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The next new target in leukemia: The embryonic stem cell gene *SALL4*

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Abbreviations: AML, acute myeloid leukemia; B-ALL, B-cell acute lymphoid leukemia; CML, chronic myeloid leukemia; DDRS, Duane-radial ray syndrome; ES, embryonic stem; FOG-1, friend of GATA1; HPC, hematopoietic progenitor cell; HSC, hematopoietic stem cell; HSPC, hematopoietic stem/progenitor cell; iPS, induced pluripotent stem; IPSS, International Prognostic Scoring System; IVIC, Instituto Venezolano de Investigaciones Cientificas Syndrome; LSC, leukemic stem cell; MDS, myelodysplastic syndromes; MEF, mouse embryonic fibroblast; MSP, methylation-specific PCR; NuRD, nucleosome remodeling and deacetylase; PTEN, phosphatase and tensin homolog; RAEB, refractory anemia with excess blasts; SP, side population; XEN, extraembryonic endoderm.

The embryonic stem (ES) cell gene *SALL4* has recently been identified as a new target for cancer therapy, including leukemia. SALL4 is expressed in ES cells and during embryonic development, but is absent in most adult tissues. It is, however, aberrantly expressed in various solid tumors and hematologic malignancies such as myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). Aberrant expression of SALL4 is frequently associated with a more aggressive cancer phenotype, which includes high-risk MDS and its progression to AML. SALL4 contributes to leukemogenesis through multiple pathways including the repression of PTEN and the activation of HOXA9 expression. Targeting the SALL4/PTEN pathway by blocking the protein–protein interaction of SALL4 and its associated epigenetic complex, nucleosome remodeling and deacetylase complex (NuRD), might be a novel approach to treating AML and holds great potential for the treatment of other SALL4-mediated oncogenic processes such as high-risk MDS and solid tumors.

SALL4 Plays a Key Role in ES Cell Function

SALL4 is a DNA binding protein with multiple conserved C2H2 zinc finger motifs.[1] It is essential for the maintenance of pluripotency in human and murine embryonic stem (ES) cells,[2-5] and loss or overexpression of SALL4 protein can affect the "stemness" of ES cells.[6] Because of its importance in ES cell fate, SALL4 expression needs to be reactivated during the reprogramming process by which mouse embryonic fibroblast (MEF) cells are converted to induced pluripotent stem (iPS) cells.[7] In addition, SALL4 is used to enhance iPS cell reprogramming efficiency in a cell fusion approach,[8] and inclusion of SALL4 in the Oct4, Sox2, and Klf4 (OSK) reprogramming system can ensure a more consistent and stable induction of iPS cells with a higher efficiency.[9]

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The ES Cell Gene *SALL4* Plays an Important Role in Development

SALL4 is crucial for human and murine organ development, and mutations in *SALL4* are often linked to developmental defects. In humans, *SALL4* mutations have been linked to the familial Duane-radial ray syndrome (DDRS, also named Okihiro syndrome),[10] Instituto Venezolano de Investigaciones Cientificas syndrome (IVIC),[11] Holt-Oram syndrome, and acro-renal-ocular syndrome.[12] In mice, Sall4 has been reported to be involved in neural tube closure and in limb and heart development.[13] Germ line homozygous *Sall4* deletion is lethal at the peri-implantation stage.[6,14]

SALL4 is Silenced in most Adult Tissues, but is Aberrantly Expressed in Cancers Including Hematologic Malignancies

In mice, *Sall4* expression is mostly restricted to germ cells after birth. It is highly expressed in undifferentiated spermatogonia[15,16] and oocytes in primordial, primary, and secondary follicles.[17] Similarly, in humans SALL4 is undetectable in most normal adult tissues with the exception of germ cells[15-19] and hematopoietic stem/progenitor cells (HSPCs).[20,21] During normal human hematopoiesis, SALL4 is expressed highly in $CD34^+CD38^-$ hematopoietic stem cells (HSCs), at a lower level in $CD34^+CD38^+$ hematopoietic progenitor cells (HPCs), and is absent in $CD34^-$ cells.[21] The expression of SALL4 in these rare adult tissues seems to be correlated with its function. In adult mouse testis, Sall4 plays a role in regulating spermatogonial proliferation.[15] In human adult bone marrow, SALL4 plays a key role in hematopoietic differentiation.[21]

In contrast to its absence in most adult tissues and its restricted expression in HSCs, SALL4 is re-expressed in various human cancers including hematologic malignancies[22-27] and subsets of liver,[28-30] gastric,[31] lung,[32] endometrial,[33] and breast[34,35] cancers. In hematologic cancers, SALL4 expression can be detected in most subtypes of AML and B-cell acute lymphoid leukemia (B-ALL), but not in lymphoma or multiple myeloma.[22] Previous studies have shown that the expression of SALL4 is higher in drug-resistant primary AML patients than in drug-responsive cases,[27] suggesting that SALL4 may be a potential biomarker to monitor the treatment status of AML patients. Increased SALL4 expression can also be detected in the blastic stage of chronic myeloid leukemia (CML). In CML patients SALL4 is expressed in the blast-crisis phase but not the chronic phase,[23] implying a potential role for SALL4 in predicting the disease progression of CML.

SALL4 was also studied in MDS, a group of preleukemic hematologic disorders characterized by dysplasia and refractory cytopenias[36-41] with an incidence of 5–15 cases per 100,000 people annually worldwide.[36-41] Approximately 30–40% of MDS cases progress to AML, an advanced disease stage that is associated with poor prognosis and short survival time because AML that develops from MDS is more difficult to treat than *de novo* AML.[36-41] The clinical presentation of preleukemic MDS can range from indolent conditions such as mild anemia that persists for many years to the most severe presentation of AML transformation within months of MDS diagnosis. While most patients with low-grade MDS with mild cytopenias are treated with more conservative supportive care, MDS patients with a high risk of AML progression are treated more aggressively with AML regimens and bone marrow transplants. Because of the heterogeneity of this disease and the lack of molecular markers that effectively monitor disease progression, clinical management of MDS patients is currently challenging. The International Prognostic Scoring System (IPSS) is widely used to predict the prognosis of MDS;[36-41] however, the prognostic information it provides is not always correlated with the clinical outcome. A molecular biomarker able to predict which MDS patients will progress to AML at the time of diagnosis would be extremely helpful for choosing the appropriate treatment. Among patients with MDS, SALL4 is mainly expressed in those with refractory anemia with excess blasts-1 (RAEB-1) and RAEB-2.[26] Survival analysis based on SALL4 expression showed that MDS patients with high SALL4 expression had a worse survival rate than those with low SALL4 expression. These results suggest that SALL4 expression can be used as an additional confirmation and/or refinement marker for the World Health Organization (WHO)/IPSS system. Furthermore, it can even be used alone or as a part of a combinatorial molecular test to help predict the prognosis of MDS.[26]

Regulation of SALL4 Expression in Normal and Cancer Cells

How SALL4 expression is regulated during embryonic development and in normal adult tissues is not well understood. In ES cells, in which SALL4 is most abundantly detected, its expression can be positively regulated by OCT4, which is encoded by the ES cell gene *Pou5f1*. In addition, studies have demonstrated that SALL4 negatively regulates its own expression by binding to its own promoter and repressing transcriptional activities as measured by luciferase reporter assays.[3] A SALL4/OCT4 regulatory feedback network is proposed in which OCT4 activates the *SALL4* gene and SALL4 activates *Pou5f1*; the self-repressive effect of SALL4 thus acts as a brake for this positive feedback loop, leading to a balanced expression level of SALL4 and OCT4 in ES cells.

In addition to the regulation of SALL4 expression by OCT4 and its autoregulation in ES cells, studies using *in vitro* promoter reporter assays in cell lines and *Xenopus* neural tissues have demonstrated that the Wnt/β-catenin pathway can also regulate SALL4 expression.[42,43] In the latter study, researchers identified TCF/LEF-binding sites within the *SALL4* promoter and intragenic regions, and showed that canonical Wnt signaling can promote SALL4 activation. In breast cancer cells, STAT3 has been shown to be an upstream transcriptional activator of SALL4.[44]

The mechanism underlying aberrant SALL4 expression in leukemia is not known although recent studies have suggested DNA demethylation as one possibility.[25] In a study using B-ALL cell lines and primary B-ALL patient samples, hypomethylation of the *SALL4* CpG islands spanning the exon1-intron1 region was observed to correlate with its high expression. Furthermore, treatment of low SALL4-expressing B-ALL cell lines with a DNA methylation inhibitor led to demethylation of the *SALL4* CpG region and increased SALL4 expression. Independent studies also found that hypomethylation of the same *SALL4* CpG islands is a common event in MDS and AML patients, and is correlated with high SALL4 expression. Using methylation-specific PCR (MSP), hypomethylation of these *SALL4* CpG islands was found in 21.7% (18/83) of MDS cases and 17.8% (15/84) of AML cases that were analyzed.[45,46]

Different Functions of SALL4 in Normal and Leukemic Hematopoiesis

SALL4 is expressed in both normal $CD34^+$ HSPCs and leukemic cells; however, the functional role of SALL4 in these 2 cell types seems to be different (**Fig. 1**). In normal $CD34^+$ HSPCs, downregulation of SALL4 leads to a loss of the stem/progenitor cell marker CD34 and affects the expression of self-renewal and differentiation genes such as *RUNX1*, *HOXA9*, *TPO*, *BMI-1*, and *PTEN*. This is further supported by reduced myeloid colony-forming ability *in vitro* and impaired engraftment *in vivo* using cells with decreased SALL4 expression.[21] Consistent with these findings, overexpression of SALL4 in $CD34^+$ cells leads to a >10,000-fold expansion of human HSPCs in the presence of appropriate cytokines *in vitro*, and these expanded HSPCs contribute to enhanced stem cell repopulation capacity *in vivo*.[47] Taken together, these results suggest that in normal hematopoiesis SALL4 plays a role in HSC proliferation and myeloid differentiation.

In contrast, in leukemia and other cancers, downregulation of SALL4 expression results in increased cell apoptosis and cell cycle arrest without notable effects on differentiation, which supports its

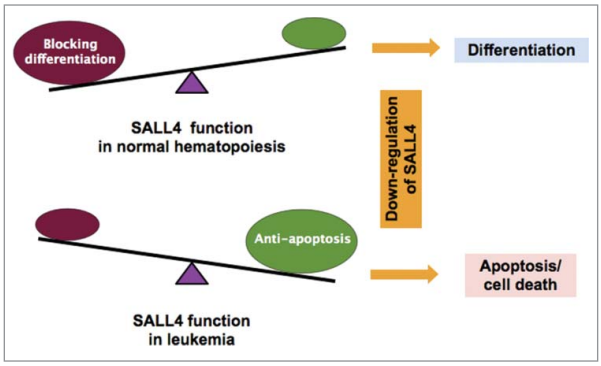

Figure 1. Differential functions of SALL4 in normal and leukemic hematopoiesis. While the main functional role of SALL4 in normal hematopoiesis is to maintain hematopoietic stem/progenitor cell (HSPC) proliferation and to block myeloid differentiation, the major function of SALL4 in leukemic cells is to promote cell survival. Downregulation of SALL4 expression in normal HSPCs induces differentiation; however, downregulation of SALL4 in leukemic cells causes apoptosis and cell death.

important role in tumors as a cancer cell survival factor.[23,25,28,33,48] Global analysis of gene promoters that are directly or indirectly bound by SALL4 in the human acute promyelocytic leukemic cell line NB4 showed that genes involved in cell death, cancer, DNA replication/repair, and the cell cycle were highly enriched. These genes included 38 important apoptosis-inducing genes (e.g., *TNF*, *TP53*, *PTEN*, *CARD9*, *CARD11*, *CYCS*, *LTA*) and apoptosis-inhibiting genes (e.g., *Bmi-1*, *BCL2*, *XIAP*, *DAD1*, *TEGT*).[49]

With respect to underlying mechanisms, a global target search performed on the SALL4-expressing leukemic cell line NB4 and normal $CD34^+$ cells showed little overlap.[21] The NB4 AML cell line and CD34+ cells only have 258 SALL4-bound targets in common (18.2% of targets in $CD34^+$ cells; 9.8% of targets in leukemic cells). Some targets that are differentially bound by SALL4 during normal and leukemic hematopoiesis are members of important signaling pathways, including JAK/STAT, p53, NF-kB, and Wnt/β-catenin pathways. This limited overlap suggests that SALL4 targets are cell-context dependent, which is consistent with a similar observation reported for 2 types of SALL4-expressing stem cells, mouse ES cells and extraembryonic endoderm (XEN) cells, in which genome-wide analysis revealed that only 25% of SALL4-bound regions were common to mESC and XEN.[50]

In addition, gain-of-function and loss-of-function studies of SALL4 suggest that it plays an important role in cancer initiation, drug resistance, and tumor metastases. The *SALL4B* transgenic mouse model developed MDS-like features and subsequent AML that was transplantable,[24] indicating that SALL4 contributes to leukemia initiation and maintenance. Supporting the observation that SALL4 expression is higher in drug-resistant than drug-responsive primary AML patients, knocking down SALL4 expression in a myeloid leukemic line, KG1a, led to increased drug sensitivity compared with untreated cells. Further exploration revealed that SALL4 is involved in the maintenance of side population (SP) cells by regulating the expression of the ATP-binding cassette drug transport genes *ABCG2* and *ABCA3*, thus facilitating drug resistance in these cells.[27]

SALL4 Contributes to Leukemogenesis Through Multiple Mechanisms

Experiments to determine the mechanisms by which SALL4 contributes to leukemogenesis revealed that SALL4 can interact with other proteins and can recruit different epigenetic modulators, both activator and repressor complexes depending on the cellular context, to the promoter regions of its downstream target genes for either transcriptional activation or repression. Multiple pathways are known to be involved in SALL4-mediated leukemogenic process.

As a co-activator of Wnt/β-catenin pathway

In *SALL4B* transgenic mice, the downstream target genes of Wnt/β-catenin, such as *c-Myc* and *CyclinD1*, were upregulated at both preleukemic and leukemic stages.[24] The Wnt/β-catenin pathway plays an important role in the self-renewal of leukemic stem cells (LSCs).[51-53] Both SALL4A and SALL4B isoforms can bind the β-catenin protein and synergistically enhance the transcriptional output of the Wnt/β-catenin signaling pathway,[24] suggesting that constitutive expression of SALL4 causes MDS/AML, at least partially, through the Wnt/β-catenin pathway (**Fig. 2**). Another recent study in an endometrial cancer model showed that SALL4 can bind to the *c-Myc* promoter and directly active its expression.[33] A TCF/LEF consensus sequence is present near the SALL4-binding site within the *c-Myc* promoter, suggesting that SALL4 might be part of the TCF/β-catenin complex. Since the Wnt signaling pathway can be a potential active regulator of *SALL4* gene expression, and SALL4 protein can act as a co-activator of this pathway, a positive feedback between SALL4 and Wnt/β-catenin may exist. Further work is needed to test this model and its functional relevance in normal and leukemic hematopoiesis.

As a transcriptional activator

As a potential DNA binding protein, SALL4 has been shown to activate genes such as *Bmi-1* and *HOXA*. Bmi-1 is a member of the polycomb group of proteins that plays an essential role in regulating the self-renewal of adult normal HSCs and LSCs.[54-56] The expression of Bmi-1 is increased in *SALL4B* transgenic mice compared to control wild type (WT) mice, both at preleukemic and leukemic stages.[57] In human MDS and AML, high expression of SALL4 is also associated with the expression of high levels of Bmi-1.[26,57] In addition, SALL4 can bind the Bmi-1 promoter and upregulate its expression, suggesting that Bmi-1 is a direct transcriptional target of SALL4.[57]

A SALL4/HOXA9 pathway has also been shown to mediate murine and human myeloid leukemogenesis. Gene expression profiles of leukemic cells from *SALL4B* transgenic mice identified *Hoxa9* as one of the top upregulated genes compared to WT mice.[48] HOXA9 is a homeobox domain-containing transcription factor that is important for myeloid leukemogenesis and a key cell survival factor for human leukemia subtypes with mixed-lineage leukemia (MLL) gene translocations.[58-60] Functional studies showed that downregulation of HOXA9 expression in SALL4B-overexpressing leukemic cells led to decreased replating capacity

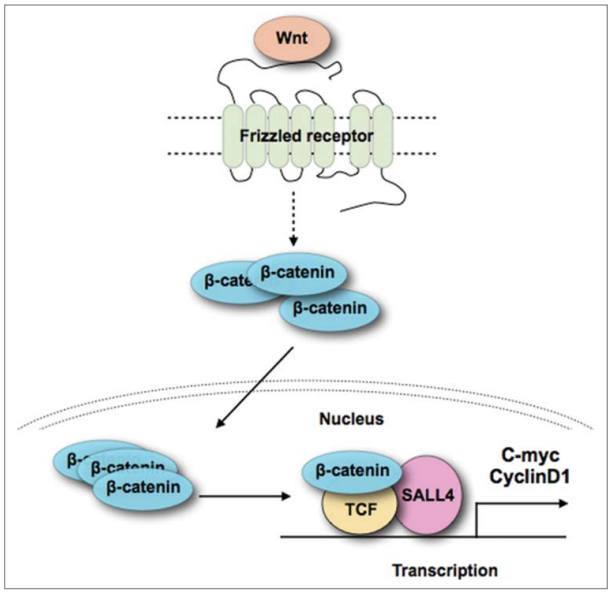

Figure 2. SALL4 contributes to leukemogenesis as a co-activator of the Wnt/ β-catenin pathway. SALL4 can bind β-catenin and form a complex with β-catenin/TCF. The SALL4/β-catenin/TCF complex can further activate downstream target genes *c-Myc* and *cyclin D1*.

of these cells *in vitro* and delayed AML development when they were transplanted into recipient mice. In human AML cells, HOXA9 expression is regulated by SALL4 through its interaction with MLL protein and their co-occupation of the *HOXA9* promoter region (**Fig. 3**).[48] As SALL4, MLL, and HOXA9 have all been implicated in the development of leukemia, this unique SALL4/MLL/HOXA9 pathway provides new insights into the pathogenesis of AML.

As a transcriptional repressor

Recent studies have demonstrated that a conserved N-terminal 12 amino acid (N-12aa) domain present in SALL1 and friend of GATA1 (FOG-1) is sufficient for recruiting the NuRD complex and mediating transcriptional repression.[61,62] SALL4 protein contains the same N-12aa motif,[8,61] and was also found to associate with the NuRD complex and function as a strong transcription repressor.[63] Phosphatase and tensin homolog protein (PTEN) was found to be downregulated in *SALL4B* transgenic mice. PTEN is encoded by a tumor suppressor gene that is essential for both normal hematopoiesis and leukemogenesis.[64,65] The

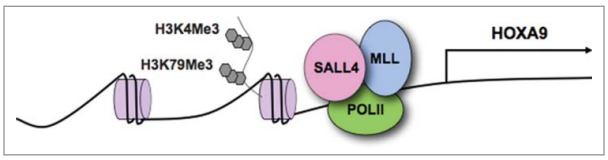

Figure 3. SALL4 contributes to leukemogenesis as a transcriptional activator. SALL4 interacts with MLL and co-occupies the *HOXA9* promoter region with MLL. This results in increased enrichment of activating histone markers such as H3K4 and H3K97 trimethylation and POLII binding in the region, which in turn leads to upregulation of HOXA9 expression.

subsequent observation that SALL4 co-occupies the *PTEN* promoter regions with a NuRD component, HDAC2, to repress *PTEN* expression[63] suggests that SALL4 represses PTEN through the epigenetic repressor NuRD. These results suggest a separate mechanism by which SALL4 functions in leukemogenesis through its association with the MLL/HOXA9 pathway (**Fig. 4A**).

In summary, SALL4 can regulate its downstream genes through multiple pathways and mechanisms. However, how SALL4 balances its functions as a transcription repressor and an activator remains unknown.

Targeting SALL4 by Blocking its Protein–Protein Interaction: A New Approach to Treat AML

The unique expression pattern of SALL4 in cancer cells and its essential functional role in cancer cell survival make it an ideal candidate for therapeutic targeting of cancer cells. Targeting transcription factors in cancer by blocking the formation of oncogenic complexes is an exciting new approach.[66-68] One important mechanism of SALL4 in the promotion of leukemogenesis is mediated by its transcriptional repression of the tumor suppressor PTEN through the HDAC-containing NuRD complex. The conserved 12 amino acids at the N-terminus of SALL4 directly interact with the complex. This binding, as well as PTEN repression, can be reversed using a peptide corresponding to the N-12aa of SALL4 that competes with and inhibits the SALL4–NuRD interaction (**Fig. 4B**). Treatment of SALL4-expressing malignant cells with the N-12aa peptide leads to cell death that can be rescued by a PTEN inhibitor. The antileukemic effect of this peptide was further confirmed in primary human leukemia cells in culture and *in vivo*, and is identical to the effect of downregulation of SALL4 in these cells using an RNAi

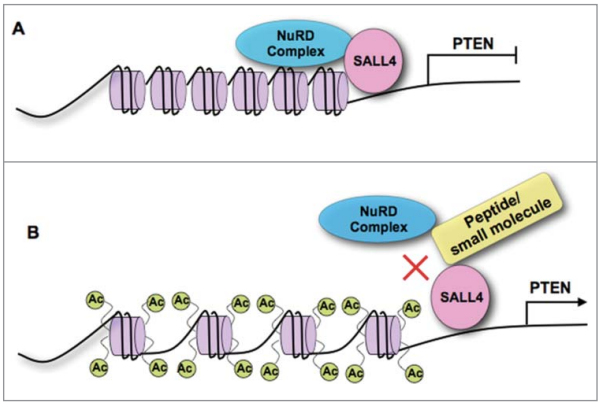

Figure 4. SALL4 contributes to leukemogenesis as a transcriptional repressor and targeting this pathway provides a new approach to targeting leukemia. (**A**) SALL4 recruits the NuRD complex to a specific region of the *PTEN* promoter, resulting in histone deacetylation, a more compact chromatin structure, and transcriptional repression. (**B**) A peptide derived from the 12-amino acid of SALL4 (or a small molecule) can compete with SALL4 for its interaction with the HDAC complex and reverse its effect on PTEN repression that leads to cell growth inhibition.

approach.[69] The antiproliferative activity of the SALL4-derived N-12aa peptide was also validated in hepatocellular carcinoma both *in vitro* and *in vivo* in a xenotransplant model.[28]

Interestingly, when the same SALL4 peptide was tested in CD34+ cells in *ex vivo* culture conditions, the expected increased in cell death was not observed. The differential effects of peptide treatment in SALL4-expressing leukemic cells and normal $CD34^+$ cells could be due to a differential functional role of SALL4 in these cells. Further studies are needed to elucidate the mechanisms by which the SALL4 peptide can induce different phenotypes in these 2 cell types.

Closing Remarks: The Future Direction of SALL4 as a New Cancer/Leukemic Target

In conclusion, aberrant expression of the oncofetal transcription factor SALL4 is generally associated with a more aggressive cancer phenotype and drug resistance. Targeting the SALL4/PTEN pathway by blocking the protein–protein interaction of SALL4 and its associated epigenetic NuRD complex is a novel approach to treating AML, and holds great potential for treating other SALL4-mediated oncogenic processes such as high-risk MDS and solid tumors. In the future, diagnostic analysis of the SALL4 expression level may be helpful not only for determining the patient prognosis but also for identifying candidates for SALL4-specific treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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