Scl binds to primed enhancers in mesoderm to regulate hematopoietic and cardiac fate divergence

Tõnis Org1,†, Dan Duan1,†, Roberto Ferrari2,3, Amelie Montel-Hagen1, Ben Van Handel1, Marc A Kerényi4, Rajkumar Sasidharan1, Liudmilla Rubbi1, Yuko Fujiwara4, Matteo Pellegrini1, Stuart H Orkin4, Siavash K Kurdistani2,3 & Hanna KA Mikkola1,3,*

Abstract

Scl/Tal1 confers hemogenic competence and prevents ectopic cardiomyogenesis in embryonic endothelium by unknown mechanisms. We discovered that Scl binds to hematopoietic and cardiac enhancers that become epigenetically primed in multipotent cardiovascular mesoderm, to regulate the divergence of hematopoietic and cardiac lineages. Scl does not act as a pioneer factor but rather exploits a pre-established epigenetic landscape. As the blood lineage emerges, Scl binding and active epigenetic modifications are sustained in hematopoietic enhancers, whereas cardiac enhancers are decommitted by removal of active epigenetic marks. Our data suggest that, rather than recruiting corepressors to enhancers, Scl prevents ectopic cardiogenesis by occupying enhancers that cardiac factors, such as Gata4 and Hand1, use for gene activation. Although hematopoietic Gata factors bind with Scl to both activated and repressed genes, they are dispensable for cardiac repression, but necessary for activating genes that enable hematopoietic stem/progenitor cell development. These results suggest that a unique subset of enhancers in lineage-specific genes that are accessible for regulators of opposing fates during the time of the fate decision provide a platform where the divergence of mutually exclusive fates is orchestrated.

Keywords cardiac specification; enhancer; hematopoiesis; mesoderm diversification; transcriptional regulation

Introduction

Specification of cell types is dictated by few master regulators that activate lineage-specific transcriptional networks. This concept is underscored by studies in which overexpression of a small set of transcription factors can reprogram fibroblasts (or other cells) into a variety of cell types that closely resemble pluripotent cells, neurons, pancreatic beta cells, cardiomyocytes or hematopoietic cells (Takahashi & Yamanaka, 2006; Ieda et al, 2010; Huang et al, 2011; Kim et al, 2011; Pereira et al, 2013). Hematopoietic and cardiovascular systems are important targets for cell-based therapies due to the high morbidity and mortality associated with blood and heart diseases; however, so far, in vitro generation of transplantable cells for treating these diseases has not been successful. Blood cells, vasculature and the heart share not only an intimate functional relationship, but also a common origin in Flk1+ mesoderm (Fehling et al, 2003; Huber et al, 2004; Iida et al, 2005; Kattman et al, 2006). Although many regulators of the blood and circulatory system have been discovered, it is unclear how the divergence of these lineages in multipotent cardiovascular mesoderm is orchestrated and how their cell identity is solidified. Although several specific epigenetic modifications have been associated with activation or repression of the regulatory regions of genes (Cui et al, 2009; Creyghton et al, 2010; Zentner et al, 2011), to what degree they cause, or are the consequence of, gene activation/repression by cell type-specific transcription factors is still unclear.

The embryonic hematopoietic system is established in multiple waves, starting with the generation of lineage-restricted progenitors in the yolk sac and culminating in the emergence of multipotent hematopoietic stem/progenitor cells (HS/PC) in the major vessels in the yolk sac (Li et al, 2005; Yoshimoto et al, 2011), aorta-gonad mesonephros (AGM) region (North et al, 1999; De Bruijn et al, 2000; Zovein et al, 2008; Chen et al, 2009; Bertrand et al, 2010; Boisset et al, 2010; Kiss & Herbomel, 2010) and the placenta (Rhodes et al, 2008). The Ets factor Etv2/ER71/Etsrp first
Scl represses cardiac fate via primed enhancers

Tõnis Org et al

Scl represses cardiac fate via primed enhancers. To understand how Scl specifies cardiac repression while simultaneously repressing the cardiac fate, we defined Scl target genes by combining gene expression and chromatin immunoprecipitation sequencing (ChIP-seq) analysis on Flk1+ mesodermal cells differentiated from mouse ES cells (Supplementary Fig S1A). Scl binding associated genes (4,393 binding sites associated with 4,158 genes within 200 kb from transcriptional start sites) (Supplementary Table S1A) were intersected with Scl-dependent genes (592 and 553 genes that were significantly up- or down-regulated in mesoderm upon Scl expression (P-value < 0.05, fold change < 1.5, see details in Supplementary Fig S1A-C, Supplementary Table S1B and C, and Supplementary Materials and Methods). Integrated analysis showed Scl binding to 57% of the activated and 29% of the repressed genes in mesoderm (Fig 1A, Supplementary Table S1B and C), which is significantly higher than would be expected by random chance (P-values 4.08 \times 10^{-103} and 3.91 \times 10^{-10}, respectively). The genes in activated/bound group were enriched for GO term hematopoiesis, including key hematopoietic transcription factors Runx1, Gata2, cMyb and Cfbat21/El02 (Fig 1B–D). The genes in repressed/bound group were enriched for GO term heart development, including cardiac transcription.
Gata4, Gata6 and Tbx3 (Fig 1B–D). Quantitative RT–PCR and ChIP–PCR verified Scl-dependent expression and Scl binding with both activated and repressed genes (Fig 1E and F).

As the expression analysis in day 4 EB Flk1+ cells was limited to genes that were immediately activated or repressed upon Scl induction in mesoderm, we extended the analysis of Scl-dependent genes to those that became differentially expressed in endothelium in the yolk sac or the placenta, or endocardium in the heart in Scl+/− embryos (Van Handel et al., 2012). This analysis yielded an extended list of 3,709 Scl-dependent activated and 2,839 Scl-dependent repressed genes, of which 1,100 (29.6%) and 715 (25.2%) (Supplementary Fig S1A, Supplementary Table S1D).

Figure 1.
Scl represses cardiac fate via primed enhancers

Tõnis Org et al

Published online: January 6, 2015

The EMBO Journal

Scl represses cardiac fate via primed enhancers

To verify that the ES cell in vitro differentiation system recapitulates ectopic cardiogenesis in Scl-deficient endothelial precursors, SclKO and wild-type ES cells were differentiated toward mesodermal lineages and assayed for differentiation potential and gene expression. SclKO ES cells with doxycycline-inducible Scl overexpression (SclKOiScl) were included to assess whether reintroduction of Scl is sufficient to reverse the phenotype. As expected, both wild-type and SclKOiScl EBs, but not SclKO EBs, could generate CD41+CD31+ hematopoietic progenitors by day 11 (Supplementary Fig S1D). Likewise, Tie2+CD31+ endothelial precursors isolated from day 4.75 EBs from wild-type and SclKOiScl cells, but not SclKO cells, robustly generated CD45+CD11b+/+ hematopoietic cells on OP9 stroma (Supplementary Fig S1E). SclKO Tie2+CD31+ endothelial precursors readily differentiated to troponin T-expressing cardiomyocytes, whereas re-expression of Scl abolished the ectopic cardiogenic potential in ES cell-derived endothelial cells (Supplementary Fig S1E). These data were reinforced by qRT–PCR analysis that verified the lack of expression of hematopoietic transcription factors and ectopic induction of cardiac factors in Scl-deficient endothelium, and rescue of these molecular defects by Scl overexpression (Supplementary Fig S1F). These data validate the ES cell in vitro differentiation system as a suitable model to study Scl-dependent cardiac repression and ectopic cardiogenesis from endothelial precursors.

Scl regulates mesodermal fate diversification via pre-established enhancers

Analysis of genomic locations of Scl binding showed that the majority of Scl binding sites in Flk1+ mesoderm reside away from transcriptional start site (TSS) (Fig 2A), suggesting that Scl functions through enhancer elements. This was most pronounced in the repressed genes, where only 3% of Scl binding sites were found within 5 kb of TSS. We thus correlated Scl mesodermal binding sites with published datasets for cardiac enhancers, including the Vista Enhancer database that contains in vivo experimentally verified enhancers (Visel et al, 2007) and ChIP-seq analysis for co-activator p300, a marker of enhancers (Visel et al, 2009), in E11.5 mouse hearts (Blow et al, 2010). Intersecting these data with Scl binding sites in Flk1+ mesoderm revealed Scl binding in 10–16% of all putative cardiac enhancers, which is significantly (P < 8.6E–5) higher than the overlap with enhancers in other tissues (Fig 2B and C).

Analysis of well-established enhancers of cardiac transcription factors Myocardin and Nkx2.5 (Fig 2D) (Lien et al, 1999; Creemers et al, 2006) and hematopoietic regulators Runx1 (+3 enhancer) (Nottingham et al, 2007) and Gata2 (+9.5 enhancer) (Wozniak et al, 2007; Gao et al, 2013) (Supplementary Fig S2A) showed clear overlap with Scl binding.

To assess the epigenetic state of Scl-bound enhancers, 4,393 Scl mesodermal binding sites were assessed for the average levels of histone modifications associated with enhancers (H3K4me1) and active enhancers (H3K4me1 and H3K27ac) (Creighton et al, 2010; Wamstad et al, 2012) during ES cell differentiation to cardiomyocytes. In ES cells, Scl mesodermal binding sites were largely devoid of H3K4me1 and H3K27ac; however, in Flk1+ mesoderm they had acquired high levels of H3K4me1 and some H3K27ac, which were in part retained during differentiation into cardiac progenitors and cardiomyocytes (Fig 2E and F). Analysis of the average H3K4me1 and H3K27ac levels around Scl binding sites in other mesodermal tissues (limb, fibroblasts) showed no enrichment (Fig 2F). This indicates that the regulatory regions to which Scl binds become epigenetically primed for activation specifically in Flk1+ mesoderm.

We next asked whether Scl is required for depositing active histone marks at hematopoietic and/or cardiac enhancers or whether these enhancers have been epigenetically pre-established prior to Scl binding. ChIP-seq analysis for histone H3K4me1 and
Tõnis Org et al. Scl represses cardiac fate via primed enhancers

The EMBO Journal

Published online: January 6, 2015

Figure 2.
H3K27ac in WT and Scl\textsuperscript{EKO} Flk1\textsuperscript{+} mesoderm evidenced co-localization of the active marks across all Scl binding sites (Fig 2G) including cardiac and hematopoietic genes (Fig 2H) in both cell lines. Although H3K27ac levels were generally lower and this mark was not present in all hematopoietic or cardiac enhancers, there was no significant difference in H3K27ac levels at Scl mesodermal binding sites in WT and Scl\textsuperscript{EKO} Flk1\textsuperscript{+} mesoderm (Fig 2G and H). These results show that Scl is not required for the establishment of active enhancer marks in mesoderm, suggesting that these enhancers have become primed for activation in multipotent cardiovascular mesoderm to pave the way for Scl action.

To investigate whether the epigenetically primed status of enhancers is necessary for Scl binding, we induced Scl expression ectopically in Scl\textsuperscript{EKO}Scl ES cells and compared Scl binding in these cells to that in Flk1\textsuperscript{+} mesoderm in wild-type and Scl\textsuperscript{KO}Scl cells. While inducible Scl was faithfully bound to the hematopoietic and cardiac enhancers in mesoderm, minimal binding was observed in ES cells (Supplementary Fig S2). Notably, the few binding sites where ectopically expressed Scl was able to bind in ES cells also harbored H3K4me1, whereas the key hematopoietic and cardiac enhancers were devoid of both Scl binding and H3K4me1 (Supplementary Fig S2). These data imply that Scl binding is tightly developmentally controlled, and at least in part determined by epigenetic status of the enhancers.

Repression of cardiac enhancers by Scl occurs transiently during mesoderm diversification

Since Scl is required only during a brief developmental window in midgestation to repress cardiogenesis in hemogenic tissues (Van Handel et al., 2012), we assessed whether Scl binding to cardiac enhancers is maintained in hematopoietic cells later in development. Intersecting Scl binding sites in Flk1\textsuperscript{+} mesoderm with previously published Scl binding sites in ES cell-derived hematopoietic progenitor cell line (HPC7) (Wilson et al., 2010) or fetal liver erythroblasts (Kassouf et al., 2010) showed that many Scl binding sites are developmental stage specific (Fig 3A). GO enrichment analysis revealed enrichment of heart-related terms only with Flk1\textsuperscript{+} mesoderm in wild-type and Scl\textsuperscript{KO}Scl cells. While inducible Scl was faithfully bound to the hematopoietic and cardiac enhancers in mesoderm, minimal binding was observed in ES cells (Supplementary Fig S2). Notably, the few binding sites where ectopically expressed Scl was able to bind in ES cells also harbored H3K4me1, whereas the key hematopoietic and cardiac enhancers were devoid of both Scl binding and H3K4me1 (Supplementary Fig S2). These data imply that Scl binding is tightly developmentally controlled, and at least in part determined by epigenetic status of the enhancers.

ChIP-seq on MEL cells (mouse erythroleukemia line) and verified the absence of Scl binding in cardiac genes (Fig 3C, Supplementary Fig S3B). In comparison, Scl binding sites shared between different stages were enriched for hematopoiesis-related terms, and included major hematopoietic transcription factors (e.g. Gata1, Gata2, Lyl1, Gfi1b, Runx1 and Myb) (Fig 3C, Supplementary Fig S3A and B). While Flk1\textsuperscript{+} mesoderm-specific peaks were rarely found within 5 kb of TSS (Fig 3D), 46% of the HPC7 or 34% of the erythroid cell-specific peaks were located within 5 kb to TSS. These data suggest that Scl binding extends from distant enhancers to promoters as hematopoiesis progresses.

Since Scl was no longer bound to cardiac enhancers in hematopoietic cells, we assessed whether the epigenetic landscape in Scl binding sites was modified upon differentiation of mesoderm to hematopoietic cells. Combinatorial clustering of Scl mesodermal binding sites based on H3K4me1 patterns in mesoderm, hematopoietic progenitors (HPC7) and erythroid cells (MEL) revealed two major clusters. Although the majority of Scl binding sites harbored H3K4me1 in mesoderm, this enhancer mark was lost in Scl binding sites in cluster L (= Loss of H3K4me1) but retained in cluster R (= Retention of H3K4me1) as hematopoietic development progressed. The gradual loss of H3K4me1 enrichment from HPC7 to MEL in cluster L strongly correlated with the loss of H3K27ac and Scl binding, while Scl binding sites in cluster R retained H3K4me1, H3K27ac and Scl binding (Fig 3E). The peaks in cluster L were enriched for cardiac GO terms and included key cardiac regulators Gata4, Gata6 and Myocardin (Fig 3E, Supplementary Fig S3B), while Scl peaks in cluster R were enriched for hematopoietic-related GO terms and included hematopoietic regulators Gata1, Gata2, Lyl1 and Runx1 (Fig 3E, Supplementary Fig S3B). The opposite was observed in the cardiomyocyte cell line HL1, in which the enhancers of key hematopoietic regulators lost H3K4me1 and H3K27ac, while the enhancers of regulators of cardiomyocyte differentiation maintained these active marks (Supplementary Fig S3B). These results imply that the epigenetic landscape becomes dynamically remodeled in unused enhancers upon mesodermal fate diversification to blood and heart.

Decommissioning of Scl-regulated cardiac enhancers is associated with loss of active epigenetic marks rather than gain of repressive marks

We next investigated whether the active epigenetic marks in Scl-regulated cardiac enhancers are replaced by repressive marks in
Comparison of Scl binding sites between different developmental stages

GO enrichment analysis of Scl developmental stage-specific binding sites

Scl binding sites in different hematopoietic developmental stages

Clustering of Scl MES binding sites based on H3K4me1 enrichment in different hematopoietic developmental stages

Figure 3.
**Figure 4.**

The EMBO Journal
Vol 34 | No 6 | 2015

Tõnis Org et al

Scl represses cardiac fate via primed enhancers

Histone modifications in FL erythroblasts

- H3K4me1
- H3K27ac
- H3K27me3
- H3K9me3

**Average histone modification profiles at TSS**

- Ery H3K27me3
- Ery H3K27ac

**Average Ezh2 profiles at Scl MES binding sites**

- WT MES Ezh2
- SclKO MES Ezh2

**Average Ezh2 profiles at TSS of Scl target genes**

- WT MES Ezh2
- SclKO MES Ezh2

**Lsd1 enrichment**

- ESC
- WT MES
- SclKO MES
- Gr

**H3K27me3 profiles around Scl target genes**

- chr18: 11,070,000 - 11,090,000
- chr5: 120,250,000 - 120,300,000
- chr14: 63,950,000 - 63,950,000

**Ezh2 profiles around Scl target genes**

- chr18: 11,070,000 - 11,090,000
- chr5: 120,250,000 - 120,300,000
- chr14: 63,950,000 - 63,950,000

**Lsd1 profiles around Scl target genes**

- chr18: 11,070,000 - 11,090,000
- chr11: 65,070,000 - 65,130,000

Published online: January 6, 2015
hematopoietic cells to solidify the fate choice. Analysis of Ter119 erythroid cells for several histone marks (ENCODE project and Kowalczyk et al., 2012) in Scl mesodermal binding sites confirmed a strong correlation between Scl binding and retention of the active histone marks H3K4me1 and H3K27ac in Cluster R, and loss of these marks in Cluster L (Fig 4A), also in erythroid cells in vivo. A candidate for a repression mechanism was Polycomb-mediated deposition of H3K27me3, which has been associated with silencing of both promoters and enhancers (Harmston & Lenhard, 2013). Moreover, Scl has been shown to interact with Ezh2, a core component of the Polycomb repressive complex 2 (PRC2) (Pinello et al., 2014). Analysis of H3K27me3 ChIP-seq data provided no evidence of H3K27me3 acquisition at Scl binding sites in erythroid cells in either Cluster R or L (Fig 4A). ChIP-seq tracks around cardiac genes Gata4, Gata6 and Tbx5 confirmed the lack of H3K27me3 at Scl binding sites in ES cells, mesoderm and erythroid cells (Fig 4B). However, H3K27me3 was observed at the TSS of some Scl-regulated genes (Fig 4B). Comparison of H3K27me3 levels in Scl-bound extended activated and extended repressed genes (see Fig 1, Supplementary Table S1D and E) in erythroid cells revealed an increase in H3K27me3 at the TSS of repressed genes, while H3K27ac was more enriched at the TSS of activated genes (Fig 4C). These data suggested that Polycomb may contribute to the silencing of Scl-regulated genes at promoters, and raised the question whether Scl binding at enhancers is required for the gain of common repressive histone marks in cluster L.

Moreover, Scl has been shown to interact with Ezh2, a core component of the Polycomb repressive complex 2 (PRC2) (Pinello et al., 2014). Analysis of H3K27me3 ChIP-seq data provided no evidence of H3K27me3 acquisition at Scl binding sites in erythroid cells in either Cluster R or L (Fig 4A). ChIP-seq tracks around cardiac genes Gata4, Gata6 and Tbx5 confirmed the lack of H3K27me3 at Scl binding sites in ES cells, mesoderm and erythroid cells (Fig 4B). However, H3K27me3 was observed at the TSS of some Scl-regulated genes (Fig 4B). Comparison of H3K27me3 levels in Scl-bound extended activated and extended repressed genes (see Fig 1, Supplementary Table S1D and E) in erythroid cells revealed an increase in H3K27me3 at the TSS of repressed genes, while H3K27ac was more enriched at the TSS of activated genes (Fig 4C). These data suggested that Polycomb may contribute to the silencing of Scl-regulated genes at promoters, and raised the question whether Scl binding at enhancers is required for the gain of common repressive histone marks in cluster L.

We next assessed whether the repressive histone modification H3K9me3 is acquired at the decommissioned enhancers during hematopoietic differentiation. The average levels of H3K9me3 were slightly higher in cluster L binding sites than in cluster R binding sites in three hematopoietic cell types (Ter119 erythroid cells, Fig 4A, Supplementary Fig S4B, and G1E embryonic erythroid cell line and Ch12 B lymphoid cells, Supplementary Fig S4A and B). However, analysis of H3K9me3 around key cardiac genes did not show specific enrichment for H3K9me3 at Scl-bound enhancers (Supplementary Fig S4C). These data imply that although regions around some Scl mesodermal binding sites acquire H3K9me3 during hematopoietic differentiation, deposition of H3K9me3 is unlikely the predominant mechanism that initiates Scl-mediated cardiac repression.

As DNA methylation can restrict transcription factor binding to its target DNA (Blattler & Farnham, 2013), we asked whether changes in DNA methylation mediate the inactivation of Scl-regulated cardiac enhancers in hematopoietic cells. Comparison of the average DNA methylation levels at Scl mesodermal binding sites associated with extended list of Scl activated or repressed genes in genome-wide methylation datasets from mouse ES cells (Habibi et al., 2013), bone marrow, heart, pancreas and skin (Hon et al., 2013), showed that Scl binding sites are on average hypomethylated compared to the surrounding regions (Supplementary Fig S4D). Nevertheless, both Scl’s extended activated and repressed target genes showed a general increase in DNA methylation within 200 kb of Scl binding sites from ES cells to all adult cell types analyzed (Supplementary Fig S4E). The methylation levels in Scl extended activated genes were lower in the bone marrow compared to other tissues, while the extended repressed genes showed lower methylation in the heart (Supplementary Fig S4E).

To investigate whether Scl initiates differential methylation in mesoderm, we performed RRBS (reduced representation bisulfite sequencing) in ES cells, WT and SclKO Flk1+ mesodermal cells, and MEL cells. Global covariance analysis showed that major changes in DNA methylation occur later in hematopoietic development rather than in mesoderm (Supplementary Fig S4F). Intersection of RRBS data with Scl mesodermal binding sites showed no difference in methylation levels in ES cells and WT and SclKO Flk1+ mesoderm; only MEL cells had higher DNAmethylation (Supplementary Fig S4G). Thus, while DNA methylation may contribute to the silencing of Scl target genes in differentiated cells, this occurs later in development and is not centered at Scl-bound enhancers. These data imply that DNA methylation is not a primary mechanism that silences Scl-regulated cardiac enhancers during hematopoietic specification.

Our analysis of known repressive histone marks did not provide evidence that Scl-regulated enhancers would be actively silenced during mesodermal lineage diversification, but rather implied that gain and loss of active epigenetic marks is the key determinant

© 2015 The Authors

The EMBO Journal Vol 34 | No 6 | 2015 767
that governs tissue-specific access to these enhancers. We thus assessed whether the recruitment of Lsd1, a lysine-specific demethylase 1 that decompensations enhancers by removing H3K4me1 (Whyte et al., 2012), to cardiac enhancers is interrupted in the absence of Scl. Comparison of ChIP-seq data of Lsd1 mesodermal binding to published Lsd1 binding datasets in ES cells and myeloid cells (Whyte et al., 2012; Kerenyi et al., 2013) showed robust colocalization of Lsd1 at Scl-bound enhancers (Fig 4G and H) in both cluster L and R in Flk1+ mesoderm, which is a heterogeneous population of cells committed to hematopoietic or cardiac fates. In myeloid cells, cardiac enhancers were devoid of both Lsd1 and H3K4me1 (Fig 4H), documenting a short temporal window for Lsd1 action. Nevertheless, analysis of Lsd1 binding in Scl-deficient mesodermal cells did not show evidence of impaired Lsd1 binding in cardiac enhancers in Cluster L (Fig 4G and H). These results imply that, although Lsd1 is likely involved in the decompensation of unused enhancers upon mesodermal lineage diversification, defective cardiac repression in Scl-deficient mesodermal derivatives is not caused by an inability to recruit Lsd1 to primed cardiac enhancers.

**Binding by Scl and cardiac Gata4 and/or Hand1 define enhancer subgroups that may determine fate choice between opposing mesodermal fates**

As the analysis of repressive marks and corepressors provided no evidence for direct Scl-dependent corepressor recruitment to enhancers or promoters in mesoderm, we considered the possibility that Scl inhibits cardiac fate by interfering with the activation of these genes by cardiac regulators. To identify candidate factors that could also bind to Scl-regulated enhancers, we performed DNA motif enrichment analysis for known transcription factor binding sites in Transfac and Jaspar databases. This analysis identified the composite Tal1(Scl)_Gata motif and Gata motifs as the most prevalent motifs in both activated and repressed genes (Fig 5A).

There are six known Gata factors, of which Gata1, 2 and 3 act in hematopoietic cells and Gata4, 5 and 6 regulate cardiac development (Chlon & Crispino, 2012). Gata4 can regulate cardiac development both indirectly via endoderm and directly by binding to cardiac genes in Flk1+ mesoderm (Holtzinger et al., 2010; Oda et al., 2013). Analysis of a published dataset for Gata4 binding in Flk1+ mesoderm revealed that Gata4 binding overlaps with a large fraction (41%) of Scl binding sites (Fig 5B). Moreover, as other bHLH factors can also bind to E-box motifs similar to Scl, we also performed ChIP-seq for Hand1, a key cardiac bHLH factor that can interact with cardiac Gata factors. 13.2% all Scl-bound enhancers were co-occupied by Scl and Hand1 in Flk1+ mesoderm (9.7% Scl, Hand1 and Gata4 altogether, and 3.5% Scl and Hand1 alone). Analysis of the regulatory regions for key cardiac (Gata6, Tbx5 and Myocardin) and hematopoietic (Runx1, Gfi1b and Gata2) transcription factors identified at least one enhancer in each gene bound by Scl, Hand1 and/or Gata4 (Fig 5C). The sites that can be occupied by all three factors (424 sites) included both that remain active (cluster R, 167 sites) and sites that become decommissioned during hematopoietic development (cluster L, 201 sites), and they associated with both Scl-dependent activated (128) and repressed (79) genes. Similar distribution was observed for Scl binding sites that were shared with Gata4 or Hand1 alone. These data suggest that a distinct subset of Scl-bound enhancers may be used by both hematopoietic and cardiac bHLH and Gata transcription factors to activate their own lineage or repress a competing lineage.

We next assessed whether there are any unique features that sets the ‘dually accessible’ enhancers apart from enhancers bound by Scl alone. The enhancers bound by both Scl and Gata4 and/or Hand1 were more evolutionarily conserved as compared to other Scl mesodermal binding sites (Fig 5D) and showed higher levels of Scl binding (Fig 5E), H3K4me1, H3K27ac (Fig 5F and G) and Lsd1 enrichment (Fig 5H). Moreover, Scl target genes that harbor an enhancer that can be bound by Scl and Gata4 and/or Hand1 showed higher average gene expression change in Scl-expressing WT versus SclKO Flk1+ mesoderm as compared to Scl target genes that do not have a dually accessible enhancer (Fig 5I). The genes that contain a dually accessible enhancer included many hematopoietic (27/31, 87%) and cardiac (10/15, 67%) transcription factors (Fig 5J).

We next assessed whether similar developmental stage-specific binding observed with Scl is also seen with Gata4, for which several published datasets from fetal and adult stages are available (He et al., 2014). Analysis of Gata4 binding in different stages of heart development showed gradual loss of overlap between Scl mesodermal binding sites and Gata4 binding from Flk1+ mesoderm to E12.5 hearts to adult hearts (Supplementary Fig S5A and B). While Gata4 binding was observed in both hematopoietic (Gfi1b and Myb) and cardiac enhancers (Tbx5 and Gata6) in mesoderm, hematopoietic enhancers had lost Gata4 binding in fetal hearts by E12.5. Many cardiac enhancers also lost Gata4.
Figure S.

A

Enriched DNA motifs in Scl MES binding sites

<table>
<thead>
<tr>
<th>Motif</th>
<th>Name</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAL1_Gata</td>
<td>GATAAA</td>
<td>&lt;1E-20</td>
</tr>
<tr>
<td>Gata</td>
<td>GATAA</td>
<td>&lt;1E-20</td>
</tr>
<tr>
<td>Ets1</td>
<td>CGGA</td>
<td>&lt;1E-20</td>
</tr>
<tr>
<td>Ev1</td>
<td>CACCC</td>
<td>2.22E-16</td>
</tr>
<tr>
<td>Tal1</td>
<td>8.17E-7</td>
<td></td>
</tr>
</tbody>
</table>

Repressed bound genes (186)

<table>
<thead>
<tr>
<th>Motif</th>
<th>Name</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAL1_Gata</td>
<td>GATAAA</td>
<td>&lt;1E-20</td>
</tr>
<tr>
<td>Gata</td>
<td>GATAA</td>
<td>&lt;1E-20</td>
</tr>
<tr>
<td>Ets1</td>
<td>CGGA</td>
<td>&lt;1E-20</td>
</tr>
<tr>
<td>Ap4</td>
<td>CACCC</td>
<td>&lt;1E-20</td>
</tr>
<tr>
<td>Tal1</td>
<td>8.17E-7</td>
<td></td>
</tr>
</tbody>
</table>

B

Scl binding sites in Flk1+ mesoderm (4393)

C

Cardiac genes

<table>
<thead>
<tr>
<th>chr:11</th>
<th>65 050 000</th>
<th>65 100 000</th>
<th>65 150 000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scl</td>
<td>Myocd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gata4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hand1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

chr:18 | 11 070 000 | 11 090 000 |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Scl</td>
<td>Gata4</td>
<td></td>
</tr>
<tr>
<td>Hand1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

chr:11 | 57 650 000 | 57 690 000 |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Scl</td>
<td>Gata4</td>
<td></td>
</tr>
<tr>
<td>Hand1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hematopoietic genes

<table>
<thead>
<tr>
<th>chr:21</th>
<th>28 460 000</th>
<th>28 470 000</th>
<th>28 480 000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scl</td>
<td>Gata4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hand1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

chr:6  | 88 145 000 | 88 165 000 | 88 165 000 |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Scl</td>
<td>Gata4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hand1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

chr:16 | 92 800 000 | 92 950 000 | 93 100 000 |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Scl</td>
<td>Gata4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hand1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure S.
binding in adult hearts (Supplementary Fig S5C). These data show that the key genes regulating cardiovascular fate determination harbor dually accessible enhancers that can be bound by both hematopoietic and cardiac factors in mesoderm, but are dynamically remodeled during lineage diversification and maturation. Our data propose that this subset of enhancers may be critical for initiating the fate choice between competing cardiovascular lineages.

**Figure 6. Gata1 and 2 are dispensable for cardiac repression, but essential for the emergence of HS/PCs from hemogenic endothelium.**

A. FACS analysis of day 7 EBs with markers CD41 and c-Kit shows efficient generation of HS/PCs from WT, but not SclKO or Gata1&2KO cells. Average of six independent experiments with SEM is shown.

B. FACS analysis of day 5.25 EBs shows the generation of hemogenic endothelial cells from WT and Gata1&2KO cells, but not SclKO cells. Average of five independent biological experiments with SEM is shown.

C. Assessment of the developmental potential of day 4.75 CD41+CD31+Tie2+ endothelial cells from WT cells but not Gata1&2KO or SclKO cells, and troponin T+ cardiomyocytes from SclKO cells, but not WT or Gata1&2KO cells. Average of four independent experiments with SEM is shown.

D. Heatmaps show gene expression differences in subsets of hematopoietic genes between Gata1&2KO and WT or SclKO day 4.75 CD41+CD31+Tie2+ endothelial cells. Cardiac derepression is observed only in SclKO cells. *designates non-functional transcripts.
Gata1 and/or Gata2 are dispensable for cardiac repression but are essential for emergence of HS/PCs from hemogenic endothelium

Since Scl and Gata4 share common binding sites in both hematopoietic and cardiac enhancers and Scl acts together with hematopoietic Gata factors 1 and 2 in blood forming cells, we investigated whether hematopoietic Gata factors are required for Scl-induced gene activation and/or repression during mesoderm diversification. ChIP-PCR for Gata2 showed that, similar to Scl, Hand1 and Gata4, Gata2 binds to dually accessible enhancers in both hematopoietic and cardiac transcription factors that are activated or repressed by Scl (Supplementary Fig S5D).

To investigate whether hematopoietic Gata factors function in Scl-mediated gene activation and repression, we assessed the developmental potential of Gata1 and 2 double knockout (Gata1&2KO) ES cells, which eliminates possible redundancy between Gata1 and 2. No c-Ki67+CD41+ hematopoietic progenitors were detected in day 7 Gata1&2KO EBs or SclKO EBs (Fig 6A). However, FACS analysis on day 5.25 EBs identified a subpopulation of cells in Gata1&2KO EBs that expressed the early HS/PC marker CD41, while SclKO EBs were devoid of this population. Nevertheless, all CD41+ cells in Gata1&2KO EBs co-expressed Flk1 and the majority also expressed endothelial markers CD31 and Tie2, raising the hypothesis that HS/PC development becomes stalled at hemogenic endothelium in the absence of Gata1 and 2 (Fig 6B).

To assess the differentiation potential of Gata1- and 2-deficient endothelial cells, EBs were differentiated for 4.75 days, after which CD41+CD31+Tie2+ endothelial cells were sorted and cultured for 14 days in hematopoietic and cardiac conditions on OP9 stroma. Neither Gata1&2KO nor SclKO endothelial cells generated CD45+ hematopoietic cells (Fig 6C). In contrast, only SclKO, but not Gata1&2KO or WT endothelial cells, robustly differentiated into troponin T+ cardiomyocytes. These data show that Gata1 and/or 2 is required for hematopoiesis, but not for the repression of cardiovascular gene expression in endothelial precursors.

To investigate the effect of Gata1 and 2 on endothelial gene expression, CD41+CD31+Tie2+ endothelial cells from day 4.75 EBs were sorted for RNA sequencing. No significant derepression of cardiac regulators was observed in Gata1&2KO endothelial cells, unlike SclKO endothelial cells which showed induction of several cardiac genes (Fig 6D, Supplementary Table S2). Notably, expression of many hematopoietic transcription factors that were down-regulated in SclKO endothelial cells (e.g. Ly6H, Hhex, Cbfa2t3/Eto2, Erg, Flt1, Sox7) was not significantly decreased in Gata1&2KO endothelial cells. Likewise, many surface markers of hemogenic endothelium and HS/PCs (e.g. CD41/Igα2b, VE-cadherin/Cdh5, Esam, lcam2) were expressed in Gata1&2KO endothelial cells, further supporting the notion that Gata1&2KO cells can specify hemogenic endothelium. However, the expression of key HS/PC transcription factors Runx1, Myb, Gfi1b, Pu.1/Sfpi1 and Klf1 was down-regulated in Gata1&2KO endothelial cells (Fig 6D), implying that Gata1 and/or 2 is required for activation of the hematopoietic transcription factor network that enables the emergence of HS/PCs from hemogenic endothelium.

To exclude the possibility that Gata3, a hematopoietic Gata factor that functions in T cells (Hosoya et al, 2010) and HSC maintenance (Ku et al, 2012) compensates for Gata1 and 2 in cardiac repression, Gata1-, 2- and 3-deficient (Gata1&2KO/Gata3KO) ES cells were generated using lentiviral knockdown of Gata3. Similar to Gata1&2KO, Gata1&2KO/Gata3KO endothelial cells showed neither up-regulation of cardiac program nor generation of ectopic cardiomyocytes in culture, and the hematopoietic program was similarly stalled at hemogenic endothelium with intact expression of many Scl-dependent hematopoietic vascular genes (Hhex, Ets2, Lyt1, Igα2b/CD41, Cadh5/Ve-Cad etc) and defective expression of key HS/PC transcription factors (e.g. Runx1 and Myb) (Supplementary Fig S6). Collectively, these data show that hematopoietic Gata factors are critical for full activation of Scl-dependent hematopoietic transcriptional network, but not for cardiac repression.

Gata factors 1 and 2 recruit Scl to specific binding sites in key hematopoietic transcription factors

To investigate whether loss of Gata1 and Gata2 compromises Scl binding to its targets during mesoderm specification, we determined genome-wide Scl binding sites in Flk1+ mesoderm from Gata1&2KO EBs using ChIP-seq (Fig 7A, Supplementary Table S3). The GO terms enriched among the genes associated with overlapping Scl binding sites included both hematopoiesis and cardiogenesis, while the peaks lost in Gata1&2KO cells were associated with genes related to hematopoiesis (Fig 7A). Analysis of the individual genes confirmed intact Scl binding to key cardiac enhancers (Myocardin, Gata4 and Gata6) in the absence of Gata1 and 2 (Fig 7B, Supplementary Fig S7B). Likewise, a majority of hematopoietic transcription factors (Ly6H, Flt1) that were expressed in Gata1&2KO endothelium retained Scl binding (Fig 7C). The subset of genes that lost an Scl binding site in Gata1&2KO Flk1+ cells included transcription factors Runx1, Myb, Pu.1/Sfpi1 and Gfi1b, whose expression in endothelium was Gata1 and 2 dependent (Fig 7D, Supplementary Fig S7B). Remarkably, while several other Scl binding sites were preserved near Runx1 gene, the binding site that was lost in Gata1&2KO cells corresponds to +23 enhancer that has been shown to be essential for Runx1 regulation during HSC emergence (Nottingham et al, 2007) and becomes the strongest Scl binding site in HPc7 hematopoietic progenitor cells (Supplementary Fig S3B). These results imply that although Gata1 and 2 are not essential for Scl binding to the majority of its activated or repressed target genes, they are required for the recruitment of Scl to specific regulatory regions that govern the expression of key hematopoietic transcription factors. ChIP-PCR for Gata1&2KO/Gata3KO mesoderm confirmed similar binding for Scl in key hematopoietic and cardiac enhancers in the absence of all three hematopoietic Gata factors (Supplementary Fig S7A).

We also assessed whether the hematopoietic Gata factors function by modifying the epigenetic landscape to enable access of Scl complex to key hematopoietic enhancers. Analysis of H3K4me1 and H3K27ac in Gata1- and 2-deficient mesodermal cells did not reveal differences in the establishment of primed state in key hematopoietic and cardiac enhancers, including those that could not be activated in Gata1&2KO cells (Supplementary Fig S7B). Likewise, there was a strong correlation of global H3K4me1 and H3K27ac levels between WT and Gata1&2KO Flk1+ mesoderm (Supplementary Fig S7C). Collectively, these data show that Scl and hematopoietic Gata factors bind to enhancers that have been epigenetically primed for activation in mesoderm prior to their binding. While the repression of the cardiac fate by Scl can occur in the absence of hematopoietic
Gata factors, they help recruit Scl to new regulatory regions to enable activation of key hematopoietic transcription factors that secure the emergence of HS/PCs from hemogenic endothelium.

**Discussion**

The ability to execute developmental fate decisions with proper temporal and spatial control is critical for embryonic development, as well as for harnessing the power of stem cell and reprogramming technologies for therapeutic applications. The bHLH factor Scl has emerged as a true master regulator of mesoderm fate as it not only governs the establishment of the entire hematopoietic system, but it also is critical for preventing ectopic cardiomyocyte development in hemogenic tissues (Van Handel et al., 2012). Here we show that Scl directly binds to enhancers regulating a broad network of hematopoietic and cardiac transcription factors to specify the hematopoietic lineage and override the cardiac fate and thus has a dual function in coordinating the segregation of multipotent cardiovascular mesoderm to downstream cell fates.

We discovered that Scl regulates mesodermal fates by binding to enhancers that have been primed for activation specifically in Flk1⁺ mesoderm. A previous study with DNA binding mutant of Scl showed that 80% of Scl peaks in erythroid cells were lost in the mutant and could neither be bound by Scl’s interaction partner Gata1, raising the question whether Scl acts as a pioneer factor at these sites (Kassouf et al., 2010). Our finding that the establishment of the active histone modifications, H3K4me1 and H3K27ac, at the enhancers of Scl’s hematopoietic and cardiac target genes occurs independent of Scl and its complex partners Gata1 and 2 implies that these factors do not act as a pioneer factors but rather exploit pre-established enhancers in mesoderm. These results suggest that yet another factor(s) is responsible for establishing primed enhancers that can attract Scl and Gata factors to their cell type-specific binding sites. Our data are consistent with a model that the acquisition of a primed, or provisionally activated, state at hematopoietic and cardiac enhancers precedes Scl and Gata1 and 2 binding and determines the available fate options in mesoderm.

Several recent studies have documented highly cell type-specific, functional Scl binding during later stages of hematopoietic development (Calero-Nieto et al., 2014; Pimkin et al., 2014; Wu et al., 2014). Our study documents the dynamic nature of Scl binding already in...
mesoderm. In contrast to the key hematopoietic enhancers that retained Scl binding and active epigenetic marks throughout hematopoietic development, Scl binding to cardiac enhancers was restricted to Flk1+ mesoderm and correlated with the transient presence of active histone marks. These data imply that Scl represses the cardiac fate during a brief window of developmental plasticity before mesodermal fates become solidified, providing a molecular basis for the inducible loss-of-function studies that narrowed down the temporal requirement for Scl function in cardiac repression to mesoderm/early angioblasts (Van Handel et al., 2012). Moreover, these data suggest enhancer decommissioning as a key mechanism that extinguishes the cardiac fate option in hematopoietic cells.

Our studies propose a model that Scl suppresses cardiogenesis by interfering with the activation of key cardiac genes until the ‘window of opportunity’ for their activation is closed, rather than directly recruiting corepressors. Analyses correlating Scl binding with major corepressors and repressive epigenetic marks (H3K27me3, H3K9me3 and DNA methylation) did not provide a mechanism by which transient Scl binding to these enhancers results in the repression of cardiac genes, but rather suggests that these repression mechanisms contribute to the overall gene silencing later, once the Scl-driven lineage choice decision has already been made.

Lsd1 emerged as a key candidate for modulating enhancer activity during mesoderm diversification as it decommissions cell type-specific enhancers by demethylating H3K4me1 in several developmental contexts including ES cells and myeloid cells (Whyte et al., 2012; Kerenyi et al., 2013), and interaction of Scl with Lsd1 has been implicated in erythroid progenitors and T-ALL (Hu et al., 2009a,b; Li et al., 2012). Our analysis verified a strong correlation between Lsd1 and Scl binding sites and H3K4me1 in mesoderm. However, Lsd1 binding to cardiac enhancers was not impaired in Scl-deficient cells. Thus, although Lsd1 is involved in decommissioning of unused enhancers during mesoderm diversification, its recruitment to cardiac enhancers is not Scl dependent. These data do not, however, exclude the possibility that the function of Lsd1, or the COREST complex Lsd1 is part of, may be influenced by Scl. As acetylation can impede Lsd1 function (Amente et al., 2013), it is plausible that Scl binding at hematopoietic enhancers reinforces a fully active state, possibly by chromosomal looping to promoter, and thereby indirectly impairs Lsd1 function. On the other hand, Scl binding at cardiac enhancers may interfere with chromosomal looping and/or the establishment of a proper cardiac activation complex, enabling Lsd1 to decommission cardiac enhancers. Future studies are required to directly test these hypotheses.

Our findings that there is substantial overlap with Scl and Hand1 and/or Gata4 mesodermal binding sites, and essentially all major hematopoietic and cardiac transcription factors that are regulated by Scl contain at least one enhancer that can also be bound by Hand1 and/or Gata4, support the model that repression of an alternative fate choice may be the result of preventing activation of these genes by competing lineage-specific regulators. The subset of Scl-bound enhancers that can also be bound by Hand1 and/or Gata4 showed higher evolutionary conservation, higher H3K4me1 and H3K27ac enrichment and Lsd1 binding in mesoderm, and stronger correlation with Scl-dependent gene expression change. We propose that these dually accessible enhancers that can be regulated by factors of two opposing lineages act as a platform where the fate choice is determined. Future studies will determine which cardiac factor(s) is most critical for activating these cardiac enhancers and whether they are also critical for suppressing the hematopoietic program.

We found that Gata2 can bind together with Scl to both hematopoietic and cardiac enhancers; however, we discovered that hematopoietic Gata factors (1, 2 and 3) are dispensable for Scl-mediated cardiac repression, and only necessary for recruiting Scl to specific binding sites to activate hematopoietic factors required for HS/PC emergence and differentiation (Runx1, Pu.1/Sfpi1, Klf1, Gfi1b). These data suggest that although the core Gata/Scl complex can bind to both activated and repressed genes, Gata/Scl interaction becomes critical principally in gene activation. Similar to hematopoietic Gata factors, Runx1 can redistribute Scl to new binding sites as development progresses from endothelium to HS/PCs (Lichtinger et al., 2012). Thus, Scl establishes the blood lineage by binding to a broad set of hematopoietic enhancers; it then induces downstream transcriptional network with the help of its target genes, which enable Scl to relocate to new binding sites to build a functional hematopoietic system.

The findings that Scl targets Gata1, Gata2 and Runx1 (Van Handel et al., 2012), which are critical for modulating Scl activity in hematopoietic genes, are not essential for cardiac repression underscore the unique role of Scl as a dual regulator of hematopoietic versus cardiac fate choice. Apart from the studies with Scl, ectopic cardiogenesis in hemato-vascular mesoderm has only been observed upon ablation of Etv2, the upstream regulator for Scl (Ren et al., 2010; Palencia-Desai et al., 2011; Rasmussen et al., 2011; Liu et al., 2012; Wareing et al., 2012). Future studies will be required to determine whether Etv2 has a specific function in silencing cardiac fate or whether it contributes indirectly by inducing Scl expression.

Our finding that Scl directs mesoderm diversification via enhancers is concordant with the data that transcription factor binding at enhancers is a key determinant of tissue-specific gene expression (Heintzman et al., 2009; Visel et al., 2009; Xu et al., 2012). Our discovery that Scl directly binds to enhancers of key transcription factors of two alternative fates suggests a mechanism by which lineage-specific transcription factors secure a mutually exclusive lineage choice by both activating their own lineage and preventing the establishment of alternative fates. Coordination of both processes by the same factor enables accurate fate specification and prevents the generation of a cell with mixed characteristics. As reprogramming of induced pluripotent stem cells is also initiated at enhancers (Soufi et al., 2012), understanding the repressive function of master regulators may have broader implications in regenerative medicine as the presence of a factor that could block lineage-specific enhancers during lineage reprogramming may influence the efficiency and outcome of reprogramming. Thus, better understanding of the prerequisites for gene activation and repression by master regulators, and how the epigenetic boundaries are created between cell types, will help develop more effective protocols for directing cell fates for therapeutic applications.

Materials and Methods

ES cell culture and differentiation

Standard ES cell culture conditions were used to maintain SclPACTM (Chung et al., 2002), SclBIO (Porcher et al., 1996), SclKOiScl, WT,
ChIP with ES, Flk1+ mesodermal (MES), HPC7, MEL or HL1 cells. 

Chromatin immunoprecipitation sequencing analysis (Roche). Primer sequences are listed in Supplementary Table S4. For were merged using Cuffmerge, and differential expression (polymerase chain reaction (qRT-PCR) was determined using Cuffdiff. Quantitative reverse-transcriptase

SclKO

RRBS libraries were generated from genomic DNA of mouse ES cells. The EMBO Journal

Gene expression analysis

RNA preparation, hybridization to Mouse Genome 430 2.0 arrays, and analysis was conducted as described previously (Van Handel et al., 2012). Libraries for RNA sequencing were constructed using Encore Complete RNA-Seq DR Multiplex System 1-8 (Nugen). Libraries were sequenced using Hiseq-2000 (Illumina). Reads were mapped to mouse genome (mm9) using TopHat v2.0.4 (Trappe1 et al., 2009). Abundance estimations (FPKMs) were performed with Cufflinks v2.0.1 (Trappe1 et al., 2010). Assemblies for all samples were merged using Cuffmerge, and differential expression (P < 0.01) was determined using Cuffdiff. Quantitative reverse-transcriptase polymerase chain reaction (qRT–PCR) was performed with LightCycler 480 SYBR Green I Master (Roche) using LightCycler 480 (Roche). Primer sequences are listed in Supplementary Table S4. For further details, see Supplementary Materials and Methods.

Chromatin immunoprecipitation sequencing analysis

ChIP with ES, Flk1+ mesodermal (MES), HPC7, MEL or HL1 cells were performed as described previously (Ferrari et al., 2012; Kerenyi et al., 2013). Libraries were generated using Nugen Ovation ultralow kit and sequenced using Hiseq-2000 (Illumina). Mapping was performed using Bowtie (Langmead et al., 2009) as described (Ferrari et al., 2012). Peak identification was performed with MACS v1.3.7.1 (Zhang et al., 2004). For published ChIP-seq datasets used and more detailed methods, see Supplementary Materials and Methods.

Reduced representation bisulfite sequencing

RRBS libraries were generated from genomic DNA of mouse ES cells, WT and SclKO Flk1+ mesoderm (MES) and MEL cells as described previously (Meissner et al., 2005) with minor modifications. RRBS data were aligned with BS-Seeker2 (Guo et al., 2013). Differentially methylated cytosines were calculated with methylKit (Akalin et al., 2012). For detailed methods and the published genome-wide methylation datasets used in this study, see Supplementary Materials and Methods.

Analysis of endothelial differentiation potential

After 4.75 days of EB induction, CD31+ Tie2+ cells were sorted from WT, SclKO, SclKO:Scl, Gata1&2KO and Gata1&2KO Gata3KD EBs and 20,000 cells were plated on irradiated OP9 stroma in 8-well chamber slides (354118 BD Falcon®). 1 mg/ml doxycycline (1:1,000) is added to SclKO:Scl culture and is kept on since day 2 of EB induction. The media contained α-MEM, 20% FBS, 1% penicillin, streptomycin and 1% glutamine. For hematopoietic differentiation, 50 ng/ml SCF, 5 ng/ml IL3, 5 ng/ml IL6, 5 ng/ml TPO and 10 ng/ml Flt3L were added. For cardiac differentiation, 5 ng/ml hVEGF, 30 ng/ml mFGF, 50 ng/ml bBMP4 and 1 μM Wnt/Beta-Catenin Inhibitor XAV939 (Sigma) were added. After 14 days, FACS staining or immunostaining was performed as described (Van Handel et al., 2012). See Supplementary Materials and Methods for details.

Acknowledgements

The authors thank the Broad Stem Cell Research Center (BSCRC) Flow Cytometry Core for FACS sorting and BSCRC Sequencing core for next-generation sequencing. This work was funded by the California Institute for Regenerative Medicine (CIRM) New Faculty Award (RN1-00057), the Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research at UCLA Research Award, American Heart Association (#14GRNT120480340), and Leukemia Lymphoma Society Scholar Award to HKAM (2013S778). TO was supported by Leukemia Lymphoma Society postdoctoral fellowship (7537-13) and by the European Union through the European Social Fund (Mobilitas Grant No. MJD284). DD was supported by the government of P.R.C through the State Scholarship Fund (File No. 2011624028). AMH was supported by a fellowship from HFSP and American Society of Hematology. BVH was supported by the NIH/NIHLBI T32 HL69766. Contributions of YF and SHO were supported by P30DK092161.

Author contributions

TO and DD were involved in project design, experimental work, data analysis and interpretation and manuscript preparation. RF and RS participated in data analysis and interpretation, and manuscript editing. AM-H, BVH and MAK were involved in experimental work and manuscript editing. LR was involved in experimental work. YF and SHO provided essential reagents and edited the manuscript. MP and SKK interpreted the data and edited the manuscript. HKAM was involved in project design, data analysis and interpretation, and manuscript preparation.

Conflict of interest

The authors declare that they have no conflict of interest.

References


Scl represses cardiac fate via primed enhancers


Scl represses cardiac fate via primed enhancers  Tönis Org et al


© 2015 The Authors

The EMBO Journal Vol 34 No 6 2015

776
Scl represses cardiac fate via primed enhancers

The Authors

The EMBO Journal