCL+LMO2, however, SU5402 treatment from HH2/3 promoted robust ectopic *rho* expression in the mesoderm (Figure 2E) (18/22 embryos). These embryos were nevertheless still growth arrested, suggesting that failure to enhance erythropoiesis by SU5402-only treatment from HH2/3 is independent of its influence on growth. We therefore analyzed the effect of SU5402 on endogenous *SCL* and *LMO2* expression. *SCL* or *LMO2* intensity was not altered by SU5402 treatment after HH5, whereas both genes failed to be expressed when embryos were treated with SU5402 from HH3 (Figures 3A and 3B) (*LMO2* control treatment: five out of five embryos; *LMO2* SU5402 treatment: 0/12 embryos; *SCL* control treatment: eight out of eight embryos; *SCL* SU5402 treatment: zero out of nine embryos). These data suggested that FGF signaling is required for the initiation of *SCL* and *LMO2* and, when supplied with exogenous SCL and LMO2, FGF inhibition enhances their erythropoietic potentials. When HH3 embryos were grafted with control-electroporated HH3 posterior streak mesoderm (Figure 3C, arrow) or SCL+LMO2-electroporated HH3 posterior streak mesoderm (Figure 3C, arrowhead) and the host embryos were cultured postgraft in the presence of SU5402 to HH6 equivalent, SCL+LMO2-electroporated graft showed strong *rho* positivity (Figure 3C, arrowhead), whereas control electroporated graft remained *rho* negative (Figure 3C, arrow). Taken together, these data indicated that erythroid differentiation in the mesoderm can be efficiently induced by a combined FGF inhibition and exogenous SCL+LMO2.

Combination of Five Transcription Factors, SCL+LMO2+GATA2+LDB1+E2A, and FGF Inhibition Directly Induces Erythroid Differentiation in the Epiblast

SCL+LMO2 overexpression, however, was insufficient to differentiate epiblast-located cells (fated for neural and nonneural ectoderm tissues) into the erythroid lineage. We reasoned that additional hemangioblast regulators may be required to initiate globin gene expression in the ectoderm. We analyzed our transcriptomic data sets (Nakazawa et al., 2009) of extraembryonic tissues from stages HH4–9 for genes encoding transcription factors with expression profiles similar to those of *SCL* and *LMO2*. We uncovered two such genes, *LDB1* and *E2A*, as potential regulators of primitive erythropoiesis. Both have been reported to play a role in mammalian hematopoiesis (Li et al., 2013; Mylona et al., 2013; Semerad et al., 2009; Song et al., 2007; Wadman et al., 1997). In situ hybridization analyses confirmed that chicken *LDB1* and *E2A* are expressed in the extraembryonic mesoderm, with *LDB1* initiating its expression at HH5 and *E2A* at HH7− (Figures 3D and 3E, arrows). Unlike *SCL* or *LMO2*, however, both *LDB1* and *E2A* are also strongly expressed in the neural ectoderm (Figures 3D and 3E).

LDB1 and E2A have been proposed to form a functional complex with SCL, LMO2, and GATA proteins to regulate erythroid differentiation in vitro (Figure 2A) (Song et al., 2007; Wadman et al., 1997). Hemangioblast expression of GATA genes has been reported previously (Minko et al., 2003; Nakazawa et al., 2006; Sheng and Stern, 1999). To test whether this complex is sufficient to initiate primitive erythropoiesis in nonmesoderm cells, we generated expression constructs for *LDB1* and *E2A* and for *GATA2* (Figure 2A). Electroporation of SCL+LMO2+GATA2+LDB1+E2A at HH2/3, followed by growth to HH5/6, however, was still unable to induce *rho* in the epiblast (0/20 embryos). In the mesoderm, they induced *rho* only slightly better than with SCL+LMO2, suggesting that SCL+LMO2+GATA2+LDB1+E2A were not able to bypass the requirement for FGF inhibition at these stages. However, when SCL+LMO2+GATA2+LDB1+E2A electroporated embryos were treated with SU5402, robust induction of *rho* was observed in the epiblast (37/37 embryos) (Figure 3F). Moreover, *FECH* (*Ferrochelatase*), a gene essential for heme biosynthesis and upregulated during primitive erythropoiesis (Dailey and Meissner, 2013; Nakazawa et al., 2009), was also induced strongly in the epiblast after the same treatment (13/13 embryos) (Figures S2D and S2E). Induced cells were unable to mature into functional erythrocytes owing

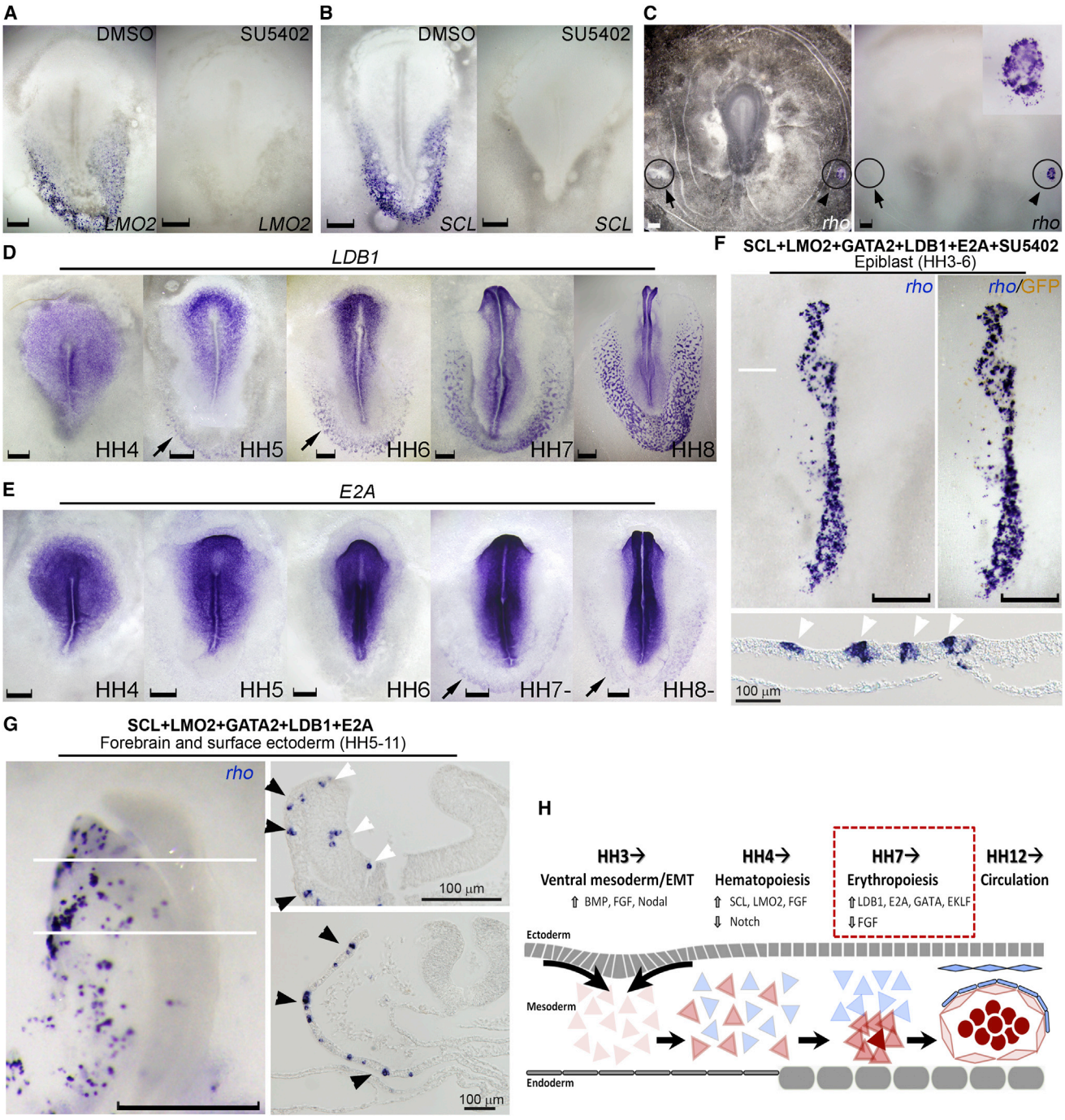

Figure 3. Induced Erythroid Lineage Differentiation in the Epiblast by SCL+LMO2+GATA2+LDB1+E2A and FGF Pathway Inhibition

(A) Embryos were cultured in the presence of control DMSO (left) or SU5402 (right) from HH3 to HH5 and stained for *LMO2*. SU5402 inhibited the initiation of endogenous *LMO2* expression.

(B) Embryos were cultured in the presence of control DMSO (left) or SU5402 (right) from HH3 to HH5 and stained for *SCL*. SU5402 inhibited the initiation of endogenous *SCL* expression.

(C) Left: dark-field view; right: bright-field view. HH3 embryos were electroporated with either control or SCL+LMO2-expressing constructs in the posterior streak. The electroporated posterior streak tissues (control: left circle/arrow; SCL+LMO2: right circle/arrowhead) were grafted to the extraembryonic territory of an HH3 host embryo. The host was further grown in the presence of SU5402 to the equivalent of HH6 and stained for *rho* expression. Both the host and control electroporated graft tissue were negative for *rho*. SCL+LMO2 electroporated graft tissue expressed *rho* robustly (arrowheads, inset).

(legend continued on next page)

to SU5402-induced growth arrest. However, these electroporated epiblast cells, when grafted to the *area vasculosa* of an HH7 host embryo and grown postgraft to HH12/13 (Figures S2F–S2P), were able to differentiate into cells with erythrocyte-like morphology and strong *rho* expression (Figures S2S–S2U), whereas control epiblast cells remained as a compact group *rho*-negative cells (Figures S2Q and S2R).

Although these five transcription factors constitute core components of the transcriptional complex regulating erythroid differentiation, it is unclear whether all five factors are required exogenously or whether some of them can be substituted by other known hematopoietic regulators. When electroporated individually, none of these five transcription factors could induce *rho* in the epiblast (Table 1). Neither could GATA1 or EKLF, two hematopoietic regulators not included in our initial analysis (Table 1). As the earliest-expressing hematopoietic-specific transcription factors, SCL and LMO2 together induced *rho* in the epiblast only very weakly (Table 1). Progressive addition of more transcription factors, as a combination of three to seven, all of which contained SCL and LMO2, led to a gradual increase in their inducing ability (Table 1). The SCL+LMO2+GATA2+LDB1+E2A combination was among the ones with the highest inducing abilities. These data suggested that efficient erythroid induction of epiblast cells requires the FGF pathway to be inhibited and hematopoietic regulators to function cumulatively and cooperatively. Interestingly, the requirement of FGF pathway inhibition appeared to be needed only because of the high endogenous FGF activities in the epiblast (Lunn et al., 2007). When SCL+LMO2+LDB1+E2A+GATA2 electroporated embryos were grown to later stages (HH10/11) without SU5402 treatment, ectoderm cells in the forebrain and surface epidermis which have low endogenous FGF activities (Bailey et al., 2006; Chambers and Mason, 2000) were able to express *rho* strongly (Figure 3G) (43/43 embryos). The chicken epiblast loses its pluripotency at HH5 (Shin et al., 2011), when neural markers become strongly expressed (Uchikawa et al., 2003). The late erythroid induction in the ectoderm therefore represents a process of transdifferentiation, the molecular mechanism of which awaits further elucidation.

In summary, primitive erythropoiesis follows a stereotypic developmental progression in vivo (Figure 3H). We showed in this work that the conversion of nascent mesoderm to the erythroid lineage requires FGF inhibition together with two early hematopoietic transcription regulators, SCL and LMO2. In the epiblast, direct erythroid differentiation of pluripotent cells, without them undergoing mesoderm induction, epithelial-mesenchymal transition, or migration, requires concerted action of five hematopoietic regulators (SCL, LMO2, GATA2, LDB1, and E2A) and FGF inhibition. In cells with low endogenous FGF signaling activities, these five factors alone are sufficient for erythroid transdifferentiation. Whether induced erythroid cells can mature in vivo into functional erythrocytes and whether the combination of five factors and FGF inhibition has the same potent erythropoiesis-inducing ability in vitro await future investigation.

EXPERIMENTAL PROCEDURES

Hen's eggs were purchased from Shiroyama Farm and incubated at 38.5°C to desired stages. SU5402 (#572630, Calbiochem) was dissolved in albumen at 85 μM. Standard protocols were followed for new culture, grafting, SU5402 treatment, RNA in situ, electroporation, tissue section, and imaging analyses (Alev et al., 2013; Nakazawa et al., 2006). DNA fragments for in situ hybridization probes were generated by PCR, cloned into pGEM-T vector, and confirmed by sequencing (*LDB1*: nucleotides 436–890 of NM_205070.1; *E2A*: nucleotides 104–932 of NM_204486.2; *SCL*: nucleotides 717–1,750 of NM_205352.1; *LMO2*: nucleotides 46–522 of AF468789.1; *rho*: nucleotides 31–465 of CHEST324a16)

(D) Expression pattern of chicken *LDB1* from HH4–8. Initiation of *LDB1* in hemangioblasts starts at HH5 (arrows). Expression in neural ectoderm is also prominent.
(E) Expression pattern of chicken *E2A* from HH4–8. Initiation of *E2A* in hemangioblasts starts at HH7− (arrows). Expression in neural ectoderm is also prominent.
(F) SU5402 treatment in combination with ectopic expression of SCL+LMO2+GATA2+LDB1+E2A resulted in robust *rho* induction in the epiblast (arrowheads in lower panel). Top: whole mount (left: *rho*; right: *rho*+GFP); bottom: section. Level of section indicated in the top left panel.
(G) Electroporation of SCL+LMO2+GATA2+LDB1+E2A at later stages, without SU5402 treatment, led to robust *rho* expression in neural (white arrowheads) and nonneural (black arrowheads) ectoderm tissues where endogenous FGF activities are low. Left: whole mount; right: sections.
(H) A model for normal developmental progression from hematopoiesis to erythropoiesis. Hemangioblast precursor cells are located in the posterior primitive streak. They receive ventralizing (BMPs) and EMT (FGFs and Nodal) signals and are internalized to become migratory mesenchymal-shaped mesoderm cells. Early hemangioblast regulators (SCL and LMO2) start to be expressed soon after internalization. This process requires FGF activity. Commitment of hematopoietic cells to the erythroid lineage needs additional cofactors (LDB1, E2A, GATA, EKLF, and possibly other factors regulated by core factors) and inhibition of the FGF pathway activity.
Scale bars, 500 μm except where indicated.

Table 1. Efficiency of Erythroid Induction in the Epiblast by Hematopoietic Transcriptional Regulators Either Individually or in Combination

Electroporation Combination[a]	No Induction (−)[b]	Very Weak Induction (+/−)[c]	Moderate Induction (+)[d]	Strong Induction (++)[e]
SCL	10/10 (100%)			
LMO2	10/10 (100%)			
GATA2	10/10 (100%)			
LDB1	10/10 (100%)			
E2A	13/13 (100%)			
GATA1	9/9 (100%)			
EKLF	8/8 (100%)			
GATA1+EKLF	9/9 (100%)			
SCL+LMO2	3/16 (19%)	13/16 (81%)		
SCL+LMO2+GATA2		4/4 (100%)		
SCL+LMO2+LDB1		12/14 (86%)	2/14 (14%)	
SCL+LMO2+E2A		4/9 (44%)	2/9 (22%)	3/9 (33%)
SCL+LMO2+GATA1			7/10 (70%)	3/10 (30%)
SCL+LMO2+EKLF			5/10 (50%)	5/10 (50%)
SCL+LMO2+E2A+LDB1		4/15 (27%)	8/15 (53%)	3/15 (20%)
SCL+LMO2+GATA2+E2A			4/10 (40%)	6/10 (60%)
SCL+LMO2+GATA2+LDB1			3/9 (33%)	6/9 (67%)
SCL+LMO2+GATA1+EKLF		1/9 (11%)	5/9 (56%)	3/9 (33%)
SCL+LMO2+GATA2+E2A+LDB1			9/37 (24%)	28/37 (76%)
SCL+LMO2+GATA1+EKLF+E2A+LDB1		1/9 (11%)	3/9 (33%)	5/9 (56%)
SCL+LMO2+GATA1+GATA2+EKLF+E2A+LDB1			2/10 (20%)	8/10 (80%)

[a]Electroporated embryos are treated with SU5402 in all cases. HH2/3 embryos were electroporated in the epiblast with DNA constructs expressing these regulators. Embryos were cultured after electroporation in the presence of SU5402 to the equivalent of HH6 and stained for *rho* mRNA and GFP protein expression.
[b](−) is defined as having no globin-positive signals at all in the epiblast.
[c](+/−) is defined as having weak globin-positive signals in less than 10% of electroporated, GFP-positive epiblast cells.
[d](+) is defined as having moderate globin-positive signals in 10%–50% of electroporated, GFP-positive epiblast cells.
[e](++) is defined as having strong globin-positive signals in more than 50% of electroporated, GFP-positive epiblast cells.

(Nakazawa et al., 2006; Shin et al., 2009). For electroporation, expression vector pCAGGS-2AGFP was constructed by inserting a 2A sequence 5′-TGGGCCAGGATTCTCCTCCACATCACCGCAT GTTAGCAGACTTCCTCTGCCCTCTCCACTGCC-3′ into the 5′ end of GFP. An additional 2A sequence was inserted between chicken *LMO2* (nucleotides 46–519 of AF468789.1) and murine *SCL* (nucleotides 142–1,128 of NM_011527). LMO2-2A-SCL fragment was cloned into 5′ end of 2AGFP for pCAGGS-LMO2-2A-SCL-2AGFP. LDB1-2A-E2A was generated by introducing another 2A sequence 5′-CAATGCACAAACTACGCACTACTAAAACTAGCA GGAGACGTAGAGAGTAATCCAGGACCC-3′ between chicken *LDB1* (nucleotides 61–1,293 of NM_205070.1) and chicken *E2A* (nucleotides 98–2,110 of NM_204486.2), which was then inserted into the 5′ end of 2AGFP of pCAGGS-2AGFP for pCAGGS-LDB1-2A-E2A-2AGFP. Chicken *GATA2* (nucleotides 410–1,807 of NM_001003797.1) was used to construct pCAGGS-GATA2-2AGFP. Chicken *GATA1* (nucleotides 41–952 of NM_205464.1) was used to construct pCAGGS-GATA1-2AGFP. Chicken *EKLF* (nucleotides 133–1,350 of XM_422416.4) was used to construct pCAGGS-EKLF-2AGFP. Chicken *EKLF* is regarded phylogenetically as the ortholog of mammalian *KLF17* (Antin et al., 2010), but it is the only erythroid-specific Krüppel-like factor reported in the chick so far (Antin et al., 2010; Chervenak et al., 2006) and is used in this study as the functional ortholog of mammalian

EKLF (*KLF1*). All experiments with chicken embryos were approved by the Animal Care and Use Committee of RIKEN Center for Developmental Biology.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.01.019.

AUTHOR CONTRIBUTIONS

W.W. and G.S. planned the experiments, W.W. performed the experiments, W.W. and G.S. analyzed the data, and G.S. and W.W. wrote the paper.

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