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Brief Report

Intrachromosomal Looping Is Required for Activation of Endogenous Pluripotency Genes during Reprogramming

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SUMMARY

Generation of induced pluripotent stem cells (iPSCs) by defined factors is an extremely inefficient process, because there is a strong epigenetic block preventing cells from achieving pluripotency. Here we report that virally expressed factors bound to the promoters of their target genes to the same extent in both iPSCs and unreprogrammed cells (URCs). However, expression of endogenous pluripotentcy genes was observed only in iPSCs. Comparison of local chromatin structure of the *OCT4* locus revealed that there was a cohesin-complex-mediated intrachromosomal loop that juxtaposes a downstream enhancer to the gene's promoter, enabling activation of endogenous stemness genes. None of these long-range interactions were observed in URCs. Knockdown of the cohesin-complex gene *SMC1* by RNAi abolished the intrachromosomal interaction and affected pluripotency. These findings highlight the importance of the *SMC1*-orchestrated intrachromosomal loop as a critical epigenetic barrier to the induction of pluripotency.

The discovery that somatic cells can be converted into iPSCs by defined factors (OCT4, SOX2, KLF4, and c-MYC) (Takahashi and Yamanaka, 2006) allows us to create patient-specific stem cells for regenerative therapy. However, there is relatively little data explaining how reprogramming factors remodel chromatin structure and activate the network of genes related to pluripotency. Moreover, the conversion of somatic cells to iPSCs is extremely inefficient; >99% of the transfected or treated donor cells are not converted into iPSCs (Park et al., 2008; Takahashi et al., 2007).

To discover potential epigenetic mechanisms responsible for the low efficiency of iPSC induction, we constructed polycistronic viral vectors containing *OCT4-SOX2-KLF4-c-MYC-EGFP* (OSKME) and *OCT4-SOX2-NANOG-EGFP* (OSNE) (Figure S1A available online) for inducing reprogramming into iPSCs in human fibroblasts. After their transfection with viral factors, we found that virtually all of the fibroblasts were fluorescence positive (Figure 1A, top middle panel), demonstrating efficient delivery of the viral vectors. Western blot demonstrated that each transfected gene produced a protein of the predicted size (Figure S1A). After transfer onto MEF feeder cells, some EGFP-positive cells developed into iPSCs. In HSF1 fibroblasts that had been passed for more than 15 passages, however, we very rarely observed the formation of iPSC colonies (Figure 1A, bottom middle panel). In conjunction with previous reports (Park et al., 2008; Takahashi et al., 2007), it is clear that in the vast majority of infected cells, successful expression of the defined factors did not lead to cell reprogramming.

We used EGFP fluorescence-sorting to isolate "unreprogrammed cells" (URCs) that expressed the defined factors but were morphologically similar to the parent fibroblasts. By comparing them with the characterized iPSCs (Figures S1B–S1F), we proposed to identify the epigenetic barrier accounting for the failure of URCs to reprogram. *OCT4* is a master regulator of the molecular circuitry that regulates embryonic stem cell (ESC) proliferation and differentiation. OCT4 protein contains a POU DNA binding domain and two proline-rich domains (Imagawa et al., 1991; Okamoto et al., 1990; Rosner et al., 1990). The iPSC-inducing factors function by binding and regulating a large network of target genes (Boyer et al., 2005; Kim et al., 2008; Loh et al., 2006).

We first used chromatin immunoprecipitation (ChIP) to determine whether the virus-derived factors bind to their target genes. We found that in URCs, there was an enrichment of OCT4 binding to its own promoter (autopromoter binding) (Figure 1B, left top panel, lanes 3 and 6) as well as to the *SOX2* and *NANOG* promoters (Figures S1G–S1H). This promoter binding was comparable to that observed in iPSCs (lanes 5 and 8) and in the ESC line H14 (lane 2). Similarly, we found that the viral NANOG

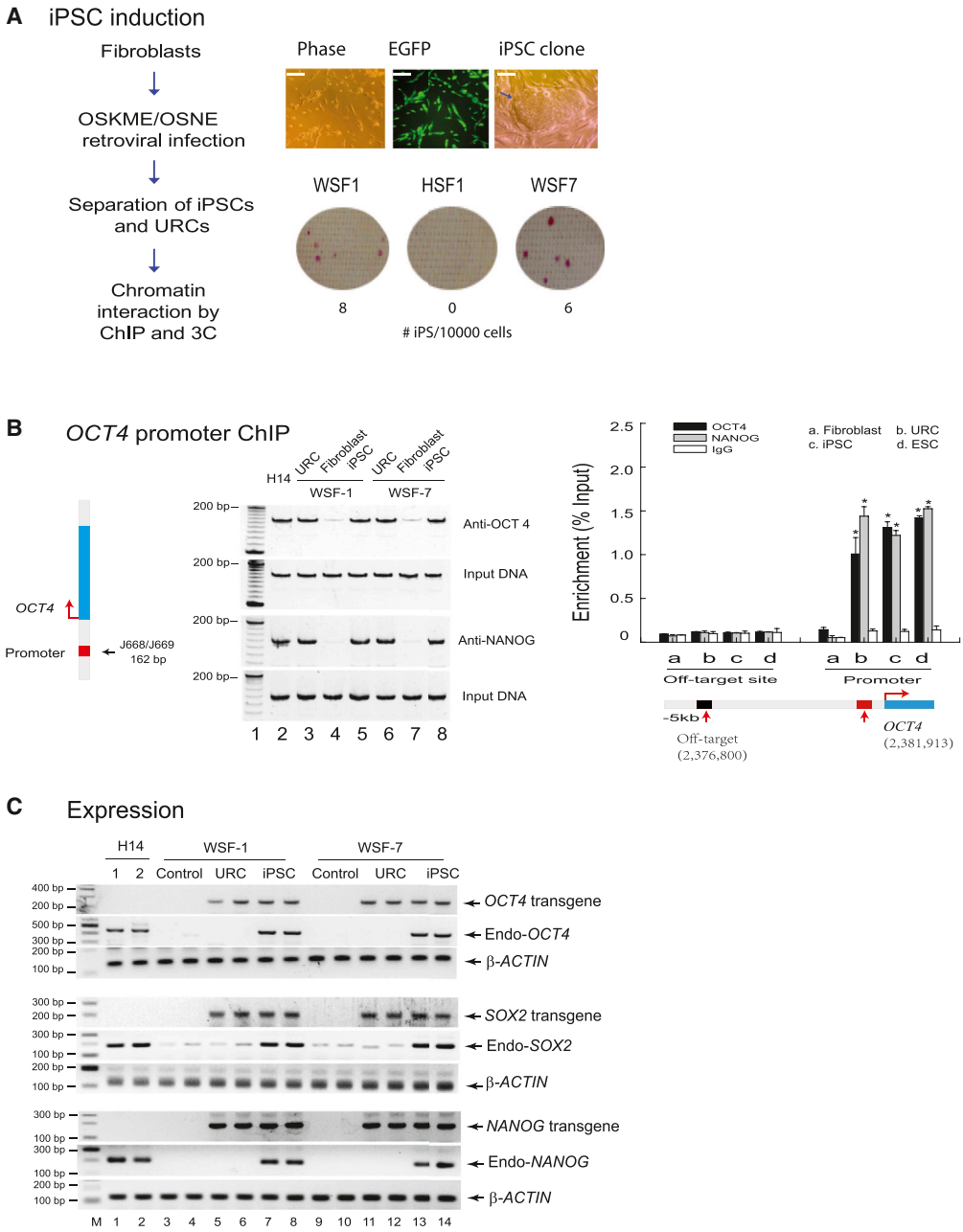


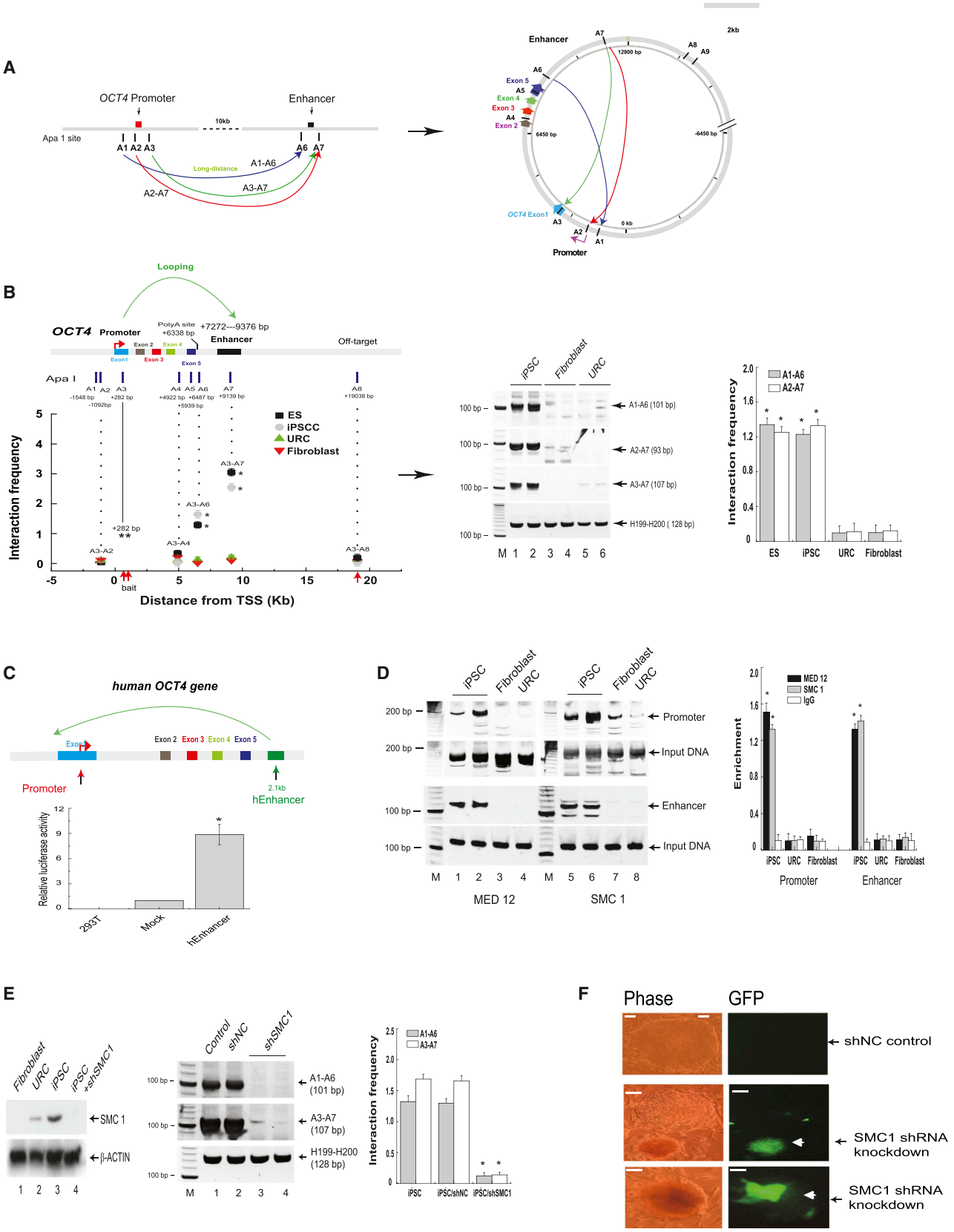
Figure 1. Virus-Derived Factors Fail to Induce Transcription of the Target Pluripotent Genes

(A) Low efficiency of iPSC induction. OSKME, *OCT4-SOX2-KLF4-c-MYC-EGFP*; OSNE, *OCT4-SOX2-NANOG-EGFP*; URCs, unreprogrammed cells. The rate of iPSC induction was given as the average number of alkaline phosphatase (AP)-positive colonies per 10,000 transduced cells from three independent experiments. Scale bar: 100 μm.

(B) Chromatin immunoprecipitation (ChIP) for *OCT4* promoter. Crosslinked DNA-protein complexes were immunoprecipitated with antiserum against OCT4 and NANOG, followed by PCR amplification with specific primers covering the promoter of *OCT4*. Input: genomic DNA collected before antibody precipitation. Left panel: RT-PCR. The arrows mark the location of ChIP-specific primers in the target gene promoter. Right panels: the qPCR measurement of the binding of viral OCT4 and NANOG factors to the *OCT4* promoter and the upstream off-target sites (vertical arrows). Numbers in parenthesis are genome gene sequences. IgG: negative binding control. Error bars represent the standard error of the average of three independent ChIP assays (each with three qPCR repeats). *$p < 0.01$ as compared with the off-target sites and fibroblast controls.

(C) Expression of the transgenic and endogenous stem cell marker genes. The mRNA transcripts of the virally transduced (exogenous) factors and the endogenous genes were distinguished, respectively, by specific primer sets covering either the vector T2A sequence or the untranslated region (UTR) RNA, which is absent in the virally induced transgenic cDNA. M, 100 bp marker; H14, embryonic stem cell line; WSF-1 and WSF-7, embryonic skin fibroblasts; Control, wild-type fibroblasts; URCs, virally infected fibroblasts that were not fully reprogrammed; Endo-, endogenous pluripotent genes; Transgene, retrovirally transduced factors. β-actin was used as an internal control.

Also see Figure S1.



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proteins derived from the OSNE retroviral vector also bound equally well to their downstream target gene (*OCT4*, *SOX2*, and *NANOG*) promoters. ChIP-qPCR data confirmed that there was enrichment of the virally expressed OCT4 and NANOG proteins bound to the three target gene promoters, but that there was no binding to the off-target site (Figures 1B, S1G, and S1H, right panels). Taken together, these data suggest that binding of virally expressed factors to the downstream target genes is not a limiting factor in the iPSC induction.

We then examined if the binding of virus-derived factors could activate the endogenous target genes, an essential step for successful iPSC induction. To distinguish the virus-derived expression from endogenous gene expression, the mRNA of the virally transduced (exogenous) factors was measured by specific primer sets that cover the viral T2A sequence. The expression of the endogenous genes that are related to cell pluripotency, on the other hand, was quantitated by primer sets covering the untranslated region (UTR) RNA, which is absent in the virally induced transgenic cDNA. Both the isolated iPSCs and URCs expressed the viral transgenes. However, endogenous *OCT4* expression, a key factor for iPSC induction, was detected in the iPSCs, but it was not detected at all in the URCs (Figure 1C). Similar results were also observed for the other two pluripotency genes *SOX2* and *NANOG*. Thus, it appears that even though the virus-derived factors bound to their target promoters, they failed to induce transcription from these genes, suggesting that the activation of endogenous stemness genes may represent a critical reprogramming block preventing iPSC generation.

To determine if epigenetic modifications, such as the remodeling of local chromatin structure, may be required for iPSC induction, we focused on the *OCT4* locus to address the mechanism underlying the failed activation of endogenous *OCT4* in URCs. It has recently been shown that DNA looping orchestrated by the cohesin-mediator complex determines the pluripotency of the stem cells (Kagey et al., 2010). We used chromosome conformation capture (3C) methodology (Dekker et al., 2002) to examine whether a different chromatin structure surrounding the *OCT4* gene is present in iPSCs compared to URCs. In iPSCs, the *OCT4* promoter DNA interacted frequently with a DNA region that is located 10 kb downstream of the promoter (Figure 2B, right panel, lanes 1 and 2 and qPCR data). These chromatin interactions were very rarely detected in URCs or in fibroblast control cells. We also used a quantitative PCR approach to compare the 3C interaction in the *OCT4* locus and a downstream off-target site (A8). For all loci we tested, there was increased interaction between the core promoter and the downstream enhancer (A3–A7) in ESCs and iPSCs (Figure 2B, left panel). We did not detect an interaction between the *OCT4* promoter and the upstream sequence where the putative mouse *Oct4* enhancer was reported (Kagey et al., 2010), suggesting a species-specific interaction in the human *OCT4*.

To address the role of the downstream DNA sequence, we cloned a 2.1 kb *OCT4* fragment (Figure 2C, top panel) and tested its enhancer activity. As seen in Figure 2C, the 2.1 kb DNA significantly augments promoter activity as measured by luciferase activity. This fragment has much stronger enhancer activity than the enhancer that was identified in the upstream mouse *Oct4* promoter (Kagey et al., 2010) (Figure S2D). These data indicate that intrachromosomal looping helps hinge the downstream enhancer in close proximity to the *OCT4* promoter, where it activates endogenous *OCT4* as an essential step in iPSC induction. In URCs, where the intrachromosomal loop is absent, endogenous *OCT4* cannot be activated because the promoter and the downstream enhancer are no longer juxtaposed. Using the same approach, we also detected intrachromosomal interactions between promoter DNAs and downstream enhancer sequences in both the *SOX2* and *NANOG* genes in the iPSCs, but not in the URCs and the uninfected fibroblasts (Figures S2A and S2B). These data demonstrate that the formation of intrachromosomal loops in these stemness genes may constitute a critical epigenetic barrier that must be overcome for cell reprogramming to occur.

In order to determine which *trans* chromatin modifying factors coordinate this chromatin looping, we focused on cohesin and mediator because both factor complexes have been reported

Figure 2. The SMC1-Mediated Intrachromosomal Interaction between OCT4 Promoter and Enhancer

(A) Schematic diagram of intrachromosomal interactions between ApaI sites (A1–A7) in the *OCT4* gene locus used for the chromosome conformation capture (3C) assay.

(B) Intrachromosomal interaction between the *OCT4* promoter (A1–A3) and enhancer (A6–A7) regions in iPSCs. Right panel: the intrachromosomal interactions between A1–A6, A2–A7, and A3–A7 sites were measured by both PCR and qPCR. H199/H200 PCR was used as the positive control. M, 100 bp marker. Left panel: qPCR was used to determine the interaction from the promoter (A3 bait, vertical arrows) to each ApaI sites and the off-target site (A8). A1–A7: ApaI sites in *OCT4* promoter and enhancer regions; numbers under A1–A7: distance from the translation start site (TSS). The interaction frequency was determined by normalizing the 3C PCR signal over that of the positive control (H199/H200 PCR). Error bars represent the standard error of the average of three independent 3C assays (each with three qPCR repeats). *$p < 0.01$ as compared to URCs and fibroblasts.

(C) Identification of the *OCT4* downstream interacting region as an *OCT4* enhancer. The enhancer activity was measured as the relative luciferase units in 293T cells. hEnhancer, *OCT4* enhancer inserted upstream of pGL2-promoter-*Luc*; Mock, empty pGL2-promoter-*Luc* vector; 293T, wild-type 293T cells. For comparison, the luciferase expression of the mock insert at 48 hr was arbitrarily set as 1 in the calculation. *$p < 0.01$ compared to mock luciferase expression by Student's t test. All data shown are mean ± SEM from three independent experiments.

(D) Binding of Mediator (MED12) and Cohesin (SMC1) to the *OCT4* promoter and enhancer regions as quantitated by both PCR and qPCR. The enrichment was determined by normalizing the PCR signal over that of the input DNA. Input: genomic DNA collected before antibody precipitation. M, 100 bp marker. All data shown are mean ± SEM from three independent experiments. *$p < 0.01$ as compared to URCs and fibroblasts.

(E) Loss of intrachromosomal loop between the *OCT4* promoter (A1–A3) and enhancer (A6–A7) regions in *SMC1* knockdown iPSCs as quantitated by both PCR and qPCR. Control, untreated control iPSCs; shNC, negative shRNA; shSMC1, *SMC1* shRNA. Data are represented as mean ± SD. *$p < 0.01$ as compared to untreated iPSCs and shNC-treated iPSCs.

(F) *SMC1* knockdown by shRNA caused the loss of self-renewal in human H14 ESCs. EGFP was used to track the expression of *SMC1* shRNA in H14 ESCs. Scale bar: 100 μm.

Also see Figure S2.

to mediate chromatin looping in mouse ESCs (Kagey et al., 2010). Both MED12 and SMC1 (components of the mediator and cohesin complexes, respectively) bound to the *OCT4* promoter and the downstream enhancer (Figure 2D) in iPSCs, but not in URCs, indicating that these factors may play a role in the formation or maintenance of the intrachromosomal interaction required for the activation of endogenous *OCT4*. A coimmunoprecipitation assay showed that SMC1 also interacted directly with MED12 (Figure S2E). Interestingly, we found that SMC1 is differentially expressed, with abundance in the order of human ESC line H14 > iPSC > URC > fibroblasts (Figure S2F). Thus, changes in the expression of SMC1 and, potentially, several other chromatin-binding factors may be needed for the formation of intrachromosomal loops in iPSC induction.

We used RNAi to knock down *SMC1* expression in iPSCs (Figure 2E, left panel). *SMC1* knockdown abolished the intrachromosomal loops (right panel), suggesting a critical role of *SMC1* in orchestrating the local *OCT4* chromatin structure required for induced pluripotency. We found that knockdown of *SMC1* in iPSCs also triggered cell differentiation (Figure S2G, top panel). Using the TurboGFP fluorescence as the tracker in shRNA vector, we observed that *SMC1*-knockdown H14 ESCs lost the capacity for self-renewal (Figure 2F). The *SMC1*-knockdown fibroblasts could no longer be reprogrammed into iPSCs (Figure S2G, bottom panel). Together, these data suggest that cellular reprogramming requires the formation of SMC1-dependent intrachromosomal looping.

We also virally transfected *SMC1* in fibroblasts and in URCs that expressed OSKM factors (Figure S2I, left panel). However, we found that *SMC1* expression for 2 weeks was not able to restore the intrachromosomal looping (right panel, lanes 2 and 5). The data suggest that other chromatin-remodeling factors expressed during iPSC induction may also be necessary for the formation of intrachromosomal loops. A recent study published online while this paper was in review (Apostolou et al., 2013) also demonstrates that several chromatin factors, including cohesin, Mediator, and pluripotency factors, are critical for induction and maintenance of pluripotency by mediating a pluripotency-specific chromatin interaction network in the *NANOG* locus. Future studies are needed to determine if overexpression of *SMC1* in OSKM-transfected fibroblasts or in URCs for a longer period of time could induce intrachromosomal looping and thus enhance the efficiency of iPSC induction.

It is also interesting to note that in URCs, where SMC1 is expressed at a very low level (Figure S2F, lane 2), the virally expressed OCT4 still binds to its target promoters (Figure 1B, lanes 3 and 6), suggesting that cohesin may not be necessary for the viral OCT4 binding to the target promoter. However, we cannot exclude the possibility that the decreased expression of *SMC1* may also affect cell division, leading to a decrease in cell reprogramming. An enhancer deletion study may be useful to examine if the *SMC1*-mediated chromatin loop is a prerequisite for the induced pluripotency.

We suggest a model whereby an existing intrachromosomal loop between the enhancer and promoter of certain stemness genes such as *OCT4* is needed for a cell to be transformed to pluripotency (Figure S2J). A certain number of cells may undergo nuclear remodeling either spontaneously or during reprogramming. This dynamic autoremodeling builds the promoter/enhancer intrachromosomal loop and thus makes cells more susceptible to reprogramming than cells that do not demonstrate these loops. In agreement with this notion is the finding that donor cell types influence reprogramming. The efficiency of iPSC generation differs dramatically among cell types used for reprogramming (Eminli et al., 2008; Kim et al., 2009; Silva et al., 2008). Generally speaking, a less differentiated cell is reprogrammed more easily than a well differentiated cell. It is possible that these less differentiated cells may have a larger subpopulation of cells that undergo the autoremodeling process, leading to the formation of intrachromosomal loops that activate pluripotent genes.

In summary, cell reprogramming factors require the formation of intrachromosomal loops that juxtapose the enhancer and promoter regions to reactivate the endogenous pluripotent genes. The participation of *SMC1* and other chromatin-modifying factors is needed to coordinate these local intrachromosomal loops. Unlike conventional somatic cell nuclear transfer and natural fertilization, where the cell undergoes chromatin decondensation and remodeling before it transits into the pluripotent stage (Burns et al., 2003; Simonsson and Gurdon, 2005), the induction of iPSCs by defined factors is a shortcut compared with these conventional approaches, and omits the usual process of chromatin remodeling, a key factor that affects iPSC induction. Thus, further studies should be focused on the identification of other factors that can organize chromatin loops in order to promote iPSC induction.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes iPSC characterization, Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.05.012.

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