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SALL4 is a key transcription regulator in normal human hematopoiesis

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Abstract

Background—Stem cell factor SALL4 is a zinc finger transcription factor. It plays vital roles in the maintenance of embryonic stem cell properties, functions as an oncogene in leukemia, and has been recently proposed to use for cord blood expansion. The mechanism(s) by which SALL4 functions in normal human hematopoiesis, including identification of its target genes, still need to be explored.

Study Design and Methods—Chromatin-immunoprecipitation followed by microarray hybridization (ChIP-chip) was used for mapping SALL4 global gene targets in normal primary CD34+ cells. The results were then correlated with SALL4 functional studies in the CD34+ cells.

Results—Over 1,000 potential SALL4 down-stream target genes have been identified, and validation of binding by ChIP-qPCR was performed for 5% of potential targets. These include genes that are involving in hematopoietic differentiation and self-renewal, such as HOXA9, RUNX1, CD34, and PTEN. Down-regulation of SALL4 expression using shRNA in these cells led to decreased *in vitro* myeloid colony forming abilities and impaired *in vivo* engraftment. Furthermore, HOXA9 was identified to be a major SALL4 target in normal human hematopoiesis and the loss of either SALL4 or HOXA9 expression in CD34+ cells shared a similar phenotype.

Conclusion—Taking together, SALL4 is a key regulator in normal human hematopoiesis and the mechanism of its function is at least in part through the HOXA9. Future study will determine

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Conflict of Interest: The author declares that she has no conflict of interest relevant to the manuscript submitted to *Transfusion*.

Author contribution

CG, NK, AL, JL, YY, SU, HT, and JH performed the experiments and analyzed the data, JY contributed partially to the ChIP-chip experiment, YM contributed to the design of ChIP-chip experiments, GK, CG, NK, DGT and LC designed the experiment, analyzed the data and wrote the paper. In addition, we want to thank Xi Tian in assistant in animal work.

whether modulating the SALL4/HOXA9 pathway can be used in cellular therapy such as cord blood expansion and/or myeloid engraftment.

Keywords

SALL4; HOXA9; normal hematopoiesis; stem/progenitor cells

Introduction

A member of the SALL gene family that encode zinc-finger transcription factors, SALL4, has been isolated based on its homology to a *Drosophila* region-specific homeotic gene, *spalt* (*sal*). Spalt is known to specify pattern elements in the terminal regions of the fly embryo^{1,2}. Human SALL4 mutations are associated with the Duane-radial ray syndrome (DRRS, or Okhiro syndrome)^{3–6}. This condition is an autosomal-dominant disorder that displays radial-sided hand anomalies in association with the Duane syndrome (DS), which is a congenital disorder of eye movement characterized by strabismus. Recently, a novel SALL4 mutation has been identified in a large IVIC (Instituto Venezolano de Investigaciones Científicas) syndrome family by the Arias group. The IVIC syndrome is an autosomal-dominant condition with DRRS that is also characterized by leukocytosis and thrombocytopenia. The latter findings suggest that SALL4 is involved in normal hematopoiesis⁷.

Sall4-null mice die at E6.5, suggesting that SALL4 plays a crucial role in embryonic development^{8–11}. We have demonstrated that murine Sall4 is essential in the maintenance of pluripotency and self-renewal properties of ES cells by interacting with two other key regulators in ES cells, Nanog and Oct4.¹¹ These interactions are characterized by Sall4 directly activating the expression of Oct4 and acting in tandem with Nanog to control the fate of ES cells^{11,12}. Sall4-deficient ES cells differentiate spontaneously and start expressing trophoblast markers such as Cdx2. Eventually these cells can contribute to the formation of trophoblast in murine blastocysts¹¹. In addition, the depletion of Sall4 in another ES cell-like cell line derived from extraembryonic endoderm, XEN cells,¹³ also disrupts self-renewal and induces differentiation.

Using immunohistological staining and quantitative RT-PCR analysis, we found that SALL4 is abnormally expressed in leukemia cell lines and primary leukemia cells^{14,15}. Furthermore, our research group has demonstrated that constitutive expression of SALL4 contributes to leukemogenesis¹⁴. Mice with transgenic SALL4B, one of the SALL4 isoforms that we identified, developed pre-leukemic myelodysplastic syndrome (MDS)-like features and subsequent acute myeloid leukemia (AML), suggesting that SALL4 is a key regulator in leukemogenesis¹⁴.

We have previously demonstrated that Bmi-1^{16,17} and Pten¹⁸, two key regulators of self-renewal of normal hematopoietic and leukemic stem cells (LSC)^{19–21}, are direct targets of SALL4. Bmi-1 was up-regulated and Pten was down-regulated in SALL4B transgenic mouse bone marrow^{16,18}, and in the LSC population in SALL4B transgenic mice.

Given the importance of SALL4 in self renewal of both ES cells and LSCs, in this study, we aimed to further explore its role in normal human hematopoietic stem and progenitor cells (HSC/HPCs). More recently, SALL4 has been proposed for HSC/HPCs expansion^{22,23}, however, the mechanism for this usage remains unexplored, and the functional role of SALL4 in normal human hematopoiesis hasn't been tested by a loss-of-functional approach. We have mapped genome-wide promoter DNA binding sites of SALL4 and tested its effects on the expression of many of its bound targets. Moreover, down-regulation of SALL4 expression using shRNA knockdown in these cells has led to decreased *in vitro* colony

formation and impaired *in vivo* engraftment, indicating that SALL4 plays an important role in normal adult human hematopoiesis. In addition, we have shown that HOXA9 is a major SALL4 target during this process.

Materials and Methods

CD34+ cells

Peripheral mobilized stem cells, bone marrow and cord blood cells were obtained from LONZA WALKERSVILLE INC (USA) or discarded samples from normal donors (protocol number 2005P002088 and 2005P002544). These cells were selected for CD34 positive cells with the EasySep Human CD34+ Selection Cocktail (StemCell Technologies).

ChIP-chip and Validation by ChIP-qPCR

A complete ChIP-chip assay protocol was provided by NimbleGen Systems, Inc. (Madison, WI) and Agilent Technologies (Santa Clara, CA). This approach has been used to identify SALL4 targets in ChIP experiments in NB4 cells and murine embryonic stem cells (ES cells)^{17,24}, and is described in detail in supplemental material. The sequences of all primers used for validation are listed in Supplemental Table 1.

Data Analysis

Protein Analysis Through Evolutionary Relationships (PANTHER, www.pantherdb.org) classification system was used to analyze the biological functions and pathways of SALL4 bound genes in CD34+ cells. Cytoscape software was used to generate figures to demonstrate the different pathways in which SALL4-bound genes are involved, as well as differential SALL4 targets for hematopoiesis, apoptosis, and oncogenesis in both normal hematopoietic and leukemic cells.

Colony-forming unit assay after SALL4 or HOXA9 Knockdown

Colony-forming unit assays were set up with 2,000 or 3,000 CD34+ cells per well (6-well plates) in the following experimental groups: wild type control with no viral infection, negative control cells infected with retroviruses or lentiviruses containing a scrambled vector, and those containing a small hairpin RNA (shRNA) specifically targeting SALL4 or those containing a shRNA specifically targeting HOXA9. The sequence for SALL4 shRNA1 (also called SALL4 shRNA7412) was: GCCGACCTATGTCAAGGTTGAAGTTCCTG. The sequence for SALL4 shRNA2 (also called SALL4 shRNA-7410) was: GATGCCTTGAAACAAGCCAAGCTACCTCA. Negative scrambled shRNA control was a shRNA obtained from OriGene Technologies (Inc. Rockville, MD). For retroviral mediated SALL4 down-regulation, we followed our previous protocol and instruction from OriGene Technologie. HOXA9 shRNA construct scrambled control pluc-sh, were purchased from Invitrogen. The sequence is listed as following: HOXA9shRNA: 5'-GTGGTTCTCCTCCAGTTGATA-3'. For lentiviral mediated SALL4 or HOXA9 knock down, Lentiviral supernatants were obtained in 293T cells by cotransfection of the shRNA plasmids and packaging plasmids containing VSV-G and pH8.9. For lentiviral infection of primary CD34+ cells, 10⁵ cells were seeded in 12-well plates (200ul per well) in the appropriate culture medium. Polybrene (hexadimethrine bromide; Sigma-Aldrich, St Louis, MO) was added at a final concentration of 8ug/ml. After adding 1ml of lentiviral particles (titer of each lentiviral shRNA construct adjusted to 1×10⁶ transducing unit/ml to achieve a multiplicity of infection of 10 transducing units/cells), spinoculation was performed at 1800rpm for 90 minutes at 37°C. Then cells were brought back to 500ul in volume with fresh CellGro media (CellGenix Technologie transfer GmbH, Maryland, USA). Two or three thousand cells were then cultured in methylcellulose medium

supplemented with SCF, IL-3, GM-CSF and EPO (MethoCult® GF H4034, StemCell Technologies), with 1 µg/ml puromycin for selection. The rest of the cells were saved for cytospin/Giemsa staining and qRT-PCR analyses to examine changes in morphology and gene expression, respectively. Cell colonies were examined at day 7 and 14 upon plating.

Xenotransplantation

5×10^5 of SALL4-shRNA or control retrovirus-infected human CD34+ cells were transplanted into sublethally irradiated (1.0 Gy) NOD/SCID/IL2r γ null mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, The Jackson Laboratory, ME USA) by tail vein injection. Peripheral blood (PB) and bone marrow (BM) chimerism was monitored at 8 weeks post transplantation. In brief, about 20 µl PB or BM was obtained for flow cytometry analysis utilizing FITC-conjugated antihuman CD45 antibody and APC-conjugated anti-mouse CD45 antibody (eBiosciences, CA USA). The percentage of human CD45+ cells was calculated as follows: % human CD45+ cells = No. human CD45+ cells/(No. human CD45+ cells + No. mouse CD45+ cells) $\times$ 100. All animal work has been conducted according to relevant national and international guidelines and in accordance with the recommendations of the Weatherall report.

Results

SALL4 is expressed in normal human hematopoietic stem and progenitor cells (HSCs and HPCs)

To investigate the expression pattern of SALL4 in normal hematopoiesis, we first sorted normal human bone marrow cells into CD34+ and CD34- subgroups. Previous studies have demonstrated that repopulating human HSC are contained in the CD34+ fraction, while the CD34- fraction includes more matured blood elements^{25,26}. Later studies suggested that the CD34+ fraction could be further subdivided and the HSC resided in the human CD34+/CD38- population, and the HPC resided in the CD34+CD38+ population²⁷. SALL4 was found to be only expressed in normal CD34+ hematopoietic stem and progenitor cells (HSC/HPCs, Figure 1A). To determine which sub-population of normal CD34+ cells expressed SALL4, we further sorted normal bone marrow cells (N=5) into HSCs (CD34+CD38-), HPCs (CD34+CD38+), and a CD34- population (Figure 1B). With isoform-specific primers for SALL4A and B, two isoforms that we identified previously¹⁴, we observed that during normal hematopoiesis, both isoforms were expressed preferentially in HSCs, down-regulated in HPCs, and absent in the CD34- population.

Genome-wide ChIP-chip identifies multiple potential SALL4 downstream target genes in normal CD34+ cells

The unique SALL4 expression pattern suggests that it may play a functional role in CD34+ cells. As an initial step to test this hypothesis, we performed a genome-wide analysis of SALL4 target genes in human CD34+ cells using chromatin immunoprecipitation followed by promoter array hybridization (ChIP-chip). SALL4-bound DNA fragments were obtained from immunomagnetically selected CD34+ cells using an affinity-purified polyclonal anti-SALL4 antibody. This SALL4 antibody has been previously used in immunohistochemistry, flow cytometry, and western blot, and has been used to identify SALL4 targets in NB4 cells and murine ES cells^{17,24}. Two separate experiments were performed with data independently analyzed by NimbleGen and Agilent arrays. The two sets of ChIP-chip results showed that SALL4 binds to the promoter regions of over 1,000 genes (Supplemental Table 2).

Whole genome analysis could potentially yield false positive binding sites; therefore, we validated our results by ChIP-qPCR. We tested 18 genes overall in our current study,

including CCNA1 as a negative control as it did not show any enrichment in our ChIP-chip data. Sixteen genes were enriched for SALL4 binding as predicted by ChIP-chip results, whereas CCNA1 was not enriched for SALL4 binding, also as predicted (Figure 2A). The information on primers used in ChIP-qPCR was summarized in Table 1. Additionally, 37 SALL4 target genes in NB4 and CD34+ cells that have been used for validation were published previously¹⁷. Overall, 94% (52 out of 55 genes) of randomly selected (are they random? I think we eyeballed and picked some that might relevant) genes that we tested showed enrichment. Therefore, the ChIP-chip data could be used with high confidence in subsequent analyses. Examples of data for positive control PTEN and negative control CCNA1 are shown in Supplemental Figure 1.

We then used PANTHER pathway and GO biological function databases (www.pantherdb.com) to analyze the whole genome data. Genes important in apoptosis, hematopoiesis, chromatin modification, nucleic acid metabolism, oncogenesis, differentiation and proliferation were found to be particularly enriched in SALL4 immunoprecipitated DNA from CD34+ cells (p-values<0.05) (Figure 2B&C). Many genes that are related to hematopoiesis, such as PTEN, RUNX1, HOXA9, and CD34 were also found to be potential SALL4 targets in CD34+ cells (Figure 2A).

Using the same approach, we have mapped global gene targets of SALL4 in a human acute promyelocytic leukemic cell line, NB4¹⁷. When SALL4 targets from leukemia NB4 and CD34+ cells were compared, many genes that were regulated by SALL4 in normal HSC/HPC populations could not be found in the datasets from leukemic NB4 cells (Figure 3A). Comparison between the two groups of targets in CD34+ and leukemic NB4 cells yielded some common, but mostly unique SALL4 targets in these cell types. Some different targets in pathways bound by SALL4 in normal and leukemic hematopoiesis included JAK/STAT, p53, NF- κ B and Wnt/ β -catenin (Figure 3B–F). This limited overlap indicates that SALL4 might function in a cell type- or cell context- specific manner. A similar observation has been reported for mouse ES cells and XEN (extraembryonic endoderm) cells, whereby lineage specific Sall4 occupancy of target genes has been observed with less than 25% of Sall4-bound regions common to both cell types¹³.

Down-regulation of SALL4 in normal HSC/HPC affects the expression of CD34 as well as self-renewal and differentiation genes

Next, we chose to test the functional role of SALL4 in normal human CD34+ HSC/HPC by attenuating its expression in these cells. SALL4-specific shRNA (SALL4 shRNA1 and SALL4 shRNA2, Figure 4A) validated by our previous studies¹⁷ were used to explore the effects of SALL4 knock down on normal HSC/HPC CD34+ cells. SALL4 shRNA reduced the expression of SALL4 by up to 65–75% as compared to the control shRNA (Figure 4A). SALL4 shRNA1 (termed as SALL4 shRNA) had a better efficiency in knocking-down SALL4 in CD34+ cells, and was chosen for the subsequent experiments. We then tested whether SALL4 could affect the expression of its target genes identified by ChIP-chip as well as genes involved in self-renewal and differentiation. We used RNA extracted from CD34+ cells 24 and 48 hours post infection. Using qRT-PCR, we measured the expression levels of HSC/HPC marker CD34 as well as genes known to be involved in self-renewal and differentiation. Down-regulation of SALL4 led to decreased expression of CD34, RUNX1, HOXA9, TPO and BMI-1 but increased expression of PTEN (Figure 4B). Notably, CD34, RUNX1, HOXA9, and PTEN were verified to be SALL4 targets in Figure 2A. In addition, genes involved in proliferation and/or apoptosis such as BCL2, P53, FYN, which were previously shown to be SALL4 targets¹⁷ were also affected when SALL4 was down-regulated (Figure 4C). In addition, the effect of SALL4 on non-direct targets was examined (Supplemental Figure 2). This gene expression profiling result suggests that knocking-down

of SALL4 in the CD34⁺ cells may lead to the loss of self-renewal and impairment of differentiation.

Down-regulation of SALL4 in CD34⁺ cells leads to reduced myeloid colony-forming ability *in vitro*

CD34⁺ cells contain both hematopoietic stem and progenitor cells, and the colony-forming unit (CFU) assay is an *in vitro* test of proliferation and/or differentiation ability for hematopoietic progenitors. If down-regulation of SALL4 in normal CD34⁺ cells leads to loss of self-renewal and/or proliferation as well as impairment of differentiation, as expression studies suggested, then the colony forming ability of SALL4-reduced cells could be attenuated. To test our hypothesis, we infected cells with lentiviruses harboring SALL4 shRNA and plated them with cytokines as described in methods. CFUs were counted 7 days or 14 days after plating, and we observed significantly decreased number of colonies from SALL4-reduced CD34⁺ cells, indicating a loss of differentiation and proliferation properties in these cells. Specifically, the numbers of CFU-granulocyte and monocyte (CFU-GM) colonies derived from SALL4-reduced CD34⁺ cells were decreased more than seven folds than those derived from control cells. Furthermore, the number of cells per colony from SALL4-reduced CD34⁺ cells was also fewer than those from the control cells, as indicated by smaller sizes of these colonies, signifying the lack of progenitor cells or the reduced proliferation ability of the cells in these colonies (Figure 5A–C).

Down-regulation of SALL4 in CD34⁺ cells leads to impaired *in vivo* engraftment

To further investigate whether down-regulation of SALL4 would affect hematopoietic differentiation *in vivo*, we performed xenotransplantation assays. 5×10^5 human CD34⁺ BM cells treated with SALL4 shRNA or control shRNA were transplanted into sublethally irradiated (1.0 Gy) NOD/SCID/IL2r γ -null mice (NSG) by tail vein injection. Peripheral blood (PB) and bone marrow (BM) chimerism was monitored at 8 weeks post transplantation. CD34⁺ cells treated with SALL4 shRNA had statistically lower engraftment compared to the control shRNA-treated cells (Figure 5D–E and Supplemental Figure 3). While the CD34⁺ cells treated with control shRNA had an average engraftment of 12.11% (12.11 ± 2.5 , N=7) in the bone marrow and 9.36% (9.36 ± 2.74 , N=7) in the peripheral blood, CD34⁺ cells treated with SALL4 shRNA had an average engraftment of 4.11% (4.11 ± 0.8 , N=10) in the bone marrow and 3.24% (3.24 ± 0.81 , N=10) in the peripheral blood (Figure 5D–E). These observations, taken together with the *in vitro* CFU results, suggest that SALL4 plays an important role in normal hematopoiesis.

In summary, down-regulation of SALL4 in normal CD34⁺ cells led to a loss of stem/progenitor marker CD34 and affected the expression of self-renewal and differentiation genes. This is further supported by decreased *in vitro* myeloid colony forming abilities as well as impaired *in vivo* engraftment of SALL4-knockdown cells. These results suggest that the proper level of SALL4 expression is important for maintaining the balance between self-renewal and differentiation of normal human hematopoietic cells.

Down-regulation of HOXA9 also leads to impaired hematopoietic differentiation, similar to SALL4 knockdown

HOXA9 is critical for normal hematopoiesis^{28–30}. Similar to human SALL4 expression, HOXA9 is enriched in normal CD34⁺CD38[–] stem cells as compared to CD34[–] cells³¹. Giving SALL4 binding is enriched at the HOXA9 promoter from our ChIP results, it is possible that HOXA9 can be a major target gene of SALL4 in normal human hematopoiesis. To test this hypothesis, we chose to knock down SALL4 or HOXA9 in CD34⁺ cells, then perform colony forming assays. The HOXA9 lentiviral shRNA was generated according to a previous report by the Armstrong's group³². For this experiment, we have inserted the

SALL4 shRNA into lentiviral vectors with either a GFP marker or with puromycin resistance for selection. The SALL4 lenti-GFP shRNA and SALL4 lenti-puromycin shRNA had similar efficiency in SALL4 knock down and had the same effect on CD34+ cells. Therefore, we combined the results from the two approaches, and termed them SALL4-shRNA. For the experiments using lenti-GFP vectors, the non-infected cells (GFP negative) cells within the same experiment were used as internal controls. The GFP negative cells showed similar CD11b, CD14 or Glycophorine A expression when compared to those cells infected with viruses (GFP positive) harboring random scrambled shRNA (Supplemental Figure 4). These results indicate that treatment of lentiviruses alone have no effects on the cells.

Both HOXA9 and SALL4 can be down-regulated by 60% using this lentiviral mediated approach (Figure 6A). Either down-regulation of SALL4 or HOXA9 could lead to impaired myeloid differentiation, demonstrated by decreased total and myeloid colony numbers ($p < 0.05$, Figure 6B and C), reduced percentage of myeloid population with the expression of CD11b (Figure 6D and supplemental table 3, $P < 0.05$) and smaller myeloid colonies (Figure 6E). In addition, down-regulation of either gene led to impaired erythroid colony forming abilities (Supplemental Figure 3 and Supplemental Table 3). In summary, SALL4 can bind to the promoter region of HOXA9 (Figure 2A) and affect its expression (Figure 4B). In addition, down-regulation of either gene led to a similar phenotype. These results suggest that SALL4 could directly activate HOXA9 to maintain the differentiation potential of human CD34+ cells.

Discussion

Hematopoiesis consists of a cascade of signaling and transcriptional events whereby pluripotent, self-renewing HSCs give rise to all blood lineages. HSCs can produce and maintain blood cells throughout the lifetime of an organism through a tightly regulated balance of either differentiation divisions that generate functional mature cells, or self-renewal divisions that propagate additional HSCs. It is vital to maintain this balance for the proper function of hematopoietic system, and disruption of the balance between self-renewal and differentiation of HSC in this delicate developmental cascade can lead to leukemic transformation.

SALL4 is an essential stemness factor for ES and XEN cells and can be potentially used for cord blood expansion²². Additionally, we have reported that SALL4 is aberrantly expressed in human AML, and transgenic SALL4B mice develop AML¹⁴. Despite these findings, and although SALL4 is highly expressed in CD34+ human hematopoietic stem/progenitor cells, its functional mechanism(s) in normal human hematopoiesis remains largely unexplored.

Here, we address the question of SALL4 role in normal hematopoiesis by connecting the biological functions of SALL4, the phenotypic and gene expression changes after SALL4 knockdown, with its global target genes identified by ChIP-chip in CD34+ HSC/HPC cells. We have demonstrated that SALL4 plays a key role in hematopoietic differentiation and down-regulation of SALL4 leads to the loss of expression of HSC/HPC marker CD34. The effect of SALL4 on differentiation in normal CD34+ cells is probably due to its ability to regulate multiple downstream target genes that are involved in HSC function and lineage specification. Among genes that are associated with HSC function are PTEN and BMI-1. Both PTEN and BMI-1 are important for self-renewal of HSCs^{19,21}. We have successfully shown that down-regulation of SALL4 leads to up-regulation of PTEN and down-regulation of BMI-1 (Figure 4B), and both have been confirmed to be a direct SALL4 target in CD34+ cells (Figure 2A for PTEN and our previous publication¹⁷ for BMI-1). Among the hematopoietic differentiation genes, HOXA9 seems to be a major down-stream target of

SALL4. SALL4 can bind to the promoter region of HOXA9 in CD34⁺ cells, and down-regulation of SALL4 leads to decreased HOXA9 expression in these cells (Figure 2A and Figure 4B). Furthermore, knocking-down of either gene can lead to a similar phenotype of impaired hematopoietic proliferation and differentiation (Figure 6, Supplemental Figure 5 and Table 3).

We have also compared the global target genes of SALL4 in normal CD34⁺ cells versus leukemic populations. Only limited number of overlapping SALL4 direct target genes were found in the two cell types. In addition, the down-regulation of SALL4 has a differential effect on some of these target genes, especial genes involved in pro- and anti-apoptotic pathways. Among these genes, TRO, ABL1 and BCL2 were differentially affected by SALL4 in leukemic versus normal CD34⁺ cells. In leukemic cells, when SALL4 was down-regulated, along with the apoptotic phenotype, the expression of TRO and ABL1 was increased while the expression of BCL2 was decreased¹⁷. In CD34⁺ cells, while there was no notable apoptosis when SALL4 was down-regulated in these cells, the expression of BCL2 was increased in SALL4-reduced CD34⁺ cells, and the expression of apoptosis induction genes TRO and ABL1 was decreased (Figure 4C). This group of studies suggests that SALL4-mediated function is cell context-dependent. This is particular important to keep in mind if we want to target SALL4 in treating leukemia without the cost of damage on normal hematopoiesis.

In summary, by correlating the biological functions of SALL4 and its global target genes in normal human hematopoietic systems, we have demonstrated that SALL4 plays a key role in hematopoietic differentiation, and the major functional role of SALL4 in normal hematopoiesis is to maintain the stem/progenitor cells in an undifferentiated stage with self-renewal capacity. We have further demonstrated that HOXA9 is a main target of SALL4 in normal hematopoiesis. Future study will determine whether modulating the SALL4/HOXA9 pathway can be used in cellular therapy such as cord blood expansion, HSC/HPC engraftment, and myeloid engraftment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1A **Figure 1B** **Figure 1. SALL4 expression in normal human hematopoietic cells**

(A) SALL4 protein expression in normal CD34+ and CD34- cells examined by confocal immunofluorescent staining. Normal bone marrow cells were sorted into CD34+ and CD34- fractions. SALL4 expression is in green, and the nuclear staining by TO-PRO-3 is in blue. Merged images showed that nuclear expression of SALL4 was only detected in the CD34+ HSC/HPCs, but not in the CD34- cells. (B) SALL4 RNA expression in normal CD34+ and CD34- cells examined by qRT-PCR. CD34+ cells were sorted into CD34+CD38-, CD34+CD38+, and CD34- fractions. Quantification of SALL4A and B mRNA levels normalized to GAPDH, and showed that both SALL4 isoforms were expressed preferentially in CD34+CD38- HSCs, but down-regulated in normal CD34+CD38+ HPCs, and not present in the CD34- cells (N=5 Error Bars: +/- SE). Y-axis: SALL4A or B relative expression.

Figure 2. Identification of potential SALL4 direct target genes in normal CD34+ cells by genome-wide chromatin immunoprecipitation (ChIP)-chip

(A) Validation of ChIP-ChIP results. 16 genes known to play a role in hematopoiesis with various ChIP-chip score were chosen for validation, with CCNA1 as a negative control. Fold enrichment of above 1.5 compared to input (no immunoprecipitation added) after GAPDH normalization was considered positive. ChIP-qPCR was performed with two biological replicates with each biological sample being done as triplicates. The results shown were the average data. (B) Gene ontology categories of SALL4 bound targets in CD34+ cells. A total of 1160 genes were found to be bound by SALL4 in CD34+ HPC/HSCs through ChIP-chip genome-wide analysis. Protein Analysis Through Evolutionary Relationships (PANTHER), a web-based software, was used to analyze the dataset. (C) GO function categories as defined by Ingenuity. The two percentages behind the gene numbers represent: in green: genes in this particular category bound by SALL4 relative to the total number of SALL4-bound gene in CD34+ cells; in red, total number of genes defined by this category relative to total number of genes represented on the array.

Figure 3. comparison of SALL4 targets in normal CD34+ cells versus leukemic cells
 (A) NB4 AML cell line and CD34+ cells have 258 SALL4-bound targets in common (CD34+ cells = 18.2%; leukemic cells = 9.8%). SALL4-bound genes in (B) P53 pathways, (C) NF-kB pathway, (D) Wnt/beta-catenin pathway, (E) FGF pathway and (F) JAK/STAT pathways in NB4 and CD34+ cells.

Figure 4. Down-regulation of SALL4 in normal CD34⁺ cells affects genes involved in differentiation and self-renewal/proliferation

(A) The abilities of the two shRNA constructs to knock down SALL4 in CD34⁺ cells were confirmed by qRT-PCR with 25–35% knockdown efficiency compared to the scrambled pRS control vectors (N=3, Error Bars: $\pm$ SD). (B) Gene expression changes upon the down-regulation of SALL4 for HSC/HPC genes CD34, and RUNX1, HOXA9, GATA-1, TPO and PTEN. (C) Gene expression changes upon SALL4 downregulation for proliferation, anti- and pro-apoptosis genes ABL1, TRO, BCL2, FYN, ATF3, MAP3K12, p53, CUL3 and TNF. Fold enrichment was calculated after normalization of both control and SALL4 knock down samples to GAPDH (N>3, Error Bars: $\pm$ SD). * P<0.05

Figure 5. Impaired colony forming abilities in vitro and engraftment in vivo upon SALL4 knock down

Colony forming unit (CFU) assays were performed on CD34⁺ cells infected with control vector and SALL4 shRNA retroviruses that were then plated on methylcellulose supplemented with myeloid-differentiation inducing cytokines for 7–14 days. **(A)** The total number of all colonies when CD34⁺ cells were infected with SALL4 shRNA, and the number and size of CFU-GM **(B&C)** in SALL4-reduced group when compared to those that were infected with the control vectors. 5×10^5 of human CD34⁺ BM cells infected with either SALL4 shRNA or control shRNA retroviruses were transplanted into sublethally irradiated (1.0 Gy) NOD/SCID/IL2 γ -null mice by tail vein injection. Peripheral blood (PB)

(D) and bone marrow (BM) (E) Chimerisms were monitored at 8 weeks post transplantation. CD34 cells with reduced SALL4 expression (n=10) had statistically lower engraftment than control cells (n=7). P-values are listed as indicated.

Figure 6. SALL4 and HOXA9 shares similar effects on myeloid differentiation in human primary CD34⁺ cells

Purified CD34⁺ cells were either infected with scrambled shRNA or shRNAs against SALL4, HOXA9, then cultured in methylcellulose medium supplemented with SCF, IL-3, GM-CSF and EPO, with 1 μ g/ml puromycin for selection. **(A)** Down-regulation of SALL4 or HOXA9 mRNA expression level to 35–40% of those treated with scrambled controls (N=3, Error Bars: $\pm$ SD), evaluated by qRT-PCR. **(B)** Total colony number and **(C)** myeloid colony number on CFU plates were scored on day 14. The numbers of colonies formed by scrambled viral infected cells is set at 100%. Data depict average and standard deviation of 3 independent experiments. The P value was obtained by comparing to the control using a paired two-tailed distribution t-test $**p < 0.01$. **(D)** Flow cytometric analysis of myeloid differentiation of cells harvested from the CFU plates. The level of CD11b expression was plotted on histograms in comparison to the control. **(E)** Morphology of CFU colonies after treatment with scrambled control and SALL4 or HOXA9 knock down (10x).