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Regulatory mechanism of Hematopoietic Stem Cell self-renewal by stem cell factor SALL4

A Thesis Presented

by

Jing Yang

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in Partial Fulfillment of the

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Stony Brook University

The Graduate School

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acceptance of the thesis.

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Second Reader

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Charles Tabler

Dean of the Graduate School

Abstract of the thesis

Regulatory mechanism of Hematopoietic Stem Cell self-renewal by stem cell factor SALL4

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Master of Science

in

Biochemistry and Cell Biology

Stony Brook University

2013

All types of blood cells in the human body are derived from Hematopoietic Stem Cells (HSCs).The self-renewal ability of HSCs ensure that blood cells replenish and sustain life. The regulatory mechanism of HSCs self-renewal, however, remains an unsolved problem. Recent studies found that the stem cell gene SALL4 has the ability to stimulate HSCs expansion and maintain their pluripotent tendencies. In this article we summarize the current research on SALL4 and its isoforms, and how they affect HSCs self-renewal capacity. To further understand the regulatory mechanism underlying this activity, researchers explored the connection between SALL4 and other regulatory molecules. They concluded that SALL4 may regulate HSCs self-renewal through recruiting epigenetic modifying enzymes and forming a transcriptional feedback loop

with the stem cell factor OCT4.

Dedication

To my parents and my friends,

for supporting me all the time.

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List of Abbreviations

HSCs	Hematopoietic Stem Cells
SALL4	sal-like 4
OCT4	octamer-binding transcription factor 4
HOXB4	homeobox B4
TAT	transactivator of transcription
SCF	stem Cell Factor
Flt3L	fms-related tyrosine kinase 3
TPO	thrombopoietin
CD34	cluster of differentiation 34
CD38	cluster of differentiation 38
Lin	lineage
Sal	spalt-like
NuRD	nucleosome Remodeling Deacetylase
ESC	embryonic stem cell
PTEN	phosphatase and tensin homolog
CHIP	chromatin immunoprecipitation
GFP	green fluorescent protein
CFU	colony-forming unit
iPSC	Induced pluripotent stem cells

SOX2	sex determining region Y-box 2
CDX2	caudal type homeo box 2
Fgf4	fibroblast growth factor 4
Fbxo15	F-box 15
Lefty1	left-right determination factor 1
DNMT	DNA methyltransferase
HDAC	histone deacetylases
HEK293	human embryonic kidney 293
HA	hemagglutinin
IP	immunoprecipitation
VPA	valproic acid
5-azac	5-azacytidine
RLU	relative light unit
FF	foreskin fibroblast
shRNA	short hairpin RNA

Acknowledgements

I would like to thank Dr. Jerell Aguila and Dr. Neta Dean for all the wise advice they give for this thesis.

1.Introduction

Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are responsible for the production of all types of blood cells. Mainly found in adult bone marrow, HSCs can differentiate to replenish mature blood cells. Furthermore, they have the ability to self-renew in order to maintain the HSC pool [1]. Because of these characteristics, HSC transplantation remains one of the most effective approaches to treat certain hematologic diseases.

However, there are currently bottlenecks restricting HSC transplantation success arising from the limited amount of transplantable HSCs acquired from the donor and the low efficiency of engraftment [2]. A possible reason for this limitation may be DNA damage that accumulates within aged HSCs. It is reported that telomere length is significantly shortened in HSCs derived from elderly people [3], which greatly compromises HSCs ability to proliferate and engraft after transplantation [4].In order to improve the success rate of HSC transplantation it will be necessary to develop methods that expand *in vitro* HSCs that retain their ability to proliferate.

HSC identity and purification

HSCs can be distinguished from other differentiated blood cells by their unique cell surface markers [10]. It is well accepted that CD34 is a reliable marker for recognizing and purifying human HSCs (although long-term self-renewal effects are also observed in the CD34- Lin- cell population [12]). Populations of CD34+CD38- and

CD34+CD38+ cells both exist in bone marrow. The CD34+CD38- population has better resistance to environmental variablity[13] and capacity to generate progeny over an extended long-term *ex vivo* culture. Thus, the CD34+ CD38- population is considered to be a highly primitive and less committed group of progenitor cells [14]. The CD34+ CD38+ population are considered later stage progenitors and are more mature and numerous than CD34+CD38- cells. Clinical evidence has proven that they are both capable of repopulating and are recognized as “true stem cells” [15].

Achievements in HSC expansion

Many proteins including transcription factors, developmental regulators, and cell cycle regulators are involved in the HSC self-renewal process [5], which suggests there are possible solutions for increasing HSC expansion speed *ex vivo*. Actually, numerous great advances are archived on expanding HSC *in vitro* through modifying related signaling pathways. One of the best characterized genes found to promote HSC self-renewal is HOXB4, whose overexpression in murine HSCs can produce a 40-fold expansion [6]. Nevertheless, a caveat in these particular studies was that the target gene was delivered by a retrovirus, which may result in unexpected insertional mutagenesis. In addition, the continuous activation of self-renewal also activates an oncogene. It is therefore preferable to introduce self-renewing factors in a controllable way. Stimulation of mouse HSCs is also possible through extrinsic addition of TAT-HOXB4 recombinant protein [7].TAT-HOXB4 treatment also has a positive effect on

long term engraftment in mice. Although the effect of extrinsic expansion is much lower compared with that of the intrinsically overexpressed protein, it offers a much safer means for clinical use. Considering the weakness of existing methods, an efficient way to expand HSC with fewer side effects still remains to be explored.

Hematopoietic cytokines

There is evidence showing that hematopoietic cytokines, which are produced by the *in vivo* HSC microenvironment, may also be involved in the regulation of HSC self-renewal [8]. Although solely using a combination of cytokines alone seems insufficient for the maintenance of HSC self-renewal, certain combinations of cytokines (SCF, Flt3L, TPO) can support HSC proliferation and maintenance. These cytokines are often used with other self-renewal factors in *ex vivo* HSC culture to improve the expansion effect [9], which suggests the existence of cytokines may facilitate HSC self-renewal.

SALL4

Human SALL4 is a zinc finger transcription factor. The original SALL4 transcription product contains 4 exons and 3159 bp of coding sequence. It encodes three C2H2 double zinc finger domains, including a NuRD binding domain at the N-terminal [20]. SALL4 exists in two isoforms, SALL4A and SALL4B. The two isoforms are the result of alternative internal splicing in exon 2. While SALL4A retains all exons and zinc finger domains, SALL4B exists without exon 2 and the two zing finger domains

within it [21]. The difference in structure lead to different roles of the two isoforms in stem cells[21].

SALL4A and SALL4B are known to be able to form homodimers and heterodimers by binding different DNA sites and have different roles in development. Genomewide analysis reveals SALL4A and SALL4B have overlapping but not identical binding sites within the ESC genome. While isoform depletion in murine ESC can lead to multilineage differentiation, only SALL4B has been shown to have the ability to maintain the ESC pluripotent state as it preferentially binds to active locations in ESC[17], which suggests the two isoforms may have different functions.

Many studies reveal that SALL4 plays a key role in the maintenance of stem cell pluripotency through its interaction with important stem cell factors. Analysis of target genes and binding sites of SALL4 using chromatin immunoprecipitation has identified more than 30 signaling pathways that SALL4 is involved in[18],including Notch, Bmi-1, and PTEN. A CHIP-chip assay also suggests that SALL4 has varying promoter binding sites implying that SALL4 may have a broad function in ESC regulation properties [19].

SALL4 stimulates HSCs expansion and self-renewal

A recent study concluded that SALL4 is a robust stimulator for HSC expansion [22]. HSC transduced with either SALL4A or SALL4B as well as cytokine supplements (SCF, TPO, and FLT-3L) in cell culture showed approximately a120-fold expansion, while control cells transduced with a control gene only expanded 13-fold. After

a14-days culture, the primitive progenitor population (CD34+ CD38-) increased more than 300 fold in SALL4 transduced HSC. During the rapid expansion period, SALL4 transduced cells maintained the stem cell immunophenotype. Flow cytometry proved the cell group retained similar percentages of stem cell populations (15% CD34+/CD38-, 17% CD34+/CD38+) after two months culture compared to 31-day-old cells, while the control stem cells differentiated rapidly.

Overall, SALL4-transduced cells exhibited a total of more than 10,000 fold CD34+/CD38- stem cell expansion over control, and there was no statistically significant difference between SALL4 isoforms. This stimulating effect is far superior to other known approaches such as Notch ligand[22], bring out that SALL4 is a very promising gene for HSC expansion.

Xenotransplantation assays further revealed that SALL4 overexpressed cells maintained the engraftment capabilities into NOD/SCID mice 4 weeks post-injection. Not only could CD45+ human leukocytes be detected from NOD/SCID mice peripheral blood, but myeloid (CD15+) and lymphoid (CD19+) lineages were also present indicating a proper differentiation process. Myeloid and lymphoid lineages were even detected in bone marrow from animals 15 weeks post injection.

Secondary and tertiary transplantations were performed and consequently proved that these cells can survive in the bone marrow niche, differentiate properly, and increase the repopulating ability of human cells in mice.

Researchers have also tried to transduce SALL4 into cells utilizing cell penetrating peptides. After adding transactivating protein (TAT) fused SALL4B twice per day for 3 days, treated cells showed an overall ten-fold CFU number increase compared to the control. All these experimental facts prove SALL4 is a strong stimulator for HSC expansion and self-renewal. In addition the stimulating effect of TAT-SALL4 protein implies SALL4 is extremely valuable in clinical use[22].

HSCs self-renewal related genes

OCT4

Well-known for being involved in reprogramming somatic cells and inducing iPSC, the POU family transcription factor OCT4 is indispensable when discussing the stem cell pluripotency regulation network. It has been reported that the self-renewal capacity of stem cells is critically regulated by a group of genes consisting of OCT4, Nanog, and SOX2 in ESCs [23]. Expression level of OCT4 is strictly controlled by several negative-feedback loops as even a slight change in OCT4 level can cause stem cell differentiation. The repression of OCT4 leads ESCs to differentiate into trophectoderm. OCT4 can prevent differentiation by interacting with Cdx2, a differentiation inducer, and forming a repressor complex working to regulate both molecules [26]. Overexpression of OCT4 can stimulate ES cells to differentiate along endoderm-like cell lineages [25]. Two stem-cell-specific enhancers, which recruit positive and negative regulators have been

identified so far [24]. The balance between these regulators helps to determine the appropriate level of OCT4 expression. SALL4 has been reported to interact with OCT4 in murine ESCs [36],which suggests that the stimulation effect of SALL4 may relate to Oct4. Nevertheless, it is still unknown that whether human SALL4 can directly regulate human Oct4 expression.

Sox2

Sox2 belongs to the Sox family of transcription factors responsible for embryonic development regulation and determination of cell fate. It also plays an important role in maintaining stem cell self-renewal and pluripotency. Similar to OCT4, the Sox2 level needs to be controlled precisely. These two proteins form an OCT4-Sox2 complex. Many stem cell specific genes have been identified to possess juxtaposed OCT4 and Sox2 binding sites. They also have been recognized and regulated by the OCT4-Sox2 complex to maintain ESC pluripotency. So far five genes have been discovered: Fgf4, Fbxo15, Nanog, Utf1, and Lefty1 [27].Given facts that decreasing Sall4 expression in ES cells induce lower expression level of Sox2[19] and SALL4 and Sox2 form complex with Sall4/Sox2-overlapping genes[39], Sox2 may play an important role in SALL4 stimulation.

Nanog

Partially regulated by OCT4 and Sox2, Nanog is also included in the transcription regulation network. Although Nanog does not seem to trigger

reprogramming, it is still necessary for stem cells to acquire pluripotency. While cells depleted of Nanog can begin reprogramming, they cannot transition to full pluripotency until Nanog expression is restored [28]. Thus Nanog is considered to function in the later stages, establishing the self-sustaining state [29]. Nanog and SALL4 are found co-occupied enhancer regions of both genes. Furthermore, Knockdown of Nanog and SALL4 can also induce enhancer activity reduction. These experimental evidence indicate Nanog is also a key factor in SALL4 regulation mechanism.

Epigenetic modification

Given the fact that stem cells and somatic cells have identical genomic DNA except for very few exceptions, epigenetic modification is one of the major influences on cell differentiation potential and is extremely important for pluripotency [30]. Chromatin, as the substrate of epigenetic modification, is subject to many modulations such as DNA methylation, histone modification, and histone variants. To maintain pluripotency, genes inducing cell differentiation in stem cells need to be repressed and specific genomic regions are modified. For instance, while acetylation of histone 3 and histone4 result in active transcription [31], DNA methylation of certain amino acids of histone 3 usually indicates gene repression [32]. These modifications rely on various epigenetic regulators such as DNA methyltransferase (DNMT) and Histone deacetylase (HDAC).

Stem cell factors also work together with epigenetic regulators to support stem cell self-renewal ability. Some factors have been proven directly regulate epigenetic

modification enzymes. For example, OCT4 and Nanog are reported to upregulate Dnmt1 through direct binding to its promoter, as the promoter activity is enhanced by overexpression of both Oct4 and Nanog [33]. The pluripotency factors also recruit epigenetic regulators to target genes that control stem cell state. Nanog and OCT4 have also been confirmed to associate with repression complex such as NuRD on their target genes to regulate stem cell differentiation [34].

Results

Sall4 and OCT4 activate each other

To determine whether SALL4 could regulate OCT4 expression in human stem cells, A plasmid was constructed that expressed the luciferase reporter and promoter sequence upstream of an OCT4 translation start site. The relationship between the SALL4 and the OCT4 promoter was studied by co-transfecting the OCT4 promoter and SALL4 to HEK293 cells. The ratio of SALL4A or SALL4B to OCT4 promoter was generally increased to see whether OCT4 promoter could be still activated (Figure 2.1 [41]). As seen in Figure 2.1, the OCT4 promoter activity is significantly improved when co-transferred with 2 fold-excess of either SALL4A or SALL4B constructs. When the ratio of SALL4 to OCT4 promoter increases to a ratio of 3:1, the OCT4 promoter is stimulated even further, and its activity reaches a very high level.

It has been reported that OCT4 also binds the SALL4 promoter site in murine cells [37].Considering it is common that transcription factors regulate each other, to confirm if OCT4 can regulates SALL4 transcription, researchers also co-transfected the SALL4 promoter construct P2102 and plasmid expressing OCT4 in HEK293 cells to evaluate the regulation effect of OCT4 on Sall4 (Figure 2.2[41]). Surprisingly, the introduction of OCT4 raised the SALL4 promoter activity 50-fold compared to the negative control whereas the SALL4 promoter activity significantly dropped when an additional SALL4 expression construct was added. The fact that the co-expression of

SALL4 fully recovered from this effect indicated that SALL4 may also have control over this regulation loop. These experiments together reveal that there exists a transcription regulation feedback loop between SALL4 and OCT4.

Self-expression effect of SALL4

SALL4 can also regulate its own expression in various types of cells including human ESCs. To confirm the role of SALL4 in regulating its own promoter, both SALL4A and SALL4B have been used to examine the self-repression effect of SALL4 by being co-transfected together with the SALL4 promoter construct, p2102 in HEK-293, COS-7, H9 and W4 cells. The results were then determined by relative luciferase assays(Figure 2.3A [41]). As the figure shows, the self-repression effect could be observed in all cell types, and is dose-dependent. (Figure 2.3B [41]) When the ratio of SALL4 to promoter of SALL4 increases, the amount of detectable RLU decreases, indicating a drop in promoter activity.

SALL4 interacts with DNMT and HDAC

Although the SALL4-OCT4 transcriptional feedback loop has been proven to exist, how SALL4 represses target gene promoters, including its own, at the molecular level remains unknown. A recent study showed that a SALL family member could recruit the Mi-2/Nucleosome Remodeling and Deacetylase (NuRD) complex and participate in epigenetic modification [35]. Given this fact, the researchers hypothesized that SALL4 may also interact with chromatin modifying enzymes, such as HDAC and DNMT, to

mediate target gene expression.

Researchers first tested if SALL4 interacted with HDAC and DNMT proteins. SALL4 isoforms with an HA tag were transfected into HEK293 cells and the lysates of the transfected cells were used for Co-IP using an HA antibody. Western blotting was then utilized to analyze the physical interaction between the proteins and corresponding antibodies (Figure 2.4A [40]). According to the figure, both SALL4A and SALL4B both interacted with DNMT1, DNMT3A, DNMT3B, DNMT3L, and HDAC.

To further confirm whether the SALL4-DNMT and SALL4-HDAC complexes contained enzymatic activity, complexes were pulled down with HA antibodies and examined (Figure 2.4B [40]). The result demonstrated that protein complexes containing SALL4 isoforms both had high DNMT enzymatic activity, which was more than 3 times higher compared to control. High HDAC enzymatic activity was also detected in SALL4-HDAC complexes (Figure 2.4C [40]). These results strongly prove that SALL4 is not only physically associated with epigenetic modification enzymes, but also functions together with the enzymes by forming a complex.

DNMT inhibitors (5-AZAC) and HDAC inhibitors (VPA) block repression effect of SALL4

Given the experimental fact that SALL4 form complexes with HDAC and DNMT, researchers firstly examine if depletion of HDAC can eliminate the expression effect of SALL4 at first.The HDAC inhibitor VPA was used to block HDAC. Firstly, full length

SALL4A or SALL4B were co-transfected with a SALL4-pGL3 promoter to a HEK293 cell line. To ensure that HDAC function was fully blocked, 20ng/ml VPA was applied to the cell culture at the same time. Relative luciferase assays were then performed on both untreated and VPA treated groups to detect the inhibition effect 24 hours after transfection (Figure 2.5a, 2.5b [40]). The repression effect of SALL4 towards promoter was decreased to ~50% compared to the untreated cells as the RLU analysis demonstrated.

Since the SALL4-regulated repression effect was only partially recovered by blocking histone deacetylation, it's possible that another epigenetic regulation mechanism, such as methylation mediated by DNMT, was also involved in the repression effect of SALL4. To confirm this speculation, full length SALL4 was co-transfected with OCT4-pGL3 promoter into a HEK293 cell line with 5-azac,a DNMT inhibitor, instead of VPA. The effect was then measured by relative luciferase units (Figure 2.5c [40]).As the figure shows, 5-azac successfully reduced the inhibition effect induced by HDAC by roughly 50%.

Conclusively, when cells are co-treated with 5-aza and VPA, the regulation repression effect of SALL4 is almost fully reversed, shown by the RLU assay result (Figure 2.5d [40]).

SALL4 expression level affects DNA methylation state

To examine if SALL4 directly mediated chromatin modification through DNMT in

primary cells, forced SALL4 expression was performed in distinct cell lines and the change of DNA methylation status was monitored by using bisulfite sequencing. Two specifically chosen cell lines were used for overexpression: HEK293 cells and primary human foreskin fibroblast (FF) cells. The FF cells do not have measurable endogenous SALL4 expression, while HEK293 cells show high-levels of endogenous SALL4 expression. The cells were separately transfected with either SALL4 or Vector. Gene sequencing was applied 24 hours post transfection. In SALL4 treated HEK293 cells, there was no obvious difference in the methylation state of the SALL4 promoter compared to the vector treated group (Data not shown). This may have been due to the relative high endogenous SALL4 expression. Nevertheless,the frequency of methylated residues in SALL4 overexpressed FF cells increased to 86% in the GC-rich regions and CPGs, which is significantly higher than the 58% methylation rate seen in the group of cells expressing empty vectors (Figure 2.6a,2.6b[40]).

While forced SALL4 expression is proved to increase DNA methylation rate, testing the effect of SALL4-knockdown on DNA methylation is also very essential.

Two short hairpin RNAs (shRNA) named #7410 and #7412, previously proved to be able to knock down SALL4 mRNA , were induced to HEK293 cells and control shRNA was applied as well. After 5 days of puromycin selection genomic DNA was harvested and then sequenced to evaluate the methylation levels of G-C rich sites (Figure 7a, 7b [40]). As expected, the HEK293 cells transfected with 7410 and 7412 shRNA showed a

dramatic increase in methylation level while the control group maintained low-methylation state. These two facts taken together revealed that SALL4 recruits DNMT to regulate the DNA methylation state.

Discussion

SALL4 has been considered to play an important role in both developmental events and hematopoietic stem cell self-renewal since it was identified as a stem cell factor. Based on the recent discovery that SALL4 significantly simulates HSCs expansion and maintain their pluripotency, researchers have decided to investigate the SALL4 regulation mechanism.

As a transcription factor, SALL4 is inevitably involved in a regulatory network and functions together with other transcription related molecules. Previous findings suggest that SALL4 interacts with the well-known stem cell factor OCT4, which cooperates with Nanog and Sox to support ES self-renewal. This relationship between these factors has shed light on the role of SALL4 in this core network.

Researchers initially investigated the effects of SALL4 on OCT4 expression. They found that SALL4 could efficiently increase OCT4 expression by activating the OCT4 promoter. At the same time, co-transfection of the SALL4 promoter and OCT4 expression construct revealed that additional OCT4 can up-regulate SALL4 expression as well. This proved that SALL4 and OCT4 form a positive transcriptional feedback loop. Additional experiments have discovered that SALL4 itself acts as the negative regulator of this loop. Researchers were additionally able to confirm that all these mechanisms are dose-dependent by manipulating the ratio of corresponding constructs. The role of SALL4 in the OCT4-Nanog-Sox2 regulatory circuit, however, hasn't been fully revealed

(Figure 3.2). For example, the detailed mechanism of how SALL4 and OCT4 activate each other still remains unknown. Given the fact that SALL4 also interacts with Nanog [38] and Sox2 [39], it's possible that SALL4 regulates their expression and is able to form a complicated network with them. Further experiments are still needed to test the specific relationship between SALL4 and other stem cell genes.

It's common for transcription factors to modulate chromatin in order to regulate DNA transcription activity and maintain stem cell pluripotency. This process includes DNA methylation and histone modification, which are associated with epigenetic modification enzymes. Naturally, researchers hypothesized that SALL4 also played a role in recruiting such enzymes. They conducted Co-IP experiments to prove that protein interactions existed between SALL4 and the two epigenetic modification enzymes, DNMT and HDAC. Purified SALL4-enzyme complexes were additionally confirmed to demonstrate high enzyme activity. This revealed that SALL4 formed functional complexes with epigenetic modification enzymes without disturbing their activity. Furthermore, while treating cells separately with VPA or 5-azaC only partially blocked the repression effect of SALL4, utilizing both simultaneously could almost fully reverse the SALL4 repression. This clearly showed that DNA methylation and histone deacetylation greatly contribute to the transcriptional repression effect of SALL4 (Figure 3.1, J Yang et al.,2012). Nevertheless, there existed a small difference between the co-treated group and negative control, suggesting that there may be some other

regulation mechanism involved. Further tests should be performed to help identify the connection between SALL4 and other key regulatory enzymes and clarify the exact mechanism. It was also deduced through the forced expression and knockdown of cellular SALL4, that a change of SALL4 level in cells directly influences the chromatin methylated state. However, additional biochemistry studies need to be carried out to uncover the specific genomic regions affected. The development of *in vivo* model would be very helpful for testing the clinical application of SALL4 since all the previous models were established *in vitro*.

Overall, these studies clarify the role of SALL4 in regulating HSCs self-renewal. SALL4 cooperates with stem factors such as OCT4 and regulates their activity in order to maintain a balance of the transcriptional network. Simultaneously, SALL4 can repress gene expression by directly recruiting epigenetic modifying enzymes.

Figures

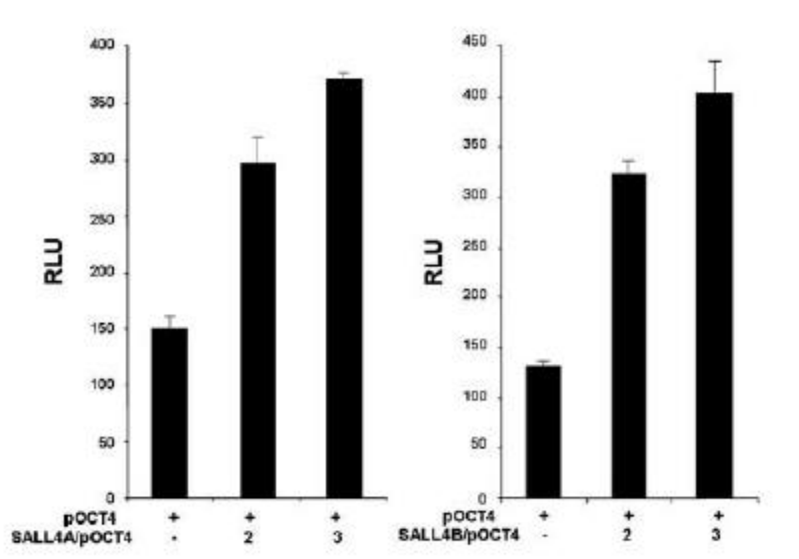

Figure 2.1: Dose-dependent activation of the OCT4 promoter by SALL4 isoforms(J Yang et al.,2010).

0.3 mg of the OCT4-Luc promoter construct was cotransfected with 0.07 mg of Renilla plasmid and increasing ratios of either the SALL4A (left) or SALL4B (right) expressing construct into HEK-293 cells, pcDNA3 was used as the control. Y axis: relative luciferase unit (RLU). Data represent three independent experiments. Error bars denote standard deviation (SD) [41]

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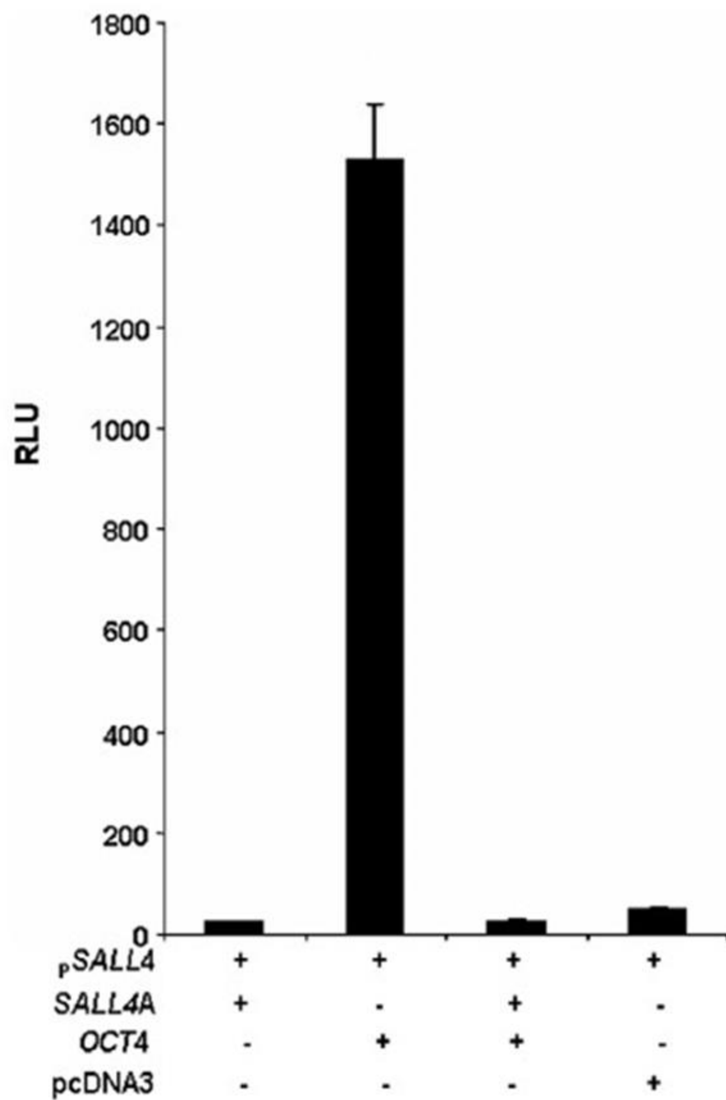


Figure 2.2: Promoter activity of SALL4 is dramatically enhanced by over-expressing OCT4, and SALL4 negatively regulates itself (J Yang et al.,2010). 0.3 mg of SALL4 P2102 was cotransfected with 0.07 mg of the Renilla reporter and 0.9 mg of either a SALL4A expression plasmid (first column), or an OCT4 expression plasmid (second column). The third column represents cotransfection of both the SALL4A and OCT4 expression plasmids. Relative luciferase activity is depicted relative

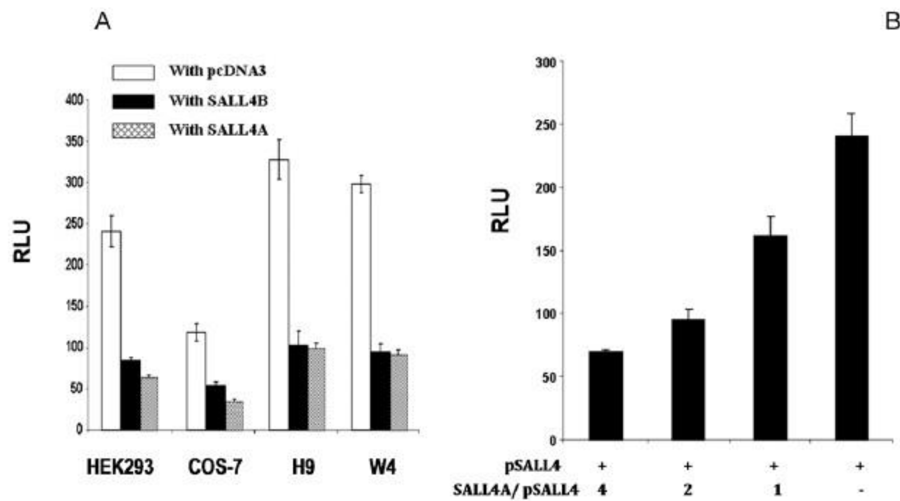
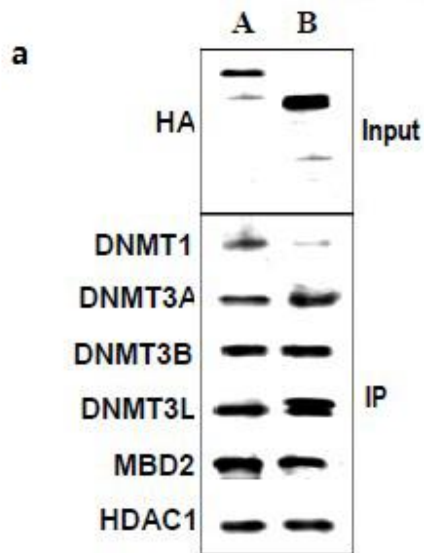
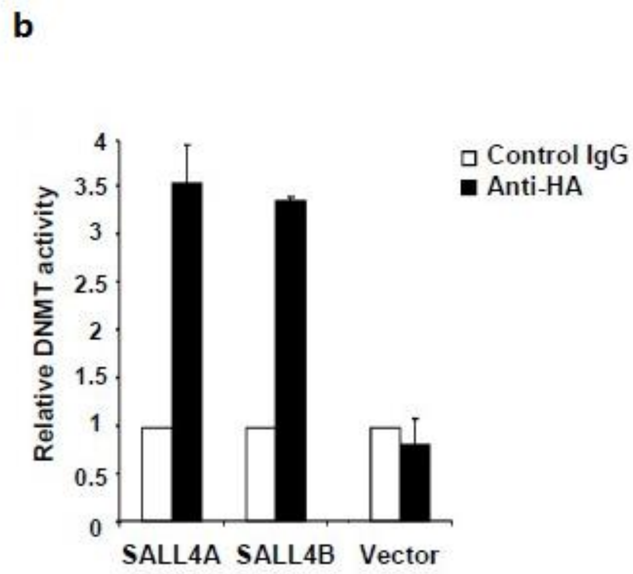


Figure 2.3: Auto-negative regulation of SALL4 isoforms (J Yang et al.,2010).

(A) Self-suppression of the SALL4 promoter by SALL4A and SALL4B in different cell types.0.3 mg of SALL4 P2102 was cotransfected with 0.07 mg of the Renilla reporter and 0.9 mg of either SALL4A (hatched bars) or SALL4B (black bars)expressing plasmid in four different cell lines (HEK-293, COS-7, human ESC H9 and mouse ESC W4). The pcDNA3 empty vector was used as control (white bars), and the luciferase activity was normalized to Renilla reporter activity. Y axis: relative luciferase unit (RLU). Data represent threeindependent experiments. Error bars denote standard deviation (SD). (B) SALL4 suppresses its own promoter activity in a dose- dependent manner inhuman embryonic stem cells. Using an approach similar to Figure 3, in human ES H9 cells, 0.3 mg of SALL4 P2102 was cotransfected with 0.07 mg of theRenilla reporter and increasing ratios of SALL4A. First bar, 4:1; second bar, 2:1; third bar, 1:1; fourth bar, pcDNA3 control. When the ratio of SALL4A toSALL4 promoter reporter reached 4:1, the promoter activity dropped approximately 3 fold when compared with the basic level. Y axis: relative luciferase unit (RLU). Data represent three independent experiments. Error bars denote standard deviation (SD) [41]


a
A
B
HA
Input
DNMT1
DNMT3A
DNMT3B
DNMT3L
IP
MBD2
HDAC1


b
Control IgG
Anti-HA
Relative DNMT activity
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3.5
3
2.5
2
1.5
1
0.5
0
SALL4A
SALL4B
Vector

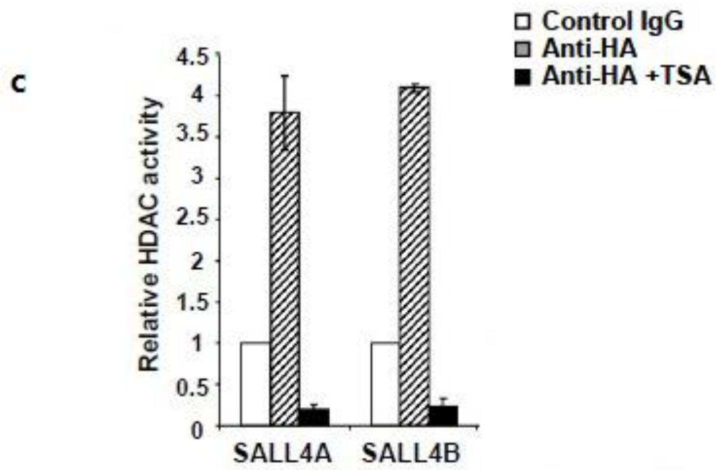
c
Control IgG
Anti-HA
Anti-HA +TSA
Relative HDAC activity
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
SALL4A
SALL4B

Figure.2.4: Interaction of SALL4 proteins with different DNMTs and HDAC1(J Yang et al.,2012).

(a) SALL4 isoforms a were transfected into 293 cells in 60mm culture dishes. After 20 hours, cells were lysed and about 100ul cell lysates per 5ug anti-HA antibody or IgG control were used for Dynabeads ProteinG immunoprecipitation. Anti-HA immunoprecipitates were analyzed by western blotting with antibodies against HDAC1 or indicated DNMT antibodies. Immunoprecipitation of SALL4 isoforms from HEK293 nuclear extracts purified different amount of DNMT (b) and HDAC (c) activities [40].

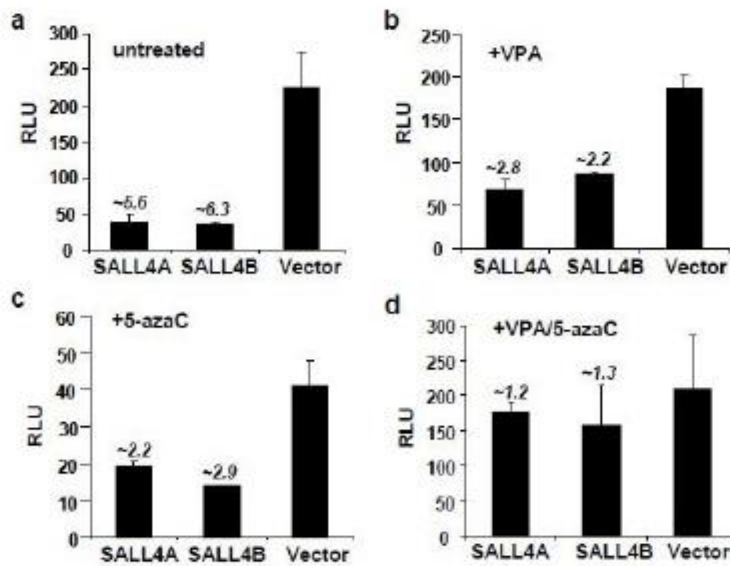

Figure. 2.5: Repression of target promoter activities by SALL4 is reversed by cotreatment of 5-azaC and VPA (J Yang et al.,2012).

A 2kb SALL4-pGL3 promoter construct was cotransfected with SALL4A or SALL4B expressing plasmids in HEK293 cells. Luciferase activity assays were performed from lysates of untreated (a), VPA only (b), 5-azaC only (c), or co-treated (d) cells [40].

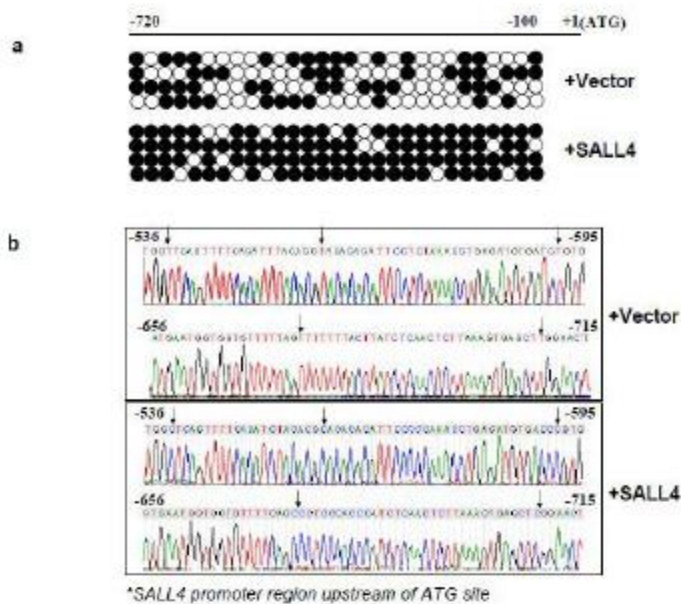

Figure. 2.6: Forced expression of SALL4 brings about increased DNA cytosine methylation of target genes (J Yang et al.,2012).

(a) Human primary FF cells were transfected with SALL4A- expressing plasmid or vector control. Bisulfite sequencing shows that the SALL4 proximal promoter is differentially methylated at specific CpGs or GC rich sites between the two groups of cells. Open circle: no methylation of cytosine; Filled circle: methylated cytosine. (b) Chromatogram of one sequenced clone derived from bisulphite-treated FF cells which were transiently transfected with SALL4 expressing plasmids or pcDNA3 vector control. Arrows indicate differentially methylated sequences between the two cell types; unmethylated C converted to T upon bisulphite treatment [40]

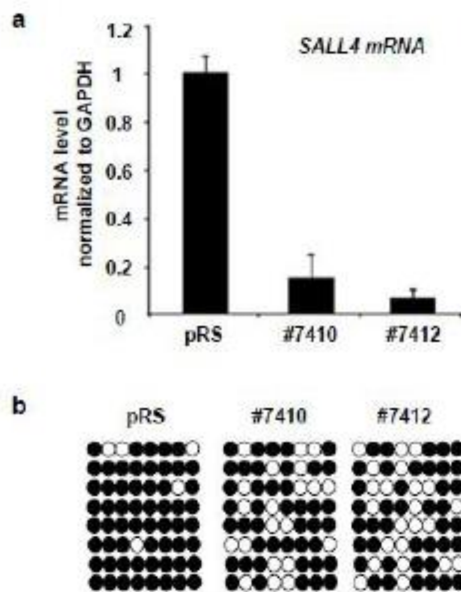

Figure. 2.7: Down-regulation of SALL4 led to decreased DNA methylation at its own promoter (J Yang et al.,2012).

(a) Detection of shRNAs interferential efficiency by two SALL4-specific oligos. Relative reduction of SALL4 mRNA by #7410 and #7412 was 83.3% and 92.9% respectively. (b) DNA methylation status of the endogenous SALL4 promoter (from -965 through -845) was examined by bisulphite genomic sequencing after control shRNA (pRS) or SALL4 specific shRNA treatment. Filled and open circles represent methylation and unmethylation, respectively [40]

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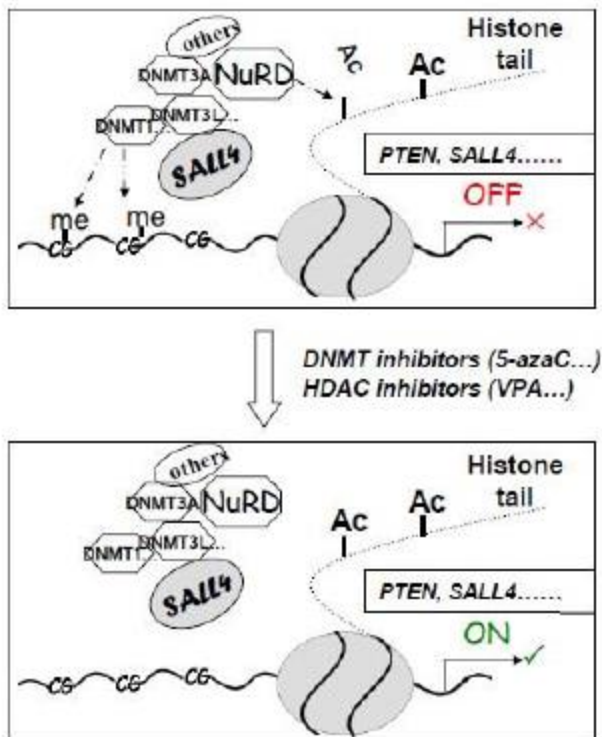

Figure 3.1: Hypothetical model of SALL4 recruits epigenetic regulators for gene suppression(J Yang et al.,2012).

SALL4 selectively recruits epigenetic modulators (DNMT1, DNMT3A, DNMT3B, DNMT3L, MBD2, HDAC1, HDAC2, and most likely others) to specific DNA sequences of its downstream targets for gene suppression, whereas both classes of epigenetic drugs may block this process and reactivate gene expression [40]

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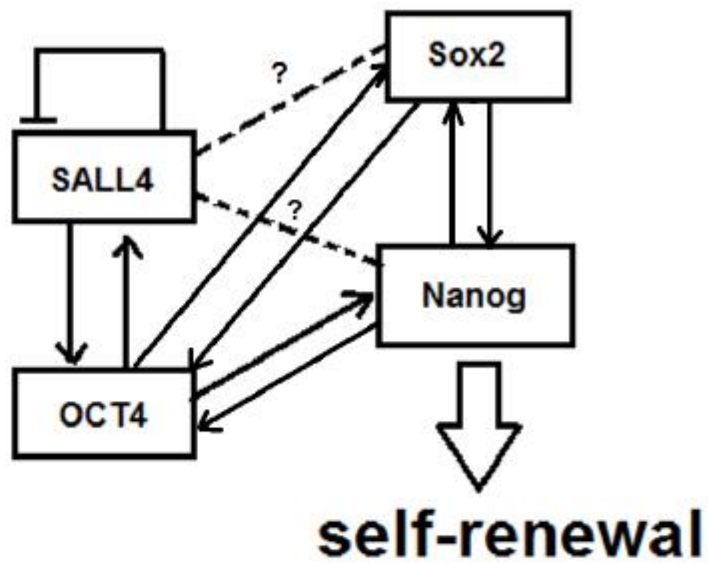

Figure 3.2: the role of SALL4 in stem cell self-renewal regulation network. Nanog, OCT4 and Sox2 regulate each other.SALL4 and OCT4 form a positive regulatory feedback loop, while SALL4 maintains self-repression.whether SALL4 regulates Nanog and Sox2 remains to be explored.

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