NIH Public Access

Author Manuscript

Stem Cells. Author manuscript; available in PMC 2012 April 7.

Published in final edited form as:
Stem Cells. 2011 April ; 29(4): 660–669. doi:10.1002/stem.623.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Promyelocytic leukemia protein in retinoic acid-induced chromatin remodeling of *Oct4* gene promoter

Ya-Shan Chuang[*], **Wei-Hong Huang**[*], **Sung Wook Park**, **Shawna D. Persaud**, **Chen-Hsiang Hung**, **Ping-Chih Ho**, and **Li-Na Wei**
Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN 55455, USA

Abstract

Promyelocytic leukemia (Pml) protein is required for *Oct4* gene expression and the maintenance of its open chromatin conformation in stem cells. In proliferating stem cells, Pml-nuclear body, along with transcription factors TR2, SF1 and Sp1 and Brg1-dependent chromatin remodeling complex (BRGC), associates with conserved region 1 (CR1) of this promoter to maintain a nucleosome-free region for gene activity. Retinoic acid (RA) rapidly down-regulates Pml, resulting in the replacement of BRGC with Brm-containing remodeling complex (BRMC), disassociation of SF1 and SP1, retaining of TR2, recruitment of RIP140, G9a and HP1γ, and sequential insertion of two nucleosomes on CR1 that progressively displays repressive heterochromatin marks. This study demonstrates a functional role for Pml in maintaining a specific open chromatin conformation of the *Oct4* promoter region for its constant expression in stem cells; and illustrates the mechanism underlying RA-induced chromatin remodeling of *Oct4* gene in differentiating cells, in which Pml plays a critical role. The study also demonstrates a novel mode of chromatin remodeling which occurs by repositioning and sequentially inserting nucleosomes into a specific region of the gene promoter to compact the chromatin in differentiating cells.

Keywords

Retinoic acid; P19; Oct4; Pml-NB; TR2; Embryonic stem cells; Chromatin remodeling

INTRODUCTION

Oct4, or POU-domain class 5 transcription factor 1 (Pou5f1), is critical for the maintenance of embryonic stem (ES) and embryonal carcinoma (EC) cells that share similar stem cell properties, as well as induced pluripotent stem cell reprogramming [1–5]. In ES or EC cell

Correspondence Information: Li-Na Wei: Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN 55455, USA. Telephone: +1 612 6259402, Fax: +1 612 6258408, weixx009@umn.edu.
[*]These authors contributed equally to this work.

Author contributions:
Y.S.C.: Experimental design, collection and/or assembly data, data analysis and interpretation, manuscript writing;
W.H.H.: Experimental design, collection and/or assembly data, data analysis and interpretation, manuscript writing;
S.W.P.: Experimental design, collection and/or assembly data, data analysis and interpretation;
S.D.P.: collection and/or assembly data;
C.H.H.: collection and/or assembly data;
P.C.H.: collection and/or assembly data;
L.N.W.: Conceptual development, experimental design, data analysis and interpretation, manuscript writing

Disclosure of Potential Conflicts of Interest
The authors indicate no potential conflict of interest.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

cultures, it is critical not only to have this gene constantly activated, but also to tightly regulate its expression level. The gene, *Pou5f1* or *Oct4*, is regulated by multiple regulatory regions including the distal enhancer (DE), proximal enhancer (PE), and proximal promoter (PP) [6]. In stem cells, PP regulates this gene through the activities of multiple activating transcription factors (TFs) including specificity protein 1 (Sp1) [7], steroidogenic factor 1 (SF-1) [8] and testis receptor 2 (TR2) [9], of which the binding sites are clustered in the most proximal Conserved Region 1 (CR1). In retinoic acid (RA)-exposed cells, TR2 is rapidly stimulated for interaction with promyelocytic leukaemia (Pml) protein and is subsequently sumoylated, then functions as a repressor for this gene [9]. RA at a physiological concentration (0.1–1 μM) suppresses this gene and triggers cell differentiation in ES and EC [10, 11]. Changes in histone methylation and acetylation on PP are found in differentiating EC cells [6]. However, neither the chromatin conformation of this gene promoter in stem cells nor its chromatin remodeling process for gene silencing during stem cell differentiation was clear. Further, since multiple TFs can activate this gene, one crucial question concerns how they are coordinated.

Pml proteins, together with other nuclear proteins including Fas death domain-associated protein (Daxx) and nuclear dot-associated Sp100 protein (Sp100), and CBP/p300, etc. [12], form Pml-nuclear bodies (NBs) [13, 14]. Pml-NBs appear near highly acetylated chromatin and can associate with certain nascent RNAs, suggesting a relationship of Pml-NBs with gene activation [15]. Our earlier studies showed RA-stimulated recruitment of TR2 to Pml for its sumoylation and repressing the *Oct4* gene in RA-exposed cells [9]; however, it was unclear if Pml played additional roles in the maintenance or regulation of this gene activity and if it had any relationship with the gene’s chromatin conformation or coordination of its multiple TFs and potential chromatin remodelers. A study of the major histocompatibility complex (MHC) locus indicated that Pml-NBs organize genes located within this locus into a high-order chromatin-loop structure [16]. Interestingly, the *Oct4* gene is found within the MHC gene cluster [17]. Other studies suggested that Pml-NBs regulate transcription by recruiting transcription factors [18] and participating in chromatin remodeling [16], but there has been no direct evidence for either Pml’s functional role or its mechanism of action in remodeling chromatin.

The current study determines the specific chromatin conformation of *Oct4* PP in stem cells, and establishes a functional role for Pml in maintaining this gene’s active chromatin conformation by recruiting specific TFs and chromatin remodelers. The study further delineates the chromatin remodeling process of this gene in RA-induced cell differentiation process where cells are rapidly depleted of Pml during early RA-induction, and uncovers a specific mode of chromatin remodeling by ordered nucleosome insertion/reposition on *Oct4* PP in differentiating cells.

MATERIALS AND METHODS

Cell culture and treatment

The P19 embryonal carcinoma (EC) cell line was purchased from ATCC. P19 cells were maintained in alpha-MEM medium containing 7.5% calf serum, 2.5% fetal bovine serum and 1% penicillin streptomycin at 37°C in 5% CO_2. CJ7 ES cells were maintained in ES medium (DMEM; Dulbecco’s modified Eagle’s medium), supplemented with 17% ES-cell-qualified fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 6 μM β-Mercaptoethanol, 2 mM HEPES, and 1000 U/ml recombinant leukemia inhibitory factor (LIF). Cells were grown on irradiated mouse embryonic feeder cells (MEFs) in 0.1% gelatin-coated plates. For RA treatment, experiments were conducted with 1 μM RA for 72 hours.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Luciferase reporter assay

Luciferase reporter assays were performed as previously described [19]. Briefly, P19 cells were cultured in 24-well plates and transfected with 0.3 μg *Oct4*-reporter plasmid, proper amount of expression plasmids (such as CMV-PML, CMV-Sp1, CMV-SF1 and CMV-TR2) and 0.1μg of SV40-LacZ by using Lipofectamine2000™ (Invitrogen). 24hr after transfection, cells were lysed and luciferase and lacZ activities were determined.

RNA interference

PML-siRNA, TR2-siRNA, Sp1-siRNA and SF1-siRNA were purchased from Qiagen. siRNAs were introduced into cells by HiPerfect (301704, Qiagen) for 72 hours and then mRNA or protein was collected for RT-qPCR or western blot.

Reverse transcription and Real-Time PCR

Total RNA was extracted from P19 EC cells using Trizol reagent according to the manufacturer's instructions (Invitrogen). RNA was reverse-transcribed by using the Omniscript RT kit (205113, Qiagen). For real-time PCR analysis, 2X Brilliant II Master Mix (600804, Agilent Technologies) was used, and PCR was performed on an MX3000P Stratagene thermocycler. The relative values were normalized to β-actin and presented as ΔΔCt methods [20]. Primer sequences are listed below.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described [21], using the following antibodies: Pml (sc-18423, Santa Cruz), Daxx (07-471, Millipore), TR2 (sc-9087, Santa Cruz), Sp1 (07-645, Millipore), SF1 (sc-10976X, Santa Cruz), BAF155 (sc-9746X, Santa Cruz), Brg1 (sc-10768X, Santa Cruz), Brm (sc-28710X, Santa Cruz), G9a (3306, Cell Signaling), RIP140 (ab42126, Abcam), H3 (ab1791, Abcam), H3.3 (ab62642, Abcam), H3K9me3 (CS200604, Millipore), H3K27me3 (ab6002-25, Abcam), AcH3 (06-599, Millipore), HP1γ (sc-10213, Santa Cruz) and RNAPII (05-623, Millipore). Primer set 8, used to monitor CR1 in most ChIP assays, was 5′-CCCACAGCTCTGCTCCTCC-3′ (forward) and 5′-AGCCCTGCTGACCCATCACC-3′ (reverse). All primers were listed in supplementary information.

MNase nucleosome mapping and restriction enzyme accessibility assays

Micrococcal nuclease (MNase) digestion was performed as described [22]. Nuclei isolated from P19 cells were digested with 20 and 80 U of MNase (Worthington) at 37°C for 5 min, followed by proteinase K treatment at 37°C overnight. The purified DNA was subjected to Southern blot analysis. Restriction enzyme accessibility assays were carried out as described [22]. Isolated nuclei from P19 cells were digested with 100 U of XbaI, StuI, HhaI, NcoI and AvrII (New England Biolabs) for 30 min. The purified genomic DNA was re-digested with 100 U of HindIII. The digested fragments were analyzed by Southern blot using ^{32}P-labeled probe 2 (Fig. 5B).

Ligation-mediated PCR (LM-PCR)

LM-PCR was performed as described [21]. Nuclei were digested with 20 U of MNase (Worthington) for 15 min at 37°C, followed by proteinase K treatment overnight at 37°C. Mononucleosomal DNA (~150 bp) was then purified from 1.5% agarose gels and 1 μg of the purified DNA was phosphorylated at 5′-termini with T4 polynucleotide kinase and then ligated to the universal linker using T4 DNA ligase. Purified DNA fragments were amplified by PCR with the universal linker oligonucleotide (25 bp) and ^{32}P-labeled *Oct4*-specific primers.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

RESULTS

RA reduces the expression of Pml, a positive regulator of *Oct4* gene

In RA-induced P19 EC cell differentiation process, Oct4 protein was reduced beginning at 24 hrs of RA treatment; whereas Pml protein level was down-regulated even before 24 hrs (Fig. 1A), revealing that RA down-regulated Pml before it suppressed Oct4 expression. We then examined the potential functional role for Pml in the regulation of *Oct4* gene expression using siRNA-mediated knockdown approaches. As shown in Figs. 1B and C left, proliferating P19 stem cells treated with siRNA of Pml (siPml), or RA, expressed significantly lower levels of Oct4 mRNA (~70% reduction), and its protein (~90% reduction). In order to decipher whether this phenomenon holds true for ES cells, we used the same strategy to differentiate and manipulate a mouse ES cell line CJ7. As shown in Fig. 1C right panel, Pml was dramatically reduced by siRNA knockdown of Pml and by RA treatment (~95% and ~80%, respectively). Similarly, Oct4 level was proportionally reduced by knockdown of Pml and by RA treatment (~85% and ~95%, respectively) (Fig. 1C). To obtain further functional evidence for Pml to support Oct4 expression, we conducted gain-of-function studies by ectopic expression. As shown in Fig. 1D, overexpression of Pml in control cells had no significant effects on Oct4 expression, but was able to rescue, at least partially, Oct4 expression in siPml- (left) and RA-treated (right) cells. It is clear that silencing Pml or RA treatment reduces both Pml and Oct4 levels. But expressing an siRNA-resistant Pml vector effectively rescues Oct4 expression (left). However, expressing the Pml expression vector could only partially rescue down-regulation of Oct4 by RA (right). This demonstrates the specific role of Pml in maintaining Oct4 level. The lack of significant effect of over-expressing Pml to further activate Oct4 in the control (scrambled siRNA transfected cells, in Scr panels) cells might be due to saturation of the system with other endogenous components that limit over-activation of Oct4. The merely partial rescue of Oct4 in RA-treated cells by over-expressing Pml could be due to the suppression of other endogenous components affected by RA. Overall, these data show a potential functional role for Pml in the maintenance of *Oct4* gene activity in stem cells including embryonic stem cells, and RA down-regulates Pml, thereby rapidly, and at least partially, contributing to early suppression of *Oct4* gene in differentiating cells. In addition, a similar pattern of down-regulation was also observed for other pluripotency-related genes such as *Nanog* and *Sox2* (Fig. S1), implying that Pml may play a general role in regulating the genes maintaining stem cell property. For a technical consideration, we set up mechanistic experiments to start the investigation of this phenomenon using P19 cell culture, in particular for studying the remodeling process of *Oct4* gene chromatin as described in the following.

Pml-NBs regulate *Oct4* gene expression by associating with its PP

To substantiate the functional role for Pml in activating *Oct4* gene, and to determine the gene's regulatory region targeted by Pml, we generated an *Oct4* transcriptional reporter containing its promoter and enhancers, and monitored the reporter activity in gain-of-function transfection studies. As shown in Fig. 2A, we detected a Pml dose-dependent activation of this reporter. Four conserved regions (named CRs) are present in the *Oct4* upstream regulatory region (Fig. 2B left). We generated a series of Oct4 reporters deleted in various CRs and determined their activities in the presence of Pml. As shown in Fig. 2B right, the most proximal CR which is the only one located in PP, pCR1, readily and fully activated this reporter in the presence of Pml, indicating that CR1 of PP is sufficient to mediate Pml's effect and probably is the physical target of Pml.

To examine Pml's binding sites on any specific *Oct4* regulatory sequences, we conducted Chromatin immunoprecipitation (ChIP) assays to scan the entire regulation region for

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

binding by Pml, as well as key proteins associated with the Pml-NB structures, such as Daxx and CBP/P300. Fig. 2C left shows that Pml itself most strongly associated with CR1 and CR4 in stem cells (Ctrl), which was apparently reduced in either siPml- or RA-treated cells. Fig. 2C middle panel shows the ChIP-PCR result of one Pml-NB marker (Daxx), and the right panel shows the ChIP-PCR result of another Pml-NB associated protein (CBP/P300). Interestingly, in stem cell cultures, both the two Pml-NB markers associated only with the CR1 region, which was drastically reduced in either siPml- or RA-treated cells. These results suggest that for *Oct4*, on which multiple regulatory sequences can be associated with Pml protein itself, only CR1 (PP) is a physical target of Pml-NBs. Importantly, RA, or siPml, treatment effectively reduces the formation of Pml-NBs on this region, likely due to its rapid down-regulation of Pml.

Pml activates *Oct4* gene along with transcription factors TR2, SF1, and Sp1

Studies in the past have shown *Oct4* gene expression activated, principally, by TR2, SF1, and Sp1 binding to their cognate binding sites, the hormone response elements (HREs) and Sp1 site, within CR1. To determine the contribution of each one of these three major transcription factors, as well as Pml, to the *Oct4* gene activity, we conducted both gain-of-function reporter assays and siRNA-mediated knockdown of these three TFs, in combination with Pml. Fig. 3A shows that all the three TF and Pml could by itself each activate the *Oct4* reporter, and the combination of each individual TF with Pml could more effectively activate this reporter than by each individual TF or Pml alone, suggesting a slightly enhancing role for these TFs in the action of Pml, and probably vice versa. Consistently, as shown in Fig. 3B, silencing Pml, or each individual TF, reduced the endogenous Oct4 level, and silencing Pml together with each TF even more effectively suppressed Oct4 expression. Interestingly, Pml silencing alone reproducibly decreased SF1 and TR2 protein levels, suggesting that Pml can also affect SF1 and TR2 protein expression (see Discussion). All together, these gain- and loss-of-function data validate the combinatorial effects of Pml with each individual TF in activating *Oct4*.

Pml recruits TFs and chromatin-remodeling complex, and is required for maintaining the open chromatin conformation of *Oct4* promoter

The stronger effect of Pml, when combined with each TF, on activating *Oct4* gene activity suggested a potential function for Pml in recruiting these TFs, and possibly, chromatin remodelers to the *Oct4* gene. In ChIP assays, we indeed detected association of the three TFs and Pml on the CR1 region (Fig. 4A left six panels & Fig. S2A). Importantly, in siPml-treated cells, none of these TFs were effectively recruited, suggesting Pml's functional role in facilitating the recruitment of these TFs to this critical region of *Oct4* promoter. Consistently, Pml, SF1 and Sp1 (except TR2, see following) were also poorly recruited to this gene in RA-treated cells, supporting that these activating TFs could no longer be recruited to *Oct4* promoter because of RA-triggered down-regulation of Pml. Of notice is the association of TR2 with this promoter even in RA-treated cells, but not in siPml-treated cells. This was due to RA-triggered conversion of activating TR2 into a sumoylated TR2 that became a repressor and could still associate with this chromatin [23]. Failure of TR2 association with this promoter in siPml cells was due to the requirement for Pml in mediating TR2 sumoylation [9]. As predicted, the recruitment of Pml complexes with TR2, SF1 and Sp1 on the CR1 region was detected in repeated ChIP (ReChIP) as shown in Fig. 4A middle four panels and Fig. S2B. But the formation of these Pml-TF complexes was dramatically reduced under Pml silencing or RA treatment. To examine whether the recruitment of these factors to the Oct4 promoter held true in ES cells, we also carried out these ChIP experiments in ES cells. We first ruled out the possibility that the MEF feeders might complicate the ChIP experiments by conducting these experiments using the MEF cells alone, which ruled out the concern (Fig. S2A bottom). Importantly, recruitment of Pml-

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NB components and these transcription factors to the Oct4 regulatory region also happens in ES cells (Fig. 4A, right), suggesting that the dynamics of these transcription regulatory molecules to regulate the *Oct4* gene are similar between P19 and ES cells.

We then determined the possible chromatin remodeling complexes on the *Oct4* gene. Mammalian Brg1/Brm-associated-factor (BAF) chromatin remodeling complexes are essential for self-renewal and pluripotency of ES cells [24]. In proliferating ES cells, the BAF complex is mainly composed of Brahma-related gene 1 (Brg1) and BAF155, and is named BRGC. During differentiation, Brm is recruited to replace Brg1 in the BAF complex that is named BRMC [25, 26]. BRGC is indispensable for *Oct4* gene expression and Brg1 deficiency in mice leads to early embryonic lethality [27, 28]. As shown in ChIP assays (Figs. 4B upper five panels and S2C), BAF155 was effectively recruited on this region in stem cells, which was only slightly reduced in siPml- and RA-treated cells. Interestingly, Brg1 associated with this region only in stem cells, but Brm associated with this region only in cells treated with siPml or RA. While this may merely be a correlative phenomenon, the mutually exclusive recruitment of Brg1 and Brm would suggest different roles for these two proteins, i.e. Brg1 functions in *Oct4* gene activation while Brm acts in its repression. The co-recruitment of both Brg1 and BAF155 to CR1 in stem cells confirms BRGC's role in *Oct4* expression for stem cell proliferation; whereas displacement of Brg1 by Brm in the BAF complex in siPml- or RA-treated cells would imply that BRMC replaces BRGC to repress *Oct4* gene when cells lose Pml and undergo differentiation. Recruiting Brg1 only in the presence of Pml is supported by ReChIP as shown in the bottom two panels of Fig. 4B and Fig. S2D. Since RA rapidly reduced Pml protein level, it should also reduce Pml/Brg1 complex formation. This is confirmed in reciprocal co-immunoprecipitation (Fig. 4C). Further, it is known that Brg1 complex usually contains actin; this has been confirmed here because actin is detected in the Brg1 complex (Fig. 4C).

All together, the results show that, in stem cells, Pml recruits TFs like TR2, Sp1, SF1 and the activating chromatin remodeler BRGC to *Oct4* PP. In differentiating cells (RA-induction), Pml level decreases, the activating TFs and Brg1 then dissociate from this promoter, but repressive TR2 and BAF155 stays on, and Brm is recruited to form BRMC complex to repress this gene. This provides the molecular explanation for Pml's action in maintaining *Oct4* gene activity in stem cells.

Loss of Pml contributes to chromatin remodeling and specific nucleosome rearrangement on *Oct4* PP

To determine the chromatin conformation of the regulatory region of *Oct4* gene in stem cells when this gene is active, we conducted Micrococcal nuclease (MNase) digestion assays using probes detecting DE, PE and PP. As shown in Fig. 5A, clear regular nucleosomal arrays were detected with all three probes in stem cells (Ctrl), siPml-treated (siPml), and RA-treated cells. This demonstrates that the *Oct4* regulatory region forms regular nucleosome arrays in both stem and differentiating cells. However, the PI probe detected apparent changes (increased intensity) of mono-and di-nucleosomes in siPml- or RA-treated cells (lower right panel), suggesting that RA treatment, or Pml silencing, has triggered some form of chromatin remodeling without drastically disturbing the nucleosomes on *Oct4* PP.

Intensification of mono- and di-nucleosomes signals suggested nucleosome addition/ insertion to this region in siPml- or RA-treated cells. We then conducted restriction accessibility (Fig. 5B) assay. It appeared that Pml silencing (lower left) clearly altered restriction sensitivity on StuI, HhaI and NcoI sites (marked with * signs on the right of the diagnostic fragments) but not on XbaI or AvrII sites, suggesting nucleosome reposition in the siPml-treated cells. RA treatment resulted in a very similar restriction accessibility pattern (lower right), suggesting that these two treatments have elicited very similar

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

chromatin remodeling processes. Therefore, in stem cells where *Oct4* gene is active, nucleosomes do assemble on its promoter and regulatory regions; in differentiating cells (such as by RA exposure or silencing Pml), PP maintains the nucleosome array but undergoes chromatin remodeling (such as nucleosome reposition or sliding) so that restriction sensitivity changes on certain regions.

We then conducted nucleosome scanning [21] to roughly map the positions of nucleosomes on *Oct4* PP before and after differentiation. The preliminary scanning results detected two nucleosomes at the two termini of PP (approximately 600 base pairs), three nucleosomes in siPml-treated cells and four nucleosomes in RA-treated cells. To determine the fine map of nucleosome positions, we carried out ligation-mediated-PCR (LM-PCR) as shown in Fig. 6. Actual sizes of diagnostic fragments (depicted on the right of each gel) were calculated by subtracting 25-bp (the length of the universal linker ligated to the purified mononucleosomal DNA and used as a reverse primer in PCR) from the lengths shown on these gels. LM-PCR using F1 amplified 75-, 53- and 45-bp fragments from stem, siPml-treated and RA-treated cells, respectively. Accordingly, the 3′-border of the 5′ terminal nucleosome in the three types of cells is assigned to −366 (stem cells), −388 (siPml treated) and −396 (RA treated) positions relative to TIS. This is confirmed by LM-PCR using F2 primer, which generated 42-bp (stem cells), 20-bp (siPml-treated) and undetectable (RA-treated, due to the small size, 12 bp, of this fragment which migrated out of the gel) fragments. Therefore, the 3′ border of this 5′-terminal nucleosome has shifted upward (toward the 5′ direction) for 22 nucleotides in Pml-silenced cells, and 30 nucleotides in RA-treated cells. This is consistent with the inaccessibility of XbaI site in all three groups (Fig. 5B) because this site remains covered by the sliding nucleosome even when cells are differentiating.

LM-PCR using R1 primer mapped the 3′-terminal nucleosome covering TIS. Its 5′-border moved from −80 to −32 and −22 in cells treated with siPml and RA, respectively. The NcoI site initially was near the border of this nucleosome, but was then entirely covered by the downward (toward 3′ direction) sliding nucleosome in cells treated with siPml or RA. This is consistent with the result of Fig. 5B showing much reduced accessibility of NcoI site in siPml- or RA-treated cells. LM-PCR using F3 or F4 primers generated no fragments in stem cells, supporting the absence of nucleosome between two terminal nucleosomes of PP in stem cells. Very interestingly, in RA-treated cells, F3 and F4 both generated specific fragments; but in siPml-treated cells, only F4 generated a specific fragment, confirming addition of one nucleosome (3′ border at −82) in siPml-treated cells and two nucleosomes (3′ border at −247 and −55, respectively) in RA-treated cells. This is consistent with the results of Fig. 5B showing accessibility of StuI and HhaI sites only in stem cells. Therefore, the initially nucleosome-free middle region (−366 to −80) of PP assembles one new nucleosome in cells depleted of Pml, and adds two nucleosomes in cells treated with RA for differentiation. In both situations, the original two terminal nucleosomes are pushed further apart. In differentiating cells, the denser nucleosome array presumably will facilitate chromatin compaction.

Both siPml and RA each can induce repressive chromatin formation on the *Oct4* gene promoter

Addition of nucleosomes into PP might trigger this region to adopt a more repressive conformation, such as heterochromatin. We then conducted ChIP to monitor repressive chromatin markers including co-repressor RIP140 (receptor-interaction protein 140) [29], HP1γ [30], H3K9me3 and H3K27me3 [31], and, for a comparison, active chromatin marker AcH3, and RNA PolII [32] (Figs. 7A & S3A). In stem cells, AcH3 and RNA PolII were both clearly detected on this region, consistent with the expression of this gene. Pml silencing and RA treatment drastically reduced AcH3 mark and RNA PolII recruitment on this region. In both siPml- and RA-treated cells, all the repressive markers monitored,

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

including HP1γ, H3K9me3, H3K27me3 and RIP140 were enhanced. Since euchromatic histone lysine N-methyltransferase 2 (Ehmt2 or G9a) [33] can mediate H3K9 methylation, and RIP140 can be involved in heterochromatin formation [22], we speculated G9a co-recruitment with RIP140 to this promoter. This is confirmed in ReChIP (Figs. 7A bottom two panels & S3B).

DISCUSSION

This study demonstrates a functional role for Pml-NBs in supporting the expression of *Oct4* gene in stem cells including EC and ES cells. The results also provide mechanistic details in maintaining a specific open chromatin conformation of *Oct4* PP in P19 EC cells, which is to provide a platform to recruit Sp1, SF1, TR2 and activating chromatin remodeler BRGC to maintain *Oct4* gene activity. In proliferating cells, this chromatin segment exists in a partially open and nucleosome-scarce conformation, and, upon RA treatment (which first lowers the Pml level), remodeling occurs through inserting and repositioning nucleosomes and forming heterochromatin. The process involves dissociating Sp1 and SF1, retaining TR2, exchanging Brg1 with Brm, and recruiting other repressive factors like RIP140 and G9a. This would explain the requirement for Pml in the maintenance of *Oct4* gene activity and its open chromatin conformation in stem cells. However, it remains to be determined whether this interesting mechanism can be generalized for all the ES cells.

RA can rapidly lower Pml thereby depleting Pml-NB structures in differentiating cells, which contributes to repressive chromatin remodeling in the early cell differentiation process. A model (Fig. 7B) is proposed that in stem cells, Pml-NBs form on CR1 to provide platforms for recruiting activating TFs and remodeler BRGC to maintain the nucleosome-free region of PP. Upon RA-treatment, Pml is rapidly lost, which destroys Pml-NBs; therefore SF1, Sp1 and Brg1 cannot be maintained. TR2, initially associated with Pml-NBs, rapidly turns into a sumoylated repressor by RA's effect and can still bind this chromatin to recruit corepressors such as RIP140 and histone deacetylases. Discrepancy on the expression level of BAF155 in RA treated cells has been reported [26, 34], which may be due to difference in the duration of RA treatment. However within the time window we examined here (day 3), BAF155 level is relatively constant, and stays on the chromatin. Brm is recruited, so that BRGC is replaced by BRMC. Together with RIP140, G9a and HP1γ are also recruited to occupy CR1. Ultimately, this facilitates the formation of a more compacted chromatin with a denser nucleosome array on CR1.

It is also of interest to explore the specific action of Pml on the CR1 region for Pml-NB formation, since Pml associates strongly with both CR1 and CR4 regions. It is possible that Pml associates with other proteins in order to facilitate the formation of NB on CR1. For example, SATB1 is a protein that can rearrange chromatin into loops, and Pml can interact with this protein to reorganize the major histocompatibility complex (MHC) class I gene locus, resulting in higher-order chromatin loops [16]. Further, we do not exclude the possibility that Pml forms a complex with other component on CR4 region to exert a different activity. It has been shown that Pml can interact with numerous proteins including Sp1 [35]. Our ChIP-ReChIP and co-immunoprecipitation data (Fig. 4) show that Pml physically associates with TR2 and SF1, as well as Brg1. It would be interesting to investigate whether these proteins all directly interact with Pml for their recruitment.

It is interesting that the effects of RA treatment (rapidly down-regulating Pml) and Pml silencing on this gene chromatin remodeling are similar, but not identical (Figs. 5 & 6). This indicates that Pml depletion recapitulates a fraction of RA's overall effects, which include nongenomic and extensive genomic activities. Presumably, early reduction in Pml initiates the first phase of repressive remodeling by inserting a nucleosome and sliding two terminal

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

nucleosomes apart to gradually cover TIS on CR1. Prolonged RA effects would induce other genes to elicit a more complete and permanent repressive remodeling by inserting the second additional nucleosome, which pushes two terminal nucleosomes further apart to completely cover TIS. This region is ultimately compacted and heterochromatinized. With regards to the detailed mechanisms, several possibilities can be tested, such as nucleosome sliding and nucleosome exchange. For instance, H2A/H2B dimer removal and H2A/H2B dimmer exchange may be examined. In the future, it would be useful to use tagged histone proteins to investigate these several possibilities.

For *Oct4* gene, Pml acts as a positive regulator, which is supported by Pml-NBs association with nascent *Oct4* mRNA (Fig. S4). But Pml-NBs can also be involved in transcriptional repression and can co-localize with HP1 [36]. Therefore, the function of Pml may be gene-specific and related to its sub-nuclear localization and associated proteins. We have shown that TR2 protein is recruited to Pml-NBs initially as an activator, but, through RA's non-genomic activity, TR2 can be sumoylated by associating with Pml and becomes a repressor that remains binding to this promoter in differentiating cells [9]. Our current data reveal another activity of Pml in down-regulating SF1 and TR2 (Fig. 3B middle). Therefore, Pml has multiple functional roles, presumably depending upon its associated proteins.

Brg1 or Brm could be involved in transcriptional activation or repression, depending upon the target gene [25, 28]. For the *Oct4* gene, Brg1 is involved in activation in stem cells whereas Brm is probably involved in chromatin compaction and heterochromatin formation after RA treatment [24, 28]. A recent study has shown that the BAF complex associates with the *Oct4* promoter and that BAF155 is required for heterochromatin formation and chromatin compaction on this promoter [34]. Since both Brg1 and Brm, along with BAF155 to form BAF complexes, it is predicted that BAF155 would be constantly detected on *Oct4* PP. This is consistent with our data (Fig. 4). Therefore, in stem cells, Brg1 associates with Pml, contributing to the recruitment of BRGC by Pml-NBs to *Oct4* promoter to maintain a nucleosome-scarce, active chromatin conformation. In differentiating cells, Brm is recruited to replace Brg1 when Pml is reduced, probably contributing to repressive chromatin remodeling. This may also be enhanced by the continuing binding of TR2 repressor, which recruits corepressor RIP140, histone deacetylases and chromatin modifying enzyme G9a, all involved in heterochromatin formation [22]. The mechanism underlying the recruitment of Brm on *Oct4* gene promoter and the insertion of the second nucleosome in RA-induced differentiating cells remains to be determined.

CONCLUSION

Maintaining a constant level of Oct4 is crucial to healthy proliferation of ES or EC cells. In RA-induced cultures, *Oct4* gene is rapidly suppressed. This study demonstrates the functional role for Promyelocytic leukemia (Pml) protein and Pml nuclear bodies in recruiting specific activating transcription factors TR2, SF1 and Sp1, as well as activating chromatin remodeler BRGC to CR1 region of the *Oct4* promoter, thereby maintaining a specific open chromatin conformation of this promoter for its constant expression in stem cells. The study also illustrates that rapid down-regulation of Pml by RA treatment initiates the repressive chromatin remodeling process, which includes sequentially inserting two nucleosomes into the initially nucleosome-scarce CR1 region, pushing one 3′ nucleosome to completely cover the transcription initiation site, and ultimately rendering heterochromatin formation on this promoter in more differentiated cells. Thus, in RA-induced cell differentiation, chromatin remodeling of the *Oct4* gene occurs sequentially, and is initiated by down-regulating Pml and condensing its proximal promoter through nucleosome insertion and sliding to cover its transcription initiation site.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Y.-C. Tsui for technical help. This work was supported by National Institutes of Health Grants DK54733, DK054733-09S1, DK060521-07S1, DK60521, DA11190, DA11806 and K02-DA13926, the Philip Morris USA Inc. and Philip Morris International, and the Distinguished McKnight University Professorship to L.-N. W.

References

1. Chambers I, Smith A. Self-renewal of teratocarcinoma and embryonic stem cells. Oncogene. 2004; 23:7150–7160. [PubMed: 15378075]
2. Gurdon JB, Melton DA. Nuclear reprogramming in cells. Science. 2008; 322:1811–1815. [PubMed: 19095934]
3. Hanna J, Markoulaki S, Schorderet P, et al. Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. Cell. 2008; 133:250–264. [PubMed: 18423197]
4. Nichols J, Zevnik B, Anastassiadis K, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell. 1998; 95:379–391. [PubMed: 9814708]
5. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science. 2007; 318:1917–1920. [PubMed: 18029452]
6. Kellner S, Kikyo N. Transcriptional regulation of the Oct4 gene, a master gene for pluripotency. Histol Histopathol. 2010; 25:405–412. [PubMed: 20054811]
7. Marin M, Karis A, Visser P, et al. Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. Cell. 1997; 89:619–628. [PubMed: 9160753]
8. Barnea E, Bergman Y. Synergy of SF1 and RAR in activation of Oct-3/4 promoter. J Biol Chem. 2000; 275:6608–6619. [PubMed: 10692469]
9. Park SW, Hu X, Gupta P, et al. SUMOylation of Tr2 orphan receptor involves Pml and fine-tunes Oct4 expression in stem cells. Nat Struct Mol Biol. 2007; 14:68–75. [PubMed: 17187077]
10. Bost F, Caron L, Marchetti I, et al. Retinoic acid activation of the ERK pathway is required for embryonic stem cell commitment into the adipocyte lineage. Biochem J. 2002; 361:621–627. [PubMed: 11802792]
11. Soprano DR, Teets BW, Soprano KJ. Role of retinoic acid in the differentiation of embryonal carcinoma and embryonic stem cells. Vitam Horm. 2007; 75:69–95. [PubMed: 17368312]
12. Zhong S, Muller S, Ronchetti S, et al. Role of SUMO-1-modified PML in nuclear body formation. Blood. 2000; 95:2748–2752. [PubMed: 10779416]
13. Bernardi R, Pandolfi PP. Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. Nat Rev Mol Cell Biol. 2007; 8:1006–1016. [PubMed: 17928811]
14. Ishov AM, Sotnikov AG, Negorev D, et al. PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. J Cell Biol. 1999; 147:221–234. [PubMed: 10525530]
15. Boisvert FM, Hendzel MJ, Bazett-Jones DP. Promyelocytic leukemia (PML) nuclear bodies are protein structures that do not accumulate RNA. J Cell Biol. 2000; 148:283–292. [PubMed: 10648561]
16. Kumar PP, Bischof O, Purbey PK, et al. Functional interaction between PML and SATB1 regulates chromatin-loop architecture and transcription of the MHC class I locus. Nat Cell Biol. 2007; 9:45–56. [PubMed: 17173041]
17. Aoto T, Saitoh N, Ichimura T, et al. Nuclear and chromatin reorganization in the MHC-Oct3/4 locus at developmental phases of embryonic stem cell differentiation. Dev Biol. 2006; 298:354–367. [PubMed: 16950240]
18. Zhong S, Salomoni P, Pandolfi PP. The transcriptional role of PML and the nuclear body. Nat Cell Biol. 2000; 2:E85–90. [PubMed: 10806494]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

19. Tsai NP, Lin YL, Wei LN. MicroRNA mir-346 targets the 5′-untranslated region of receptor-interacting protein 140 (RIP140) mRNA and up-regulates its protein expression. Biochem J. 2009; 424:411–418. [PubMed: 19780716]
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods. 2001; 25:402–408. [PubMed: 11846609]
21. Park SW, Li G, Lin YP, et al. Thyroid hormone-induced juxtaposition of regulatory elements/factors and chromatin remodeling of Crabp1 dependent on MED1/TRAP220. Mol Cell. 2005; 19:643–653. [PubMed: 16137621]
22. Park SW, Huang WH, Persaud SD, et al. RIP140 in thyroid hormone-repression and chromatin remodeling of Crabp1 gene during adipocyte differentiation. Nucleic Acids Res. 2009; 37:7085–7094. [PubMed: 19778926]
23. Gupta P, Ho PC, Huq MM, et al. Retinoic acid-stimulated sequential phosphorylation, PML recruitment, and SUMOylation of nuclear receptor TR2 to suppress Oct4 expression. Proc Natl Acad Sci U S A. 2008; 105:11424–11429. [PubMed: 18682553]
24. Ho L, Jothi R, Ronan JL, et al. An embryonic stem cell chromatin remodeling complex, esBAF, is an essential component of the core pluripotency transcriptional network. Proc Natl Acad Sci U S A. 2009; 106:5187–5191. [PubMed: 19279218]
25. Reisman D, Glaros S, Thompson EA. The SWI/SNF complex and cancer. Oncogene. 2009; 28:1653–1668. [PubMed: 19234488]
26. Yan Z, Wang Z, Sharova L, et al. BAF250B-associated SWI/SNF chromatin-remodeling complex is required to maintain undifferentiated mouse embryonic stem cells. Stem Cells. 2008; 26:1155–1165. [PubMed: 18323406]
27. Bultman S, Gebuhr T, Yee D, et al. A Brg1 null mutation in the mouse reveals functional differences among mammalian SWI/SNF complexes. Mol Cell. 2000; 6:1287–1295. [PubMed: 11163203]
28. Kidder BL, Palmer S, Knott JG. SWI/SNF-Brg1 regulates self-renewal and occupies core pluripotency-related genes in embryonic stem cells. Stem Cells. 2009; 27:317–328. [PubMed: 19056910]
29. Wei LN, Hu X, Chandra D, et al. Receptor-interacting protein 140 directly recruits histone deacetylases for gene silencing. J Biol Chem. 2000; 275:40782–40787. [PubMed: 11006275]
30. Bannister AJ, Zegerman P, Partridge JF, et al. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature. 2001; 410:120–124. [PubMed: 11242054]
31. Lund AH, van Lohuizen M. Epigenetics and cancer. Genes Dev. 2004; 18:2315–2335. [PubMed: 15466484]
32. Lee JH, Hart SR, Skalnik DG. Histone deacetylase activity is required for embryonic stem cell differentiation. Genesis. 2004; 38:32–38. [PubMed: 14755802]
33. Tachibana M, Sugimoto K, Nozaki M, et al. G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. Genes Dev. 2002; 16:1779–1791. [PubMed: 12130538]
34. Schaniel C, Ang YS, Ratnakumar K, et al. Smarcc1/Baf155 couples self-renewal gene repression with changes in chromatin structure in mouse embryonic stem cells. Stem Cells. 2009; 27:2979–2991. [PubMed: 19785031]
35. Vallian S, Chin KV, Chang KS. The promyelocytic leukemia protein interacts with Sp1 and inhibits its transactivation of the epidermal growth factor receptor promoter. Mol Cell Biol. 1998; 18:7147–7156. [PubMed: 9819401]
36. Seeler JS, Marchio A, Sitterlin D, et al. Interaction of SP100 with HP1 proteins: a link between the promyelocytic leukemia-associated nuclear bodies and the chromatin compartment. Proc Natl Acad Sci U S A. 1998; 95:7316–7321. [PubMed: 9636146]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

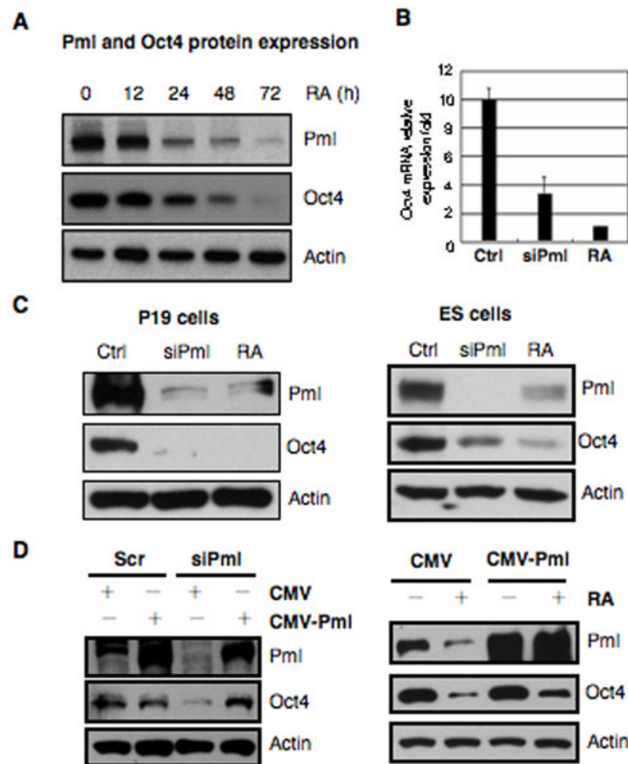

Figure 1. Pml is a positive regulator of *Oct4* gene

(A) Relative levels of Pml and Oct4 proteins in P19 cells after the indicated periods of RA treatment. Actin serves as the internal control. (B) RT-qPCR analysis of *Oct4* mRNA levels in P19 cells treated with scramble siRNA (Ctrl), siPml, or 1 μM all-trans-RA for 72 hrs. Data show Oct4 levels normalized with β-actin. SEM is indicated with the error bar. (C) Western blots detecting Pml and Oct4 protein level in different P19 and CJ7 ES cell cultures as indicated. (D) Rescue experiment by expressing an siRNA-resistant Pml in control, siPml-, and RA-treated P19 cells. Scr: scramble siRNA.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

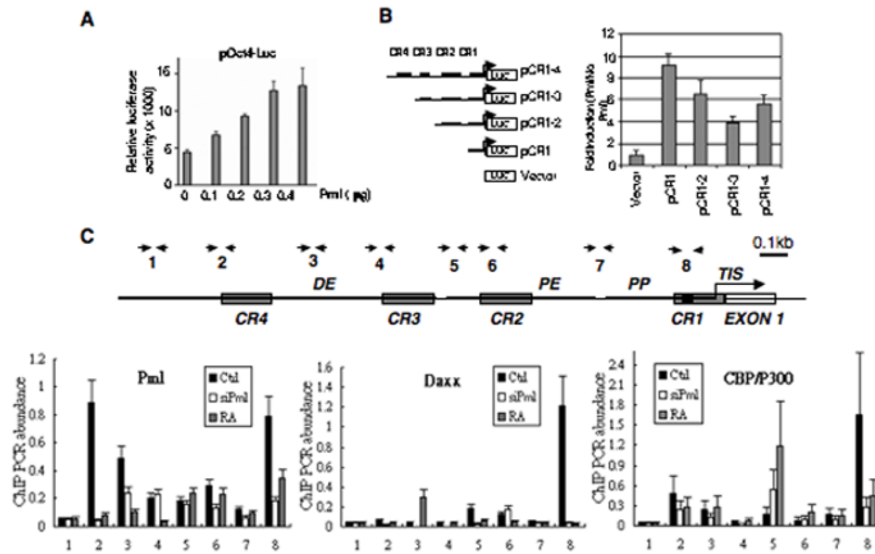

Figure 2. Pml-NBs are involved in *Oct4* activation through directly associating with the *Oct4* PP region

(A) The *Oct4*-reporter activity in P19 cells co-transfected with a Pml expression vector at various doses. (B) The activities of *Oct4*-reporters deleted in various CRs. Left panel depicts *Oct4*-reporters each deleted in different CRs. Right panel shows Pml-activated reporter activities. The activity in Pml-transfected P19 was divided by that in control transfected P19, and plotted as fold induction. (C) Upper panel: Map of four CRs (gray boxes) on the *Oct4* gene regulatory region and ChIP primer sets as indicated. The black box within CR1 depicts Sp1-binding site and hormone response elements (for TR2 and SF1 binding). Lower panels: The fold change of Pml-ChIP (left), Daxx-ChIP (middle), and CBP/P300-ChIP (right) determined by qPCR. Control cell: black bar. siPml-treated cell: white bar. RA-treated cell: grey bar. Abbreviations: CR, conserved region; DE, distal enhancer; PE, proximal promoter; PP, proximal promoter; TIS, transcription initiation site. SEM is each indicated by an error bar.

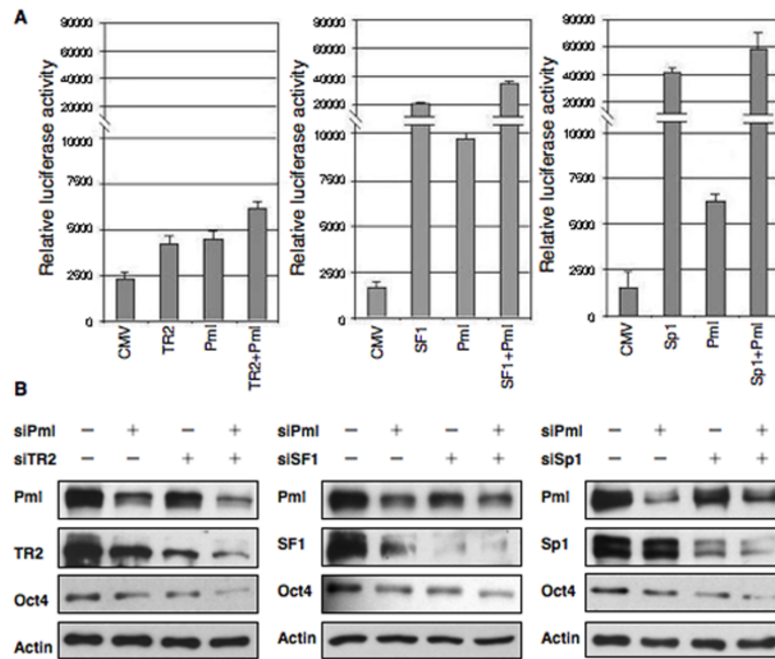

Figure 3. Pml activates *Oct4* gene along with transcription factors TR2, SF1, and Sp1

(A): Pml expression vector was transfected into P19 cells with or without TR2, SF1 and Sp1 expression vectors in the presence of the pCR1 reporter, and the normalized luciferase activity was determined. (B): P19 cells were subjected to RNA silencing individually with TR2, SF1 and Sp1 in the absence and presence of siPml. Protein levels were determined by western blot. Actin serves as the internal control.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

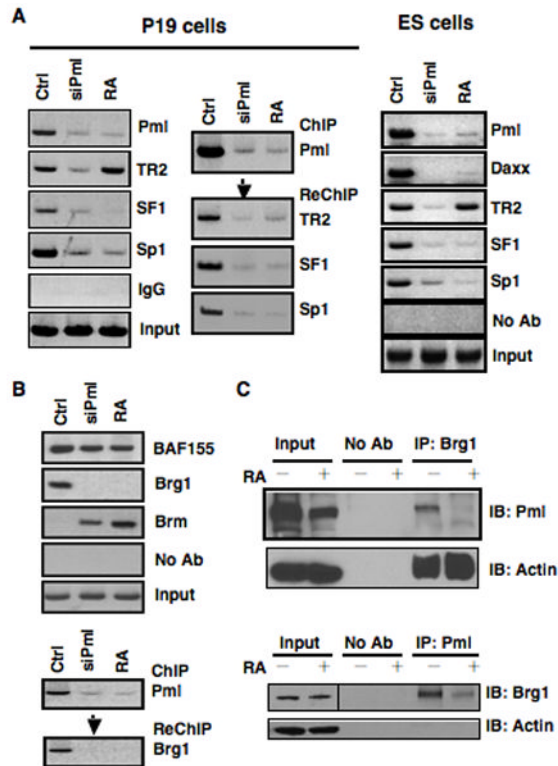

Figure 4. Pml recruits transcription factors TR2, SF1, and Sp1, and Brg1-dependent chromatin remodeling complex on *Oct4* proximal promoter

(A) Left: ChIP analysis of Pml, TR2, SF1, and Sp1, on CR1 in P19 cells. Middle four panels show ReChIP analyses of TR2, SF1 or Sp1 following Pml-ChIP in P19 cells. Right: ChIP analyses of Pml, Daxx, TR2, SF1, and Sp1, on CR1 in ES cells. (B) Top: ChIP analysis of chromatin remodeling factors on CR1. Lower: Repeated ChIP analysis of Pml recruiting Brg1 on CR1. (C) Reciprocal co-immunoprecipitation of complex formation of Pml and Brg from P19 cells treated with or without 1 μM RA. Actin serves as the internal control, as well as the control for the Brg1 complex (that is known to contain actin). No Ab: negative control. (see also Fig. S2)

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

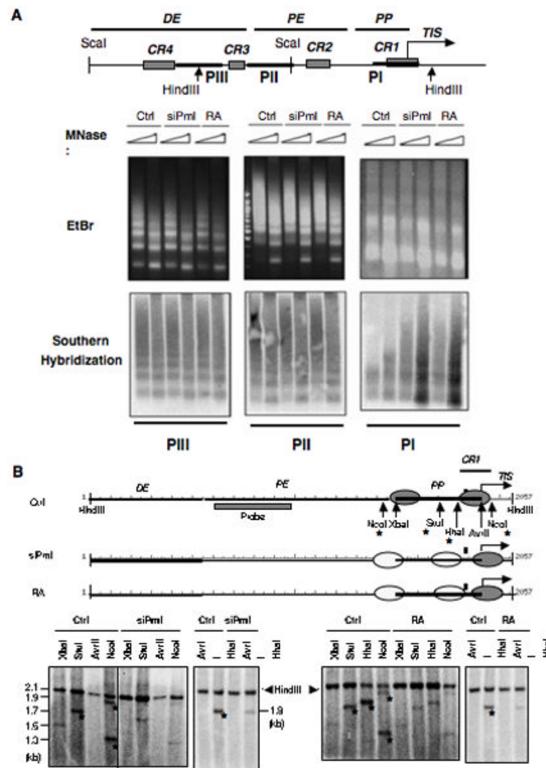

Figure 5. Pml silencing and RA treatment induce similar chromatin remodeling events on *Oct4* PP

(A) MNase mapping of the *Oct4* regulatory region. Top panel shows the map of *Oct4* gene promoter and enhancer region (DE, PE, PP) and four CRs. HindIII and ScaI sites and Southern blot probes (PI, PII and PIII for PP, PE and DE, respectively) are depicted. P19 stem, siPml- or RA-treated cells were subjected to MNase digestion for 5 minutes. Extracted chromatin DNA was separated on 1.5% agarose gels followed by Southern hybridization. The panels directly under the map show EtBr-stained gels and bottom panels show Southern blots probed with PI, PII or PIII. (B) Restriction enzyme accessibility of *Oct4* PP region in P19 stem, siPml-treated, or RA-treated cells. "*" signs mark diagnostic bands indicative of sensitive sites. The results are summarized on the map above these blots. Restriction sites are labeled under the top map. A small black box on each map depicts the Sp1-binding site and HRE cluster on CR1. Abbreviations: DE, distal enhancer; PE, proximal enhancer; PP, proximal promoter; TIS, transcription initiation site.

NIH-PA Author Manuscript

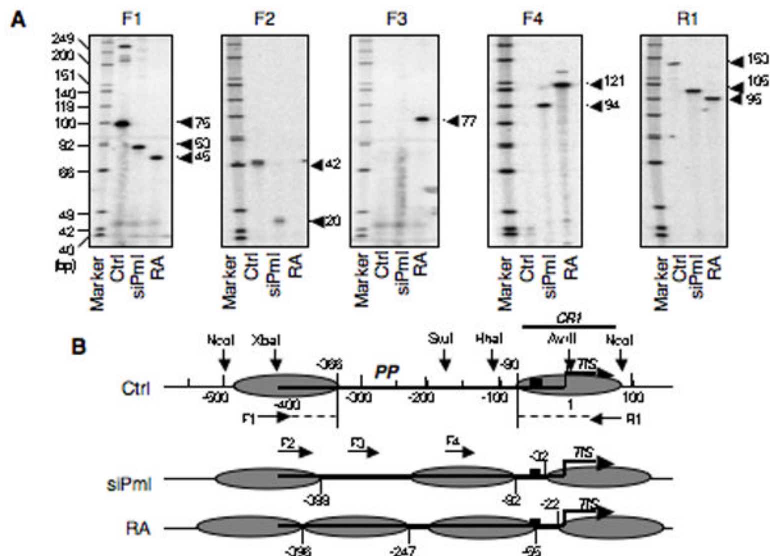

Figure 6. LM-PCR to detect nucleosome insertion and rearrangement on *Oct4* PP

(A) LM-PCR analysis of the *Oct4* PP region in P19 stem, siPml-treated and RA-treated cells. Specific primers (F1, F2, F3, F4 and R1 depicted under the control map) were each used to determine the boundary of a specific nucleosome. Data show the PCR products resolved on sequencing gels with the size maker (base pair) loaded on the left. The deduced actual size (after subtracting 25 bp linker length) of the fragment indicative of a specific nucleosome boundary is shown on the right. (B) nucleosome positions on PP in the three groups of cells according to LM-PCR results shown in panel A. Primers are indicated with arrows. The black box on the map shows the Sp1-HRE cluster.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

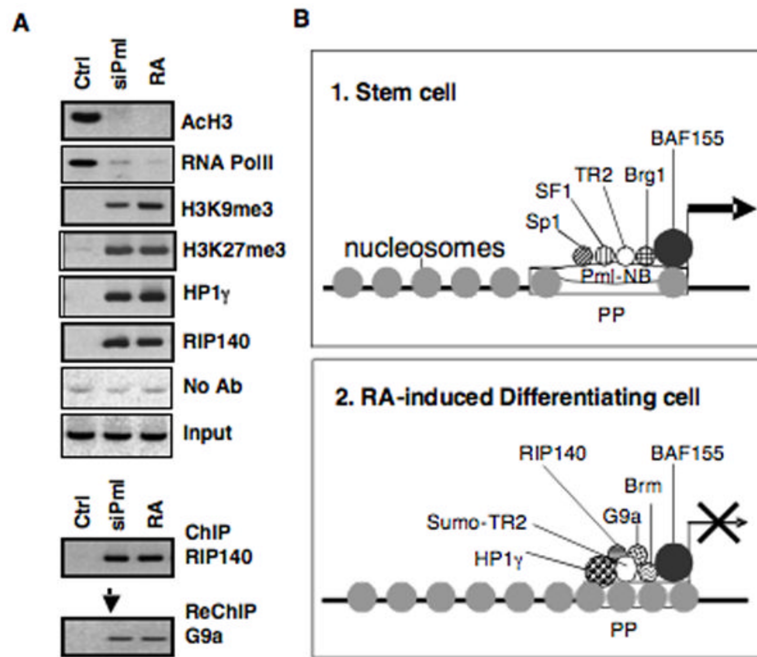

Figure 7. siPml or RA treatment each induces repressive chromatin conformation on the *Oct4* PP

(A) Top: ChIP analyses of repressive chromatin markers (H3K9me3, HP1γ and H3K27me3), active chromatin markers (AcH3 and RNA PolII), and co-repressor (RIP140) on CR1. Lower two panels show ReChIP analysis of G9a co-recruitment with RIP140. (B) A model of Pml-NB, TFs and chromatin remodelers acting on *Oct4* gene in stem (1), and differentiating (2) cells. Abbreviations: H3K9me3, histone H3 lysine 9 tri-methylation; RNA PolII, RNA polymerase II; H3K27me3, histone H3 lysine 27 tri-methylation (see also Fig. S3)