Published in final edited form as:

Crit Rev Oncog. 2011 ; 16(1-2): 117–127.

The role of stem cell factor SALL4 in leukemogenesis

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Abstract

SALL4, a member of SALL gene family, is one of the most important transcriptional regulators of stem cells. It is of particular interest to stem cell biologists because it is linked to the self-renewal of both embryonic stem cells (ESCs) and hematopoietic stem cells (HSCs), as well as involved in human leukemia. In ESCs, the Sall4/Oct4/Nanog core transcriptional network governs the self-renewal and pluripotent properties of human and murine ESCs. In normal HSCs and leukemic stem cells (LSCs), SALL4 is linked to three known pathways that are involved in self-renewal: Wnt/ β -catenin, Bmi-1 and Pten. Despite the important shared role of SALL4 in self-renewal of HSCs and LSCs, our recent studies obtained through correlating global downstream target genes and unique functional studies in normal versus leukemic cells have demonstrated that SALL4 has differential effects on both pro- and anti-apoptotic pathways in normal and leukemic cells. Targeting SALL4, particularly when combined with the use of ABT-737, a BCL2 antagonist, could lead to leukemic cell-specific apoptosis. This review summarizes our current knowledge on the SALL gene family, particularly on the role of SALL4 in stem cells, development as well as tumorigenesis, especially leukemogenesis

Keywords

Transcription factor; acute myeloid leukemia; survival factor

THE SALL GENE FAMILY

SALL4, a member of the SALL (spalt-like/sall) gene family (SALL1 to SALL4), was originally cloned based on DNA sequence homology to *Drosophila* gene spalt (sal) and has two major isoforms: SALL4A and SALL4B.¹ Sal is a non-clustered region-specific homeobox gene and is essential for the development of the posterior head and anterior tail segments of the fly.^{2, 3} Sal proteins belong to a group of C2H2 zinc finger transcription factors characterized by multiple zinc finger domains distributed over the entire protein.¹ Structural characteristics of human SALL include a single C2HC zinc finger near the N-terminus and several C2H2 zinc fingers in the middle portion of and at the C-terminus of the protein (Figure 1A).⁴ C2H2 Zinc finger domains can bind to DNA, and in some case, RNA and proteins.

THE ROLE OF SALL4 IN ESCS

Over the past few years, remarkable progress has been made in identifying stem cell factors that are essential in the maintenance of self renewal and pluripotent capacities of embryonic stem cells (ESCs). Several research groups, including us, have demonstrated that Sall4 plays an essential role in this process^{5–10} by interacting with two other key regulators in ESCs,

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Nanog and Oct4. Sall4 regulates Oct4 expression by directly binding to the highly conserved regulatory region of Oct4, as demonstrated both in vivo and in vitro. Sall4^{+/-} ESCs have significantly reduced levels of pluripotency markers (Oct4, Sox2 and Nanog) as compared to WT ESCs. These findings were consistent with the results obtained from RNAi-mediated knockdown of Sall4 using an RNAi lentiviral vector.⁵ Similar to Oct4, reduction of Sall4 in murine ESCs results in re-specification of ESCs to the trophoblast lineage.⁵

In addition, we and others have mapped the global gene targets of Sall4 using chromatin-immunoprecipitation followed by microarray hybridization (ChIP-chip).^{11, 12} Over thousands of Sall4 target genes were identified, and most were classified as genes related to stem cell maintenance and development. Co-immunoprecipitation showed that Sall4, Oct4, and Nanog formed a complex; this is consistent with the finding that Sall4 co-occupied the promoters of Oct4 and Nanog target genes.¹¹ Sall4 regulation of pluripotency appears to be tightly linked to transcriptional regulation as well as the chromatin-based epigenetic events mediated by polycomb-repressive complexes (PRCs) and bivalent domains.¹¹ This evidence suggests that Sall4 plays a central but diverse role during early embryonic development.

Most recently, we and others reported a similar role of SALL4 in human ESCs and have shown for the first time that SALL4 is required for the maintenance of human ESC characteristics.¹³ Furthermore, we found a novel SALL4/OCT4 transcription regulatory loop in balancing the proper expression dosage of SALL4 and OCT4 for the maintenance of ESC stemness. While we have observed that a positive feedback relationship is present between SALL4 and OCT4, the strong self-repression of SALL4 seems to be the “break” in this loop. In addition, we have shown that SALL4 can repress the promoters of other SALL family members, such as SALL1 and SALL3, and competes with the activation of these two genes by OCT4. Overall these studies suggest that SALL4 is a master regulator that controls its own expression and the expression of OCT4. Moreover, SALL4 and OCT4 work antagonistically to balance the expressions of other SALL gene family members.¹³

THE FUNCTION OF THE SALL GENE FAMILY IN DEVELOPMENT

Sal plays an important role in the embryonic development of the larval tracheal system and the adult wing. Sal-related genes have been identified in *C. elegans*,¹⁴ fish,¹⁵ frogs (xenopus),^{16, 17} mice¹⁸ and humans.¹ Sall1 and Sall3 are normally expressed in mouse from embryonic day 7 (E7). The expression of Sall2 in early murine development hasn't been well-studied yet. Sall1^{-/-} mice die in the perinatal period and have kidney abnormalities,¹⁹ whereas Sall2-deficient mice show no apparent abnormal phenotypes.²⁰ Sall3^{-/-} mice survive until birth, but fail to feed and eventually die on the first postnatal day.²¹ In contrast, Sall4 is visible in the 8- to 16- cell stage of the early embryo and becomes enriched in the inner cell mass,¹⁰ from which the ESCs are derived. Sall4 null mice die at E6.5,⁹ suggesting that among the Sall family, Sall4 is most critical for early embryonic development.

In humans, SALL1 is mutated in patients with Townes-Brockes Syndrome (TBS), a disorder characterized by urogenital, limb, anal and cardiac malformations.²²⁻²⁴ Defects in hematopoiesis have not been reported to date in patients with TBS. Similar to SALL1, SALL2 is expressed in the developing neuroectoderm of the brain, inner ear and urogenital ridge-derived structures such as testes, ovaries and kidneys, though Sall2-deficient mice show no apparent abnormal phenotypes and no evidence shows the association between SALL2 and developmental disorder in human. SALL3 is expressed in the developing central nervous system, heart, limb buds, kidneys, ears, and palate. SALL3 is mapped to human chromosome 18q23.²⁵ Since the Sall3-null mice have multiple developmental defects, it has been suggested that the SALL3 gene product may be involved in the phenotype of patients

with 18q deletion syndrome characterized by developmental delay, hypotonia, growth retardation, midface hypoplasia, hearing loss and tapered fingers.²⁵ The latest SALL gene member, SALL4, is mutated in human Duane-Radial Ray Syndrome (DRRS) and Instituto Venezolano de Investigaciones Cientificas syndrome (IVIC).^{26–29} Both syndromes are autosomal-dominant developmental disorders involving radial-sided hand anomalies and congenital strabismus. IVIC is also characterized by leukocytosis and thrombocytopenia, suggesting that SALL4 may be involved in normal hematopoiesis.³⁰

SALL GENE FAMILY IN CANCER

Besides genetic diseases, the SALL gene family has been reported in several types of human tumors (Table 1). Consistently high expression of SALL1 was detected in Wilms tumor, a pediatric renal cancer.³¹ Also overexpression of SALL1 mRNA was identified in the carcinoma in situ cell of testicular germ cell tumor.³² However, hypermethylation of multiple CpG islands of the SALL1 gene was reported in acute lymphocytic leukemia and breast cancer,³³ and all patients with methylation of multiple CpG islands of SALL1 had a worse overall survival rate. Therefore, the role of SALL1 in cancer still needs to be defined.

The SALL2 gene is mapped to chromosome 14q12.1–13, a region that is associated with loss of heterozygosity in 49% of ovarian cancers³⁴ and 25% of bladder cancers. SALL2 is expressed in a number of mouse and human tissues. Our previous study demonstrated loss of SALL2 expression in some solid tumors³⁵ and suggested that SALL2 may function as a tumor suppressor. Murine Sall2 was able to bind to the large T antigen of polyoma virus, a DNA virus that induces a broad variety of neoplasms in its natural host.³⁶ Overexpression of the SALL2 gene by transfection inhibits the growth and DNA synthesis of the ovarian cancer cell line SKVO3 and tumor formation in nude mice, as a result of the induction of p21^{Cip1/Waf1}.³⁷ These observations further indicate that SALL2 may have roles in suppressing tumorigenesis. There is only one study so far on the role of SALL3 in cancer. SALL3 can interact with DNMT3A, a DNA methyltransferase, in CpG island methylation in vitro. In addition, down-regulation of SALL3 results in acceleration of DNA methylation in hepatocellular carcinoma.³⁸

The most well studied SALL gene in cancer is SALL4. Recent studies have showed that SALL4 is a novel sensitive and specific diagnostic marker of ovarian primitive germ cell tumors and testicular germ cell tumors.^{39, 40} SALL4 has also been identified as a sensitive marker for AFP-producing gastric carcinoma that is especially useful in distinguishing hepatoid gastric carcinoma from hepatocellular carcinoma.⁴¹

THE ROLE OF SALL4 IN LEUKEMOGENESIS

SALL4 As A Diagnostic Marker of Leukemia

During normal hematopoiesis, SALL4 is preferentially expressed in human CD34+ hematopoietic stem/progenitor cells (HSCs) and down-regulated in CD34- cells during hematopoietic differentiation.⁴² Using immunohistological staining and quantitative RT-PCR analysis, we found that SALL4 was aberrantly expressed in many leukemia cell lines and primary leukemia cells of acute myeloid leukemia (AML) and precursor B-cell lymphoblastic leukemia/lymphomas.⁴² Recent publications from our group have shown that SALL4 expression correlates with disease progression in human chronic myeloid leukemia⁴³ and its expression in AML patients correlated with treatment status. We further explored the role of SALL4 in drug resistance, and found that SALL4 was involved in the maintenance of side population (SP) cells by regulating ATP-binding cassette drug transport genes.⁴⁴ Therefore, SALL4 may be used as a marker for diagnosis and prognosis for AML.

Functional Studies of SALL4 in Leukemia

Our research group has demonstrated that constitutive expression of SALL4 contributes to leukemogenesis in adult mice.⁴² Mice transgenic for 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 contributes to the initiation of leukemia.⁴² In addition, loss-of-function studies have demonstrated that SALL4 is a key regulator in leukemic cell survival and down-regulation of SALL4 leads to significant apoptosis of leukemic cells,⁴⁵ suggesting that SALL4 is essential for the maintenance of leukemia.

SALL4 Protein Partners

In searching for the mechanism of SALL4-mediated leukemogenesis, we found that SALL4 could bind to β -catenin and synergistically enhance the Wnt/ β -catenin signaling pathway. The expression of cyclin-D1 and c-Myc, the two known targets of the Wnt/ β -catenin pathway, were also increased in the SALL4B transgenic bone marrow cells.⁴²

We also sought to identify SALL4-associated proteins by tandem mass spectrometry. Components of a transcriptional repressor Mi-2/nucleosome remodeling and deacetylase (NuRD) complex were found in the SALL4-immunocomplexes with histone deacetylase (HDAC) activity both in ESCs with endogenously high SALL4 expression and 293T cells overexpressing SALL4.⁴⁶

Important Downstream Target Genes of SALL4 in Self-renewal in HSC and LSC

The SALL4-mediated transcriptional repression was tested on PTEN, one potential SALL4 target gene. PTEN was confirmed to be a SALL4 downstream target by chromatin-immunoprecipitation (ChIP). Moreover, the SALL4 binding site in the promoter region of PTEN was co-occupied by NuRD components, suggesting that SALL4 repressed the transcription of PTEN through its interactions with the Mi-2/NuRD complex. The *in vivo* repressive effect(s) of SALL4 were evaluated in SALL4B transgenic mice, where decreased expression of *Pten* was associated with myeloid leukemia.⁴⁷

In addition to *Pten*,⁴⁷ we and others have previously demonstrated that *Bmi-1*^{45, 48} is also a SALL4 target gene. We have shown that transcription from the *Bmi-1* promoter is activated by SALL4 in a dose-dependent manner when using a luciferase reporter gene assay. Both promoter deletion construct studies and ChIP from a myeloid stem cell line, 32D, demonstrate that SALL4 binds to a specific region of the *Bmi-1* promoter. Down-regulation of SALL4 by siRNA in the HL-60 leukemia cell line results in decreased *Bmi-1* expression. Furthermore, *Bmi-1* expression is up-regulated in SALL4B transgenic mice, and the levels of *Bmi-1* in these mice increase as they progress from normal to preleukemic (MDS) and leukemic (AML) stages.

In summary, both PTEN and *Bmi-1*, the two key regulators of self-renewal of normal HSCs and leukemic stem cells (LSCs),^{49–51} are direct targets of SALL4. *Bmi-1* is up-regulated and *Pten* is down-regulated in SALL4B murine leukemic model.^{47, 48}

Differential Effects on Pro-/anti-apoptotic Pathways Upon Down-regulation of SALL4 in Normal and Leukemic Cells

Recently, SALL4 was found to be a robust stimulator in the expansion of hematopoietic stem cells.⁵² To determine the transcriptional network that may be responsible for the function of SALL4, we performed a genome-wide analysis of SALL4 target genes in human CD34+ and myeloid leukemic cells. Chromatin-immunoprecipitated (ChIP) DNA was obtained from immunomagnetically selected CD34+ cells, leukemic blasts and leukemic cell

line NB4 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 ChIP experiments in NB4 cells and murine ES cells.^{11, 45}

When SALL4 targets from leukemia and CD34+ cells were compared, many genes that were regulated by SALL4 in normal CD34+ cells could not be found in the datasets from leukemia cells. Comparison between the two groups of targets in CD34+ and leukemia cells yielded some common, but mostly unique SALL4 targets, particularly the pro-/anti-apoptotic pathways.

We next sought to test whether SALL4 has differential effects on the pro- and anti-apoptotic pathways in these two cell types. While downregulation of SALL4 in leukemia triggered significant cell death, knocking-down of SALL4 in normal CD34+ cells didn't affect cell viability. We noticed that there were three genes that were differentially affected by SALL4 in leukemic versus normal CD34+ cells. In normal CD34+ cells, while there was no obvious apoptotic phenotypes observed, the expression of apoptosis induction genes TRO and ABL1 was decreased, and the expression of apoptosis inhibition gene, BCL2, was consistently increased in SALL4-reduced CD34+ cells. In contrast, when SALL4 was down-regulated in the leukemic cell lines KG1a, NB4 and KBM5, and in primary AML cells, along with the apoptotic phenotypes, the expression of TRO and ABL1 was increased while the expression of BCL2 was decreased.

Next, we test whether the SALL4/BCL2 pathway is essential for maintaining the survival of the leukemic cells by using the small molecule drug, ABT-737. ABT-737 is a BH3 domain mimetic that can interrupt the interaction of BCL2 and its family members and results in the inhibition of the anti-apoptotic function of BCL2. It can trigger leukemic cell apoptosis but has no effects on normal hematopoiesis.^{53, 54} Since SALL4 can regulate BCL2 expression and ABT-737 can affect its function, we hypothesized that we may observe an enhanced effect in promoting leukemic cell apoptosis if we combined the use of ABT-737 with down-regulation of SALL4. To test our theory, 2.5×10^5 primary human AML cells infected with SALL4 shRNA lentivirus, control shRNA, or no virus control were treated with either DMSO or 10nM ABT-737 for 12 hours. The cells were tested for apoptosis and cell death by Annexin V/PI staining. As reported previously by others, ABT-737 treatment alone can lead to apoptosis and cell death in primary AML samples. Interestingly, by knocking down SALL4 in these cells, in combination with ABT-737 treatment, we observed an additional 2 to 3 folds increase in apoptosis compared to cells treated with ABT-737 alone or ABT-737 with control shRNA. Cells treated with solvent control DMSO did not show significant cell death. These results show that the combination of ABT-737 and down-regulation of SALL4 could be a novel therapeutic strategy in treating AML patients.

SUMMARY/DISCUSSION

In conclusion, SALL4 is one of few genes that bridge the self-renewal properties of ESCs, normal HSCs and LSCs. In ESCs, the Sall/Oct4/Nanog core transcriptional network governs the self-renewal and pluripotent properties of human and mouse ESCs (Figure 1B). In normal HSCs and LSCs, SALL4 is involved in three known self-renewal pathways: Wnt/ β -catenin, Bmi-1 and Pten pathways (Figure 1C).

Our more recent unpublished studies by correlating global down-stream target genes and unique function(s) in normal versus leukemic cells have demonstrate that SALL4 has differential effects on both pro- and anti-apoptotic pathways in normal and leukemic cells. It has been proposed that cancer stem cells arise from dysregulated normal stem or progenitor

cells; however, the key regulator(s) responsible for the dysregulated cellular programs directing proliferation versus differentiation have not been well defined. There are very few reports of a single gene inducing differential effects on normal stem/progenitor cells and their malignant counterparts. While the main effect of SALL4 in leukemic cells is to maintain cell survival, the major functional role of SALL4 in normal hematopoiesis is to retain stem and progenitor cells in an undifferentiated stage and antagonize myeloid differentiation (Figure 1D). The differential biological effects of SALL4 in normal versus leukemic hematopoiesis correlate with the limited number of overlapping SALL4 direct target genes and its differential regulations of these target genes in the two cell types. Therefore, SALL4-mediated stem cell function is cell context-dependent. Based on our observation that SALL4 is an essential factor for survival of the leukemic cells, it is reasonable to propose future anti-cancer applications that combine the strategies targeting SALL4 and the use of ABT-737 can be used in exploiting the unique property that reduced SALL4 expression or function leads to apoptosis of leukemic but not normal hematopoietic stem cells.

Finally, many questions regarding the role of SALL4 in leukemogenesis, such as the mechanism (s) of SALL4 gene expression regulation in normal hematopoiesis and dysregulation in leukemia, still remain unanswered and require future investigation.

Acknowledgments

This work was supported in part through NIH grants RO1HL092437, RO1HL092437-A1S1 and PO1HL095489 and PO1DK080665 (to L.C). We also want to thank Kol Jia Yong, Xi Tian and Youyang Yang in assisting with the preparation of the manuscript.

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Figure 1A **Figure 1B**

Figure 1C **Figure 1D** **Figure 1. The role of SALL4 in ESC, HSC and LSC**

Figure 1A Schematic representation of the main domains present in human SALL proteins. Rectangles represent the N terminal C2HC type zinc finger (NT ZF) and C2H2 type zinc fingers (ZF 1 to 9). Ovals represent the polyQ regions. The black rectangles indicate the C terminal zinc fingers in SALL2 which are not homologous to those in other SALL proteins. The break lines represent the regions that are absent in these proteins. Figure1B Sall4/Oct4/ Nanog core transcriptional network in ESC. Sall44 contributes to the maineince of murine and human ESC stemness by regulating and interacting with Oct4 and Nanog. In addition, while we have observed that a positive feedback relationship is present between human SALL4 and OCT4, the strong self-repression of SALL4 seems to be the “break” for this loop. Black arrow: activation, black dash arrow: inhibition. Red solid line: interaction. Figure 1C The SALL4/Wnt/Bmi-1/PTEN network in normal and leukemic hematopoiesis. SALL4 contributes to hematopoietic differentiation at least, in part, through repression of PTEN, activation of Bmi-1 and interacts with Wnt /β-catenin. During normal hematopoiesis, SALL4 is preferentially expressed in HSCs, down-regulated as HSCs differentiate into

HPCs and is absent in mature myeloid populations such as neutrophils. Down-regulation of SALL4 leads to differentiation. Dys-regulation of SALL4/Wnt/Bmi-1/PTEN network can lead to leukemic development. Figure 1D Proposed model on differential effects of SALL4 in normal and leukemic hematopoiesis. While the main effect of SALL4 in leukemic cells is to promote cell survival; the major functional role of SALL4 in normal hematopoiesis is to maintain cells at the stem/progenitor state with expression of CD34 and to block differentiation. Upon down-regulation of SALL4 expression, leukemic cells undergo apoptosis and cell death, while normal hematopoietic stem/progenitor cells are induced to differentiate spontaneously.

TABLE 1**SALL GENE EXPRESSION IN CANCERS**

Gene	Tumor	Status of Gene	Reference
SALL1	Wilms Tumor and Trophoblast tumors	Upregulated	55, 56
	Sex Hormone-producing tumors	Upregulated	56
	Testicular carcinoma	Upregulated	57
	Acute lymphocytic leukemia	Methylation	58
	Breast cancer	Methylation	33
SALL2	Prostate and breast cancer	Downregulated	59
	Lung carcinoma	Downregulated	60
	Colon and Prostate adenocarcinoma	Downregulated	60
	Ovarian carcinoma	Downregulated	61
SALL3	Hepatocellular carcinoma	Methylation	38
	Bladder cancer	Methylation	62
	Acute myeloid leukemia	Upregulated	42
	Acute lymphoblastic leukemia	Upregulated	63
	Ovarian primitive germ cell tumors	Upregulated	39
SALL4	Metastatic germ cell tumors	Upregulated	64
	Testicular primitive germ cell tumors	Upregulated	40, 65
	Extragenital yolk sac tumors	Upregulated	66
	Gastric cancers	Upregulated	41
	Kidney cancer	Upregulated	67
	Breast Cancer	Up-regulated	44, 68, 69