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Sall4 overexpression blocks murine hematopoiesis in a dose-dependent manner

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Abstract

Sall4 is a transcription factor that exists in two splice isoforms – Sall4a and Sall4b – and regulates transcription in embryonic stem cells, hematopoiesis and acute myeloid leukemia. Constitutive overexpression of Sall4 in mice induces acute myeloid leukemia. Interestingly, a potential benefit of using Sall4 to facilitate *ex vivo* hematopoietic stem cell expansion has been proposed. However, distinct roles for how Sall4 contributes to normal versus malignant processes remain undefined. Here we show that Sall4b is the predominant isoform in murine hematopoietic stem cells and progenitors. Overexpression of either Sall4 isoform in HSCs or progenitors impairs hematopoietic colony formation and expansion *in vitro*. Lineage negative bone marrow overexpressing Sall4b fails to engraft and reconstitute hematopoiesis when transplanted. We found that both Sall4a and Sall4b overexpression impair hematopoiesis in part through dose-dependent repression of Bmi1. Additionally we have identified the following potential novel Sall4 target genes in hematopoiesis: Arid5b (Sall4a and Sall4b), Ezh2 and Klf2 (Sall4a). Lastly, we found that Sall4 expression is

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variable in acute myeloid leukemia, ranging from no expression to levels comparable to embryonic stem cells. These results show that Sall4 isoforms contribute to only a subset of acute myeloid leukemia and that overexpression of Sall4 isoforms impairs hematopoiesis through repression of Bmi1. Together these data demonstrate the sensitivity of hematopoiesis to appropriately balanced Sall4 expression, highlighting the importance of regulating this dynamic in potential therapeutic applications such as *ex vivo* stem cell expansion.

Keywords

hematopoietic stem cells; transcriptional regulation; Sall4; acute myeloid leukemia

Introduction

Sall4 is a highly conserved C2H2 zinc finger transcription factor that exists in two isoforms (long: Sall4a and short: Sall4b) generated by an internal splicing event in exon two [1, 2]. Sall4 is homologous to the *Drosophila spalt* gene and interacts with a core stem cell network to maintain pluripotency and self-renewal in embryonic stem cells (ESCs) [1]. Sall4 plays a similar role in normal hematopoiesis by promoting self-renewal and inhibiting differentiation of hematopoietic stem cells (HSCs) [3]. Expression of Sall4 has been demonstrated in acute myeloid leukemia (AML) samples and overexpression of Sall4b in a transgenic mouse model leads to the development of myelodysplastic syndrome and AML [4]. Interestingly, forced expression of Sall4 *ex vivo* has been proposed as a means of HSC expansion prior to bone marrow transplantation [5–7]. Naturally, delineating how Sall4 regulates normal self-renewal and differentiation in HSCs from its participation in malignant transformation is of paramount importance before Sall4 can be safely utilized for therapeutic purposes such as *ex vivo* HSC expansion.

Traditionally, it was felt that transcription factors regulate gene expression by binding DNA to exert either “on” (activation) or “off” (suppression) effects on gene transcription. Recent research increasingly suggests that transcription factors regulate gene expression in a much more dynamic fashion [8]. Transcriptional regulation exists on a continuum where variable levels and combinations of transcription factor expression may impart a range of cell fate determinations. This body of research has demonstrated that certain transcription factors may have paradoxical effects on self-renewal, proliferation and/or differentiation in a cell-specific and dose-dependent manner. For example, RUNX1 is essential for normal hematopoiesis and influences myeloid differentiation [9]. Inactivating mutations of RUNX1 are commonly seen in myeloid neoplasms such as AML and myelodysplastic syndrome [10]. It was recently demonstrated that both forced overexpression of RUNX1 as well as knockout of RUNX1 inhibits the growth of human AML cells [11]. These studies show that both normal hematopoietic cells and leukemia cells require RUNX1 for proliferation and growth, but the level of RUNX1 is important in contributing to normal hematopoietic differentiation versus malignant transformation. Alterations in the expression of PU.1 contribute to a similar situation in hematopoiesis, where low level PU.1 influences B-cell lymphoid development, higher expression leads to myeloid differentiation, while complete ablation of PU.1 is detrimental to hematopoiesis [12–14]. Thus transcriptional regulation

governing self-renewal and differentiation depends on tightly regulated and appropriately balanced levels of transcription factor expression.

The properties attributed to Sall4 in hematopoiesis ranging from transcriptional regulation of normal hematopoiesis, to a modality for *ex vivo* HSC expansion to leukemic oncogene, lead us to question whether there were isoform-specific or dose-dependent effects of Sall4 between normal and malignant hematopoiesis. Here, we utilize retroviral gene transduction to study the effects of forced overexpression of Sall4 isoforms on murine hematopoiesis. We found that overexpression of individual Sall4 isoforms neither enhanced self-renewal nor proliferation in LSK cells nor in Lin⁻ bone marrow. Rather, Sall4 overexpression leads to a dose-dependent repression of Bmi1, impairing hematopoiesis. Additionally, primary AML patient samples have a low to intermediate degree of Sall4 expression, implying that Sall4 overexpression is only tolerated in a subset of AML, likely in concert with additional permissive mutations. Our study demonstrates that hematopoiesis is sensitive to the level of Sall4. Studies such as ours highlight the importance of quantifying the level of gene expression for therapeutic applications such as HSC expansion.

Methods

All experiments involving mice were performed according to an animal use application protocol approved by the institutional animal care and use committee of the Medical College of Wisconsin. AML specimens from pediatric patients at Children's Hospital of Wisconsin (CHW), Milwaukee, WI, were collected at the time of diagnosis or at relapse. Written informed consent for cryopreservation of leukemia specimens was obtained according to CHW IRB protocol. Prior to removal of specimens from cryo-storage for this study, we obtained IRB approval to access samples for proposed analyses.

Identification of HSCs and progenitors

Bone marrow was obtained by flushing the tibiae and femurs of four to eight week old C57BL/6 mice. HSCs and progenitors were defined by surface protein expression as follows: 1) HSCs: Lin⁻, Sca1⁺, c-Kit⁺, Flk-2⁻, CD34^{lo-hi} 2) Lineage-negative, c-Kit-positive, Sca1-positive (LSK): Lin⁻, Sca1⁺, c-Kit⁺ 3) Common Myeloid Progenitor (CMP): Lin⁻, IL-7R⁻, Sca-1⁻, c-Kit⁺, FcγRII/III^{lo}, CD34⁺ 4) Granulocyte-Macrophage Progenitor (GMP): Lin⁻, IL-7R⁻, Sca-1⁻, c-Kit⁺, FcγRII/III^{hi}, CD34⁺ 5) Megakaryocyte-Erythrocyte Progenitor (MEP): Lin⁻, IL-7R⁻, Sca-1⁻, c-Kit⁺, FcγRII/III^{lo}, CD34⁻; and collected for downstream applications using fluorescence activated cell sorting (FACS) [15, 16].

Quantitative real time PCR (RT-qPCR)

RNA was extracted from bone marrow, progenitors, or patient samples using Trizol and purified using the Qiagen RNeasy microkit. Taq-polymerase primer probe assays were constructed to identify murine Sall4a, murine Sall4b, human Sall4a and human Sall4b (Table S1). Housekeeping genes murine beta-actin and human GAPDH were used for normalization controls (Table S1). Gene expression was normalized to actin (mouse) or GAPDH (human) and relative expression calculated using the Ct method [17].

Retroviral gene transduction

Sall4a or Sall4b plasmids [1] were cloned into the murine stem cell virus (MSCV) vector with a GFP reporter. 6-well plates were treated with retronectin solution and preloaded with Iscove's Modified Dulbecco's Medium (IMDM) containing 3×10^6 viral particles and centrifuged at 2,000 rpm for 60 minutes. Viral media was aspirated and wells were loaded with up to 1×10^6 Lin⁻ bone marrow, or LSK cells in fresh IMDM containing an additional 3×10^6 viral particles, 15% fetal bovine serum (FBS), cytokines IL-3, IL-6, SCF and polybrene. Spin-infection was performed at 2,000 rpm for 90 minutes followed by 6–8 hour incubation in viral media at 37°C. Following incubation, cells were replated in fresh media and incubated for 36–40 hours prior to collection for downstream applications.

Methylcellulose colony forming assays

Cells were sorted 48 hours post-infection for GFP expression and incubated at 1,000–20,000 cells/ml in methylcellulose media containing cytokines IL-3, IL-6 and SCF. Following morphologic assessment and colony counts, plates were flushed with HBSS containing 2% FBS and replated every 7–10 days out to four generations.

Murine bone marrow transplants

Syngeneic mice were irradiated with either 600 cGy (sublethal) or 900–1100 cGy (lethal) using a cesium¹³⁷ irradiator. Four hours after irradiation, 7.5×10^4 to 5.0×10^5 GFP-positive, viable MSCV-transduced cells were collected by FACS and infused retroorbitally. Mice were monitored daily and peripheral blood was obtained every other week. Moribund mice were euthanized and bone marrow was harvested for assessment of morphology and gene expression.

Primary patient samples

Peripheral blood or bone marrow specimens from pediatric patients diagnosed with AML were obtained as described above. RNA extraction and cDNA conversion for gene expression analyses were performed as above. De-identified healthy donor discard material was used for normal controls.

Results

Sall4b is the predominant Sall4 isoform in murine hematopoiesis

Sall4 expression was measured in HSCs and hematopoietic progenitors by RT-qPCR and quantified relative to murine ESCs (Figure 1). We chose to calibrate expression relative to ESCs because both isoforms of Sall4 are expressed and are critical for self-renewal and pluripotency in ESCs [1, 2]. Sall4b was the predominant Sall4 isoform in HSCs and progenitors while Sall4a expression was minimal or absent across all cell populations tested (p-value 0.03) implying that Sall4b is the hematopoietic-specific Sall4 isoform in mice.

Sall4 overexpression impairs normal hematopoiesis

To study the effects of forced Sall4 expression on hematopoiesis, Lin⁻ bone marrow was transduced using an MSCV retroviral construct to express either Sall4a or Sall4b and

cultured in methylcellulose or transplanted into syngeneic mice. Overexpression of appropriate Sall4 isoforms 48 hours post-transduction was demonstrated by RT-qPCR (Figure S1A). Protein synthesis of Sall4 isoforms was confirmed by Western blot (Figure S1B). We serially replated transduced Lin⁻ bone marrow and controls in methylcellulose every 7–10 days out to four generations. Lin⁻ bone marrow transduced with the MLL-AF9 oncogene was included as a positive control for enhanced self-renewal (Figure 2). This serial plating approach allowed us to assess long-term effects of Sall4 overexpression on *in vitro* hematopoiesis [18, 19]. Sall4a and Sall4b overexpression impaired colony formation in primary plating, with diminished recovery in the number of viable cells through secondary plating (Figure 3A, B). By the fourth round of plating there was no difference in colony formation or proliferation between Sall4a and Sall4b overexpression groups and empty vector. However, there remained subjective differences in the morphology of the Sall4a and Sall4b transduced colonies; with Sall4 colonies tending to consist of small, clumped clusters of cells, compared to much more loosely dispersed cells seen in the negative controls (Figure 2).

Because Lin⁻ bone marrow contains a heterogeneous group of cells (largely progenitors and very few HSCs) we evaluated the %LSK cells transduced to assess whether the observed phenotype was due to differences in HSC-targeting. We found that within the GFP⁺ fraction of cells in each group, the %LSK was similar between our Vector (2.4%), Sall4a (1.6%) and Sall4b (3.9%) transduced cells (Supplemental Figure S3). Next, we performed similar overexpression experiments in purified LSK cells to examine HSC-specific effects of Sall4 overexpression. As expected, LSK cells had increased colony forming and proliferative capacity compared to Lin⁻ bone marrow (5–10 fold, Figure 3). Yet, similar to Lin⁻ bone marrow, both Sall4a- and Sall4b-overexpression impaired colony formation and proliferation in LSK cells compared to empty-vector transduced cells (Figure 3C, D). Collectively, this *in vitro* phenotype over serial platings in both Lin⁻ bone marrow and LSK cells shows an impairment of self-renewal and proliferation following Sall4a or Sall4b overexpression.

Given our findings of Sall4b specificity in hematopoiesis and similar *in vitro* phenotypes with overexpression of either Sall4 isoform, we chose to focus on Sall4b overexpression for *in vivo* experiments using a syngeneic mouse hematopoietic cell transplant model [20]. C57/BL6 mice conditioned with sublethal irradiation (600cGy) showed minimal engraftment when transplanted with Sall4b-transduced Lin⁻ bone marrow (measured as peripheral leukocyte GFP expression) any GFP expression was lost by four weeks post-transplant (Figure 4A, N=5). Mice undergoing transplantation with empty vector transduced Lin⁻ bone marrow after sublethal irradiation showed low-level GFP expression out to six weeks post-transplant (Figure 4A, N=5). As positive controls, mice transplanted with MLL-AF9 transduced bone marrow developed acute leukemia three months post-transplant (2/2 mice, Figure S2A). All five mice transplanted with Sall4b transduced Lin⁻ bone marrow following lethal irradiation (900–1100 cGy) failed to engraft and succumbed to bone marrow failure 9–11 days post-transplant (Figure 4B). Four of five mice receiving empty-vector transduced Lin⁻ bone marrow following lethal irradiation demonstrated reconstitution of normal hematopoiesis with persistent GFP expression at 30 days post-transplant (Figure

4B). Together these transplantation experiments confirm our *in vitro* findings that Sall4b overexpression is detrimental to normal hematopoietic progenitors and stem cells, impairing engraftment and/or hematopoiesis.

Sall4 isoforms exert both overlapping and isoform-specific effects on hematopoietic target genes

To evaluate whether our findings were due to induction of apoptosis secondary to ectopic overexpression of Sall4a or Sall4b we measured Annexin V staining. We found no difference in Annexin V positivity at 48 hours post-infection between Vector- (31%), Sall4a- (44%) or Sall4b- (36%) transduced cells (N=3). To assess the mechanism(s) by which Sall4 overexpression may impair hematopoiesis, we reviewed the literature for Sall4 target genes in hematopoiesis or leukemogenesis. Bmi1, Pten and Meis1 have been implicated as Sall4 target genes that mediate its hematopoietic effects [21–23]. To identify potential novel targets of Sall4 in hematopoiesis we analyzed our previous isoform-specific chromatin immunoprecipitation – genome-wide location analysis (ChIP-chip) dataset of Sall4 binding sites in ESCs [1]. This dataset identified three additional DNA-binding sites in ESCs localized to promoters for genes associated with hematopoiesis and/or leukemogenesis: Arid5b (Sall4b), Klf2 (Sall4a and Sall4b), and Ezh2 (Sall4b) [24–28].

RT-qPCR analysis 48 hours post-transduction showed that Bmi1 was decreased in both Sall4a and Sall4b transduced Lin⁻ bone marrow and LSK cells (Figure 5). Interestingly, these results directly contradict the reported positive correlation between Sall4 and Bmi1 in hematopoiesis [6, 22, 29]. Pten was modestly decreased by both Sall4a and Sall4b, in keeping with previous reports that also showed modest repression of Pten by Sall4 [23]. Both Sall4a and Sall4b overexpression were associated with significantly decreased Arid5b expression. For Meis1 we observed isoform specificity with increased expression of Meis1 in Sall4b-transduced cells, while Sall4a overexpression was associated with no change in expression. We also observed isoform specific effects on Klf2 and Ezh2 expression; Sall4a overexpression was associated with increased expression of Klf2 and decreased expression of Ezh2 while Sall4b overexpression did not alter Klf2 or Ezh2 expression. To validate the previously reported Sall4-Bmi1 interaction [22], and confirm that Sall4 binds to promoter regions of Ezh2, Klf2 and Arid5b, we performed chromatin immunoprecipitation followed by quantitative PCR (ChIP – qPCR) using ESCs expressing biotinylated Sall4a or Sall4b [1]. ChIP – qPCR confirmed that Sall4 bound Bmi1 at the predicted region 450bp 5' upstream of the start codon [22]. We also confirmed Sall4a and Sall4b bound to promoters for Ezh2, Klf2, Arid5b and an additional predicted Bmi1 promoter in murine ESCs (Figure S4).

In summary, Sall4a and Sall4b overexpression are associated with both overlapping (Bmi1, Arid5b, Pten) and distinct (Meis1, Klf2, Ezh2) differences in expression levels of target genes implicated in hematopoiesis and/or leukemogenesis. Bmi1 is a member of Polycomb Repressive Complex 1 and is a critical regulator of stem cell self-renewal in both HSCs and LSCs [30]. Therefore, repression of Bmi1 would be expected to be detrimental to hematopoiesis, consistent with our observed phenotype.

Sall4 repression of Bmi1 is dose-dependent

Because we achieved robust overexpression of Sall4a and Sall4b, we sought to examine whether Bmi1 repression correlated with the level of Sall4 overexpression. In our retroviral construct Sall4 expression should correlate with GFP fluorescent intensity [12]. Retroviral transduced cells were sorted into GFP-high, medium, or low fractions 48 hours post-infection (Figure S5). Indeed, the level of Sall4a and Sall4b overexpression correlated with the level of GFP expression (Figure 6). Bmi1 expression was inversely correlated with Sall4a or Sall4b overexpression; Sall4a and Sall4b-low cells had no significant change in Bmi1, whereas in Sall4a and Sall4b-high cells, Bmi1 expression was significantly decreased compared to cells infected with empty vector alone (Figure 6).

To evaluate whether the level of Sall4 expression influenced hematopoiesis, we cultured Sall4a and Sall4b GFP-low, -medium or -high cells *in vitro* in methylcellulose as above. Impairment in colony formation and fold expansion trended towards an inverse correlation with the level of Sall4a or Sall4b overexpression. Sall4a- or Sall4b-medium and high expressing cells had lower colony formation and proliferation than Sall4a- or Sall4b-low or Vector-only cells (Figure 7A, B). Importantly these Sall4a- and Sall4b-low cells did not exhibit augmented self-renewal *in vitro*, in contrast to reports of others [4–6]. In summary, Bmi1 expression is inversely correlated with Sall4 overexpression. The level of Sall4a or Sall4b overexpression correlates with the severity of hematopoietic impairment *in vitro*.

Sall4 expression is variable in pediatric AML

Sall4 has been implicated in adult AML cases, and level of Sall4 expression correlates with outcome in hepatocellular carcinoma [4, 31]. The impaired self-renewal and proliferation observed in our *in vitro* and *in vivo* overexpression studies lead us to question whether Sall4 was truly ubiquitously expressed in AML as previously reported [4]. If Sall4 expression is variable in AML, then Sall4 may serve as a marker to aid in risk stratification in pediatric AML as has been proposed in hepatocellular carcinoma [31]. To our knowledge, Sall4 expression has not been assessed in pediatric AML, nor is not known whether there is differential expression of Sall4 isoforms in pediatric AML. Sall4a and Sall4b expression were assessed in seven pediatric AML samples by RT-qPCR (Figure 8). Patient characteristics are summarized in Table S3. For gene expression analyses, human pluripotent stem cells (hPSCs) served as a positive control; human cord blood, mobilized peripheral blood, whole bone marrow and peripheral blood were included for comparison of normal hematopoietic cells. Sall4a and Sall4b were detected in cord blood and whole bone marrow samples at low levels (less than 5% of hPSCs), but in neither peripheral blood nor mobilized peripheral blood stem cells (undetectable to 0.3% of hPSC expression). There was highly variable expression of both Sall4 isoforms across AML specimens, though in those samples that expressed Sall4 both Sall4a and Sall4b were expressed at levels comparable to hPSCs. Additionally, Sall4 expression was consistent at both initial diagnosis and relapse in the two patients with paired diagnostic and relapse samples available to study (AML-2&2R, 3&3R).

To examine Sall4 expression in murine AML, we transformed Lin- bone marrow with the MLL-AF9 fusion oncogene. Consistent with published literature, these transformed cells

formed tumor sphere-like colonies with enhanced self-renewal and proliferation *in vitro* and lead to the development of AML *in vivo* when transplanted (Figures 2, S2) [20, 32]. Sall4 was not detectable by RT-qPCR in bone marrow from mice that developed leukemia following MLL-AF9 transplant nor in MLL-AF9 transformed Lin⁻ bone marrow cultured *in vitro* and assessed at 48 hours post-infection and weekly out to 4 weeks. To study a second model of AML we transduced Lin⁻ bone marrow with the AML1-ETO fusion gene [33] and cultured in methylcellulose until they exhibited augmented self-renewal and proliferation (7–10 days). Similar to MLL-AF9, Sall4a or Sall4b were undetectable by RT-qPCR in the AML1-ETO transduced cells as well. This variable pattern of expression across AML suggests that Sall4 plays a role in only a subset of AML.

Discussion

Evaluation of endogenous Sall4 expression in murine hematopoietic stem cells and progenitors showed that Sall4b is the predominant isoform in murine hematopoiesis (Figure 1). This is in direct contrast to ESCs, where Sall4a expression is approximately 3–4 times higher than Sall4b [1]. Furthermore, Sall4 was not detected in whole bone marrow or peripheral blood, demonstrating that its expression is limited to those cells that have not yet fully differentiated and retain a degree of self-renewal (HSCs and progenitors). This suggests that splicing may regulate tissue-type expression of Sall4 and implies that Sall4b, but not Sall4a, influences murine hematopoiesis during development.

We have demonstrated that overexpression of Sall4a or Sall4b neither enhances self-renewal in HSCs (LSK cells) nor does it impart stem cell-like properties or leukemic transformation in progenitors (Lin⁻ bone marrow, Figures 3, 4). Moreover, there were persistent qualitative differences in the morphology of the Sall4a and Sall4b transduced colonies, with Sall4 overexpression associated with small, clustered colonies (Figure 2). Results of our transplantation studies are consistent with Sall4 overexpression leading to engraftment failure, which future experiments will need to address. Collectively, this suggests that hematopoiesis is sensitive to the level of Sall4 expression. Marked overexpression of individual Sall4 isoforms upsets this balance and effectively shuts down normal differentiation and self-renewal. This is in keeping with results that others have shown in ESCs, where both knockdown and overexpression of the key stem cell regulatory factor Oct4 can induce differentiation and loss of self-renewal [34–36]. And in hematopoiesis, where key regulatory genes PU.1 and Runx1 exhibit varied effects on self-renewal, differentiation and proliferation in a dose-dependent and context-specific fashion [11, 12, 37, 38]. In fact, in ESCs, Sall4 depletion induces differentiation [1], while overexpression causes cell death and/or cell-cycle arrest (data not shown).

Our results contrast previous reports in which Sall4 overexpression leads to enhanced self-renewal and *ex vivo* expansion of HSCs, and causes leukemia in mice [4–6]. One difference between our study and these previous reports are the methods of gene transduction used to overexpress Sall4. We achieved significant overexpression of Sall4 utilizing MSCV retroviral transduction, a classic method for studying the effects of genetic alterations in hematopoiesis. In contrast, other groups' over-expression experiments have utilized lentiviral transduction, TAT-conjugated recombinant protein, and a ubiquitous CMV-

promoter driven transgenic mouse, but did not quantify level of overexpression relative to a cell type that normally expresses Sall4a and Sall4b. Making a direct comparison of Sall4a/b expression levels between the studies impossible. One consideration then must be the degree of overexpression achieved by the differing methods. In our study we achieved robust Sall4a and Sall4b overexpression and both demonstrated a similar phenotype and decrease in colony formation (Figures 2, 3). Though we observed less significant impairment of proliferation and colony formation in the Sall4a- and Sall4b-low expressing groups compared to the high-expressing groups *in vitro*, these cells still had diminished proliferation and colony formation and did not exhibit enhanced self-renewal or proliferation (Figure 7). Given our findings, future experiments studying hematopoietic cell expansion must address the level of Sall4 expression achieved. Based on our experiments in LSK populations and in sorted Sall4-low, -medium and -high expressing Lin- populations, we argue that overexpression of individual Sall4 isoforms, especially at high levels, is unlikely to be a clinically useful strategy for *ex-vivo* stem cell expansion.

Our data in human hematopoietic cells and AML samples showed low level expression of both Sall4a and Sall4b in cord blood and whole bone marrow, with no detectable Sall4 in mobilized peripheral blood and whole blood (Figure 8). Sall4a and Sall4b were variably expressed in AML; however, the degree of Sall4 expression observed was comparable to hPSCs. This variable expression in AML is in contrast to the initial published report evaluating Sall4 expression in AML patient samples and cell lines [4]. A closer look reveals that this difference is due in part to how PCR data is analyzed and reported. In our study we calibrated expression relative to a cell known to express Sall4 (hPSCs) and graphed expression on a linear scale. Additionally, Sall4 was not expressed following MLL-AF9 nor AML1-ETO leukemic transformation of murine Lin- bone marrow. This suggests that only a subset of AML tolerates higher levels of Sall4, likely due to the presence of a permissive set of secondary mutations. In a 2008 publication, Yang et al. showed that shRNA-mediated knockdown of Sall4 in an AML cell line (NB4) impaired proliferation and induced apoptosis [21]. This showed that at least in a subset of AML, Sall4 isoforms are required for leukemic growth. The variable expression pattern across pediatric AML and in established murine AML models suggests that Sall4 plays a role in leukemogenic self-renewal, differentiation arrest and/or proliferation in only a subset of leukemia, presumably in the context of additional, permissive mutations. Thus, it may be that low levels of Sall4 are required in both normal hematopoiesis and a subset of leukemias, and therefore warrants further investigation. In cord blood, bone marrow and those AML samples that expressed Sall4, we did not observe the same Sall4b predominance we noted in murine hematopoiesis, rather both Sall4a and Sall4b were uniformly co-expressed. Perhaps Sall4a and Sall4b cooperate in human hematopoiesis compared to the hematopoietic specificity of Sall4b we observed in mice. Further studies will be undertaken to better elucidate Sall4 isoform-specific roles in human hematopoiesis and leukemogenesis as well as the evaluation of the coexistent genetic and epigenetic landscape in Sall4-expressing versus Sall4 non-expressing AML.

Our data, using normal bone marrow rather than cell lines or reporter systems, shows that a high degree of Sall4 overexpression suppresses Bmi1, which contradicts the reported positive correlation of Sall4 on Bmi1 expression in models where Sall4 was associated with

enhanced self-renewal [6, 22, 29]. Bmi1 is a member of Polycomb Repressive Complex 1, and has been shown to be a critical regulator of hematopoiesis and leukemopoiesis [30, 39, 40]. Bmi1^{-/-} mice are unable to sustain normal hematopoiesis beyond the fetal liver state due to a loss of self-renewal and Bmi1^{-/-} hematopoietic progenitors have greatly reduced potential to undergo leukemic transformation [30]. While our results contradict published data on the relationship between Sall4 and Bmi1, they are entirely in keeping with our observed phenotype of impaired hematopoiesis [6, 22]. Sall4a or Sall4b may be both an activator and repressor of different genes in ESCs: therefore taking our results and those of previous reports into account implies that Sall4 may act as either an activator or repressor of Bmi1 in a dose-dependent fashion in hematopoiesis as well. Collectively, this suggests that there is a threshold of Sall4 expression, above which Bmi1 is repressed and proliferation is impaired (Figure 9).

Lastly, we have identified three novel genes bound by Sall4 in ESCs through which Sall4 may influence hematopoiesis. Sall4a overexpression was associated with an increase in Kruppel-like transcription factor 2 (Klf2) and decreased expression of the Polycomb Repressive Complex 2 gene Ezh2, both of which are involved in AML [26, 28]. Both Sall4a and Sall4b overexpression were associated with marked decrease in Arid5b (Figure 5). Germline single nucleotide polymorphisms in Arid5b have been associated with increased risk of pediatric acute lymphoblastic leukemia [24, 25]. However, potential biologic mechanisms of this association are unknown and further investigation into the role of Arid5b in leukemogenesis is needed.

Collectively, we have shown that retroviral overexpression of individual Sall4 isoforms did not lead to advantages in self-renewal or proliferation in neither HSC-enriched LSK nor progenitor-enriched Lin⁻ bone marrow populations, as would be expected with HSC expansion or malignant transformation. Rather, Sall4 overexpression in our model repressed Bmi1 (Figure 5) impairing self-renewal and proliferation (Figures 3, 4). Only a subset of AML cases demonstrated an intermediate level of expression of both Sall4 isoforms (Figure 8). The variable expression of Sall4 in AML suggests that it is not a ubiquitous driver oncogene in AML. Rather, Sall4 plays a role in a subset of patients, and likely exists alongside permissive secondary mutations that allow these leukemias to tolerate higher levels of Sall4. Because significant overexpression of Sall4 by itself is detrimental to hematopoiesis *in vitro* and fails to engraft when transplanted, future experiments will need to address whether there exists a cooperative or dose-dependent role for Sall4 isoforms in hematopoiesis and/or leukemogenesis. Regardless, our model shows that care must be taken to closely regulate the level of gene expression in systems proposing to utilize *ex vivo* genetic manipulation for therapeutic purposes such as HSC expansion or regenerative medicine. Given the variety of properties ascribed to Sall4, ranging from potent oncogene to potential safe means of hematopoietic stem cell expansion, future studies carefully dissecting quantitative, context-dependent and isoform-specific differences in expression are imperative to truly determine its role in normal versus malignant hematopoiesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Sall4 overexpression in murine HSCs and progenitors impairs self-renewal and proliferation.
- Sall4 overexpression represses Bmi1 in a dose dependent manner.
- Sall4 expression is highly variable in pediatric acute myeloid leukemia.
- Sall4's hematopoietic effects are both dose- and context-dependent.

Sall4 Expression in Murine HSCs and Progenitors

Figure 1. Endogenous Sall4 expression in murine hematopoietic progenitors

Sall4a and Sall4b expression were assessed in HSCs, LSKs, CMPs, GMPs, and MEPs by RT-qPCR. Sall4b, but not Sall4a, is expressed in hematopoietic progenitors at levels similar to ESCs. Samples from biologic replicates (HSC n=2, LSK n=3, MEP, CMP, GMP n=4). Values represent mean $\pm$ the standard error of the mean (SEM).

Figure 2. MSCV transduced hematopoietic colony formation

Lin⁻ bone marrow transduced to overexpress Sall4a, Sall4b or MLL-AF9 was incubated in methylcellulose and replated every 7–10 days out to four generations. MLL-AF9 demonstrated enhanced self-renewal while Sall4a and Sall4b transduced colonies are smaller than those transduced with empty vector (size bar = 1mm).

Figure 3. Sall4 overexpression impairs hematopoiesis *in vitro*

A, C. In both Lin- bone marrow (A) and LSKs (C), colony-forming units were markedly decreased initially following Sall4 overexpression with no enhanced colony forming capacity out to four generations. **B, D.** In both Lin- bone marrow (B) and LSKs (D), cellular proliferation is significantly diminished by Sall4 overexpression, with no survival advantage demonstrated through four generations of plating. Colony assays represent results from 4 separate experiments (biologic replicates) for 1°, 2°, and 3°; and 3 separate experiments are represented in 4° data.

Survival proportions: Survival of Survival DATA

Figure 4. Sall4b overexpression impairs hematopoiesis *in vivo*

A. Sall4b overexpressing Lin⁻ bone marrow transplanted into mice following sublethal irradiation fails to achieve any meaningful engraftment as assessed by % peripheral leukocytes demonstrating GFP fluorescence. **B.** Following lethal irradiation (900–1100 cGy), all mice transplanted with Sall4b transduced Lin⁻ bone marrow fail to engraft and succumb to bone marrow failure by 11 days. Transplants were performed in 5 animals each for each condition (vector or Sall4b with lethal or sublethal irradiation) over 3 individual experiments (range 1 – 2 animals per individual experiment). Values represent the mean of 5 animals for each condition.

Figure 5. Sall4 target genes in hematopoiesis

A. Expression of the reported Sall4 target genes in hematopoiesis Bmi1, Pten and Meis1 and potential targets Ezh2, Arid5b and Klf2 identified by ESC ChIP-chip were assessed in Lin-bone marrow 48 hours post-Sall4 transduction. Bmi1, Pten and Arid5b were significantly decreased following both Sall4a and Sall4b overexpression. Sall4b was associated with increased Meis1, while Sall4a overexpression lead to increased Klf2 and decreased Ezh2 expression. **B.** Bmi1 expression was significantly decreased in LSK cells 48 hours after transduction with Sall4a or Sall4b.

Figure 6. Sorting by GFP fluorescent intensity

A. Lin- bone marrow was transduced with MSCV-Sall4b or Sall4a and sorted by GFP-low, medium, or high populations (1/3 of the total GFP+ population each). Sall4b and Sall4a expression correlates with GFP fluorescent intensity. **B.** Bmi1 expression is inversely correlated with Sall4b and Sall4a overexpression. Values represent the mean $\pm$ SEM of 2 separate experiments.

Figure 7. Level of Sall4 overexpression correlates with degree of hematopoietic impairment in vitro

Lin⁺ bone marrow was infected with Vector, Sall4a or Sall4b and sorted by GFP-low, GFP-medium or GFP-high expressing fractions, cultured in methylcellulose and replated after 10 days. Both colony formation (A) and proliferation (B) trend down as Sall4a and Sall4b overexpression increases.

Figure 8. Sall4 expression in pediatric AML

Sall4a and Sall4b expression was assessed in seven pediatric AML patient samples by RT-qPCR. Cord blood, whole bone marrow, mobilized peripheral blood, and whole blood from healthy donors were included as baseline hematopoietic controls. Samples were run in triplicate, data shown as mean $\pm$ SEM.

Figure 9. Proposed model of Sall4 in hematopoiesis

Schematic representation of dose-dependent Sall4 hematopoietic regulation. An intermediate level of Sall4 expression is necessary for normal self-renewal; loss of Sall4 leads to differentiation while marked overexpression represses Bmi1, impairing normal self-renewal and proliferation.