In the last issue of *Cell Cycle*, Bortolomai et al. isolated cervical stem cells from the A431cell line exploiting the capability of these cells to form spheres in non-adherent conditions. Following isolation, ALDH enzymatic activity and Hoechst dye exclusion were employed to further confirm the presence and size of the stem-like population. Importantly, although A431 sphere cells were characterized by "stemness" properties such as self-renewal and clone forming capability, in adherent conditions they mainly formed differentiated colonies (paraclones) compared to A431 parental cells. These data, in spite of the apparent contradiction with the assumption that loss of anchorage and serum deprivation induce *anoikis* of differentiated cells,[5] could underlay the multipotent differentiative potential of sphere cells. Indeed, A431 sphere cells could be considered an experimental strategy to select and expand in suspension "holoclones" from a heterogenous population of cancer cells. It thus became clear that in culture conditions promoting differention, sphere cells gave rise to more differentiated colonies.

Long-term cultures in suspension confirmed that sphere cells maintain an higher EGFR stimulation-independent proliferative potential than parental cells. Moreover, A431 spheres display high expression levels of self-renewal-related genes such as NANOG, NESTIN and OCT4, and, more importantly, an enrichment of podoplanin-positive cells, which were recently described as the stem-like population of A431 cells.

Cancer stem cell nature of A431 spheres was thus definitely proved by their ability to recapitulate the generation of a continuously growing tumor, which display a more undifferentiated morphology than original human tumor phenotype from the fourth passage into immunocompromised mice.

Finally, the global gene expression profile of A431 sphere cells analyzed in comparison with that of A431 parental and adherent cells rederived from A431 spheroids, revealed genes encoding for Cytokeratin 6 and Osteopontin, were highly upregulated.

From this study, Osteopontin emerges as a good stem cell marker for cervical cancer. Through its interaction with integrins and CD44v, osteopontin is known to induce metalloprotease, thus representing an important modulator of cell motility.[6] Osteopontin supports the hypothesis that cells with stem-like properties within the neoplastic mass are responsible, apart from tumor initiation, also for invasion of surrounding tissues and metastasis. If invasive and metastatic properties are typical features of these cells, the newly developed salinomycin compound, could be efficient to eradicate cervical cancer.[7] This agent regulates the migration inhibiting potassium channels and consequently interfering with epithelial-mesenchymal transition.[8]

Together with the identification of the phenotypic characteristics of cervical cancer stem cells examined by Bortolomai and coworkers a better understanding of metastatic mechanisms intrinsic to CSCs will provide essential tools for prediction and successful treatment of this malignant tumor.

Acknowledgements

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References

1. Visvader JE, et al. Nat Rev Cancer 2008; 8:755-68.
2. Martens JE, et al. Int J Gynecol Pathol 2007; 26:463-8.
3. Ye F, et al. BMC Cancer 2008; 8:108.
4. Feng D, et al. Oncol Rep 2009; 22:1129-34.
5. Charafe-Jauffret E, et al. Pathobiology 2008; 75:75-84.
6. Rangaswami H, et al. Trends Cell Biol 2006; 16:79-87.
7. Gupta PB, et al. Cell 2009; 138:645-59.
8. Lin LF, et al. Cancer Res; 70:192-201.

From Dolly to hiPS: New insights into reprogramming

Comment on: Lagarkova MA, et al. Cell Cycle 2010; 9:937-46.

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When Ian Wilmut presented in 1997 his remarkable result that a somatic nucleus could be reprogrammed when transferred into the cytosolic environment of an enucleated oocyte (SCNT, somatic cell nuclear transfer) giving rise to a full mammalian offspring (the clone sheep Dolly)[1] the new area of inducing pluripotency has been born. It is interesting that it took more than a decade to further investigate the basis of this observation. Nowadays we learn more and more on mechanisms and since the notable work of Shinya Yamanaka and colleagues[2,3,4] we can minimize the necessary factors to four transcription factors or even less.[5] A recent issue of *Cell Cycle* goes along with this development and presents the article by Maria A. Lagarkova and coworkers: "Induction of pluripotency in human endothelial cells resets epigenetic profile on genome scale." Two new aspects are demonstrated: The team around Prof. Kiselev found that induced pluripotent cells (iPS cells) can be generated from human umbilical vein endothelial cells (HUVEC) by the conventional method of overexpressing oct4, sox-2, c-myc and klf4 and secondly using a full characterization of their functional and epigenetic properties they clearly demonstrate that iPS cells generated from fully differentiated somatic cells reset their epigenetic status to pluripotency. From this as well as from other studies it becomes now quite evident that the clue of reprogramming lies in epigenetic mechanisms establishing cellular identity during differentiation by (1) DNA cytosine methylation, (2) covalent histone modifications (3) remodeling of other chromatin associated proteins such as polycomb group proteins and transcription factors, and (4) pre- and post-transcriptional gene regulation by small non-coding RNAs, such as microRNAs.[6] During epigenetic reprogramming of the somatic nucleus, these modifications, in particular DNA methylation must be reset from a fully differentiated to a pluripotent state. The work by Lagarkova et al. succeeded to obtain human iPS cells from human endothelial cell (endo-iPS) which were similar to human embryonic stem cells in morphology and gene expression. Using genome-wide methylation profiling the group shows in particular that the promoter elements of endothelial specific genes were methylated following reprogramming. On the other side, the pluripotency-related promoters were hypomethylated to levels also observed in embryonic stem cells. A similarity between endo-iPS and ES cells was seen in the genome-wide methylation analysis of CpG sites located in the functional regions of over than 14,000 genes but it is interesting to mention that the methylation levels of 46 genes were found to

be different suggesting that these sites represent endothelial genes which are resistant to reprogramming. Overall CpG methylation of promoter regions in the pluripotent cells was higher than in somatic. Within their work, the team around Kiselev also demonstrated that during reprogramming female human endo-iPS cells the somatically silenced X chromosome are obviously reactivated leading to the notion that chromosomal inactivation is epigenetically regulated and thus reversible. All these data now strongly give us the aim to reach more understanding on the underlying mechanism occurring after overexpression of the "Yamanaka-factors." Why does the before thought irreversible epigenetic inactivation of the female X chromosome comes out to be reversible while other genes obviously are not reverted? Are there additional mechanisms controlling the inactivation of genes which are not subject to reprogramming? Is this a limitation of the iPS-strategy for a later clinical usage of these cells? Is the function affected as recently demonstrated in electrophysiological studies?[7] Do we need more sufficient reprogramming techniques based on a deeper insight into the genetic programming and reprogramming? How does this relate to possible dedifferentiation and carcinogenesis? After Dolly, which obviously resulted from a complete reprogramming of a somatic cell into the pluripotency, we have reached a lot but we still need more experimental data to get insights into processes of epigenetic memory and genetic regulation of the cell fate to a specific phenotype.

References

1. Wilmut I, et al Nature 1997; 6619:810-13.
2. Takahashi K, et al. Cell 2006; 126:663-76.
3. Okita K, et al. Nature 2007; 448:313-7.
4. Nakagawa M, et al. Nat Biotechnol 2007; 26:101-6.
5. Kim JB, et al. Nature 2009; 461:649-53.
6. Wu H, et al. Pediatr Res 2006; 59:21R–5R.
7. Kuzmenkin A, et al. FASEB J 2009; 23:4168-80.

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Induction of pluripotency in human endothelial cells resets epigenetic profile on genome scale

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Reprogramming of a limited number of human cell types has been achieved through ectopic expression of four transcription factors to yield induced pluripotent stem (iPS) cells that closely resemble human embryonic stem cells (ESCs). Here, we determined functional and epigenetic properties of iPS cells generated from human umbilical vein endothelial cells (HUVEC) by conventional method of direct reprogramming. Retroviral overexpression of four transcription factors resets HUVEC to the pluripotency. Human endothelial cell-derived iPS (endo-iPS) cells were similar to human ESCs in morphology, gene expression, in vitro and in vivo differentiation capacity. Endo-iPS cells were efficiently differentiated in vitro into endothelial cells. Using genome-wide methylation profiling we show that promoter elements of endothelial specific genes were methylated following reprogramming whereas pluripotency-related gene promoters were hypomethylated similar to levels observed in ESCs. Genome-wide methylation analysis of CpG sites located in the functional regions of over than 14,000 genes indicated that human endo-iPS cells were highly similar to human ES cells, although differences in methylation levels of 46 genes were found. Overall CpG methylation of promoter regions in the pluripotent cells was higher than in somatic. We also show that during reprogramming female human endo-iPS cells exhibited reactivation of the somatically silenced X chromosome. Our findings demonstrate that iPS cells can be generated from human endothelial cells and reprogramming resets epigenetic status of endothelial cells to pluripotency.

Cellular reprogramming has been achieved by direct transfer of somatic nucleus to the cytoplasm of pluripotent cell and by fusion of somatic and pluripotent cells.[1-4] The first report of genetic reprogramming involved overexpression of the transcription factor MyoD into fibroblast cells, this forced overexpression was sufficient to convert fibroblasts into muscle cells.[5] More recently four key factors Oct4 (*Pou5f1*), Sox2, cMyc and Klf4 have been shown to re-establish the pluripotent state in adult fibroblasts.[6] This technique has been modified and improved in subsequent studies.[7-9] Several reports have demonstrated that some of these four factors can be replaced or omitted, although this leads to a slight decrease in reprogramming efficacy.[10-13] Originally induced pluripotency stem (iPS) cells were generated using viral methods of gene delivery, leading to the integration of the transgene. Subsequently, nonintegrating approaches exploiting adenoviruses, plasmids or transgene removal or even protein transduction have been developed.[14,15]

Since the first report of induced pluripotency in mouse embryonic fibroblasts, a number of cell types have been shown to be amenable to reprogramming. For example, iPS cells have been generated from multiple mouse cell types.[14] Pluripotency has also been induced in human fibroblasts, keratinocytes and blood cells.[16-19] These studies have suggested a strong influence of cell type on the reprogramming efficiency and timing.[7,18] Additionally, choice of cell type is also important in terms of the therapeutic usefulness of reprogrammed cells. For example, cell accessibility or availability as well as cell age might be the critical factors in determining the optimal cell type for therapeutic use. Although adult skin cells are readily accessible and easy to manipulate these are exposed to UV and may thus accumulate mutations limiting their therapeutic application. Additional passaging in vitro or growth factor application required to expand the starting amount of cells prior to iPS generation could further contribute to epigenetic and genetic alterations in parental cells.[20,21] The choice of cell type to be used for reprogramming is therefore important to minimize accumulation of DNA damage and to obtain complete reprogramming.

Reprogramming of somatic cells into iPS cells is accompanied by gradual loss of somatic markers followed by the reactivation of the pluripotency markers.[22,23] However, some clonally derived cells in iPS colonies were able to reactivate the Oct4-GFP reporter earlier while some did not express it at all.[9] Hence, even though iPS cells are genetically identical and display characteristic features of ESCs, their transcriptional profiles can differ. To

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obtain genuine iPS cells, appropriate gene activation or silencing must be accompanied by epigenetic events leading to the reprogramming consolidation. During reprogramming, promoters of genes expressed in differentiated cell types should be silenced by methylation, whereas promoters of pluripotency genes should become hypomethylated.[24] Global changes in histone methylation, reactivation of a somatically silenced X chromosome in female cells, and demethylation at the promoters of key pluripotency genes have all been observed during reprogramming of mouse embryonic fibroblasts.[25] Although human iPS cells are very similar to hESCs transcriptionally their epigenome is still largely unknown.[17]

Here, we present the first report of generation of iPS cells from human umbilical vein endothelial cells (HUVECs). We established endo-iPS cell lines that could be maintained in defined culture conditions without karyotype changes and exhibit pluripotency in vitro and in vivo. Our data demonstrate that ectopic expression of four transcription factors is sufficient to revert endothelial cells to an embryonic state, to reset somatic cells genome-wide DNA methylation, and to start the reactivation of the silenced X chromosome in female cells.

Results

Generation of endo-iPS cells from HUVECs. We introduced the retroviruses expressing human *Oct3/4, Sox2, Klf4* and *c-Myc* into genetically unmodified, diploid HUVECs derived from the umbilical vein of a female newborn. The homogeneity and identity of HUVECs were characterized by flow cytometry analysis using CD31 and VE Cadherin antibodies; CD90 antibodies were used to detect possible fibroblast contamination (**Suppl. Fig. S1**).[26] Additionally homogeneity of cell population was confirmed by immunohistochemical analysis with antibodies to vWF, CD-105, CD-34, CD31 (data not shown).

Our protocol for iPS cells generation is summarized in **Figure 1A**. On days 3 and 5 post-infection, cells from parallel plates were harvested to analyze induction of *Nanog* and *FoxD3* expression (**Fig. 1B**). These transcription factors, not normally expressed in HUVECs, were detected as early as on the third day after viral infection. Cells were maintained in HUVEC medium for five days, and then plated onto mitomycin-treated mouse embryonic fibroblasts (MEFs) at a density 3 x 10^5 per 100 mm dish or left on Matrigel. On the next day, cell culture medium was replaced with KO-DMEM/KSR or mTeSR1 medium respectively. Around day 12 of infection, the first colonies that were distinct from feeder fibroblasts and endothelial cells appeared (**Fig. 1C–K**). On day 16, colonies acquired a hESC-like morphology that became even more pronounced by day 20 (**Fig. 1F and G**).

We also observed a number of colonies that were distinct from hESCs in their morphology. In spite of induction of *Nanog* and *FoxD3* expression HUVECs plated on Matrigel and grown in mTeSR1 medium did not form ES-like colonies. On day 21 we picked single hES cell-like colonies from MEFs, mechanically disaggregated them into small clumps without enzymatic digestion and replated these individually on inactivated MEFs and Matrigel with the addition of the respective medium. During this passage cells became almost undistinguishable from hESCs, they formed compact flat colonies, cells within the colony exhibited morphology similar to that of hESCs (**Fig. 1I–K**). All selected colonies were maintained on Matrigel in mTeSR1 medium from the first passage because this medium better preserved cells in undifferentiated state. A total of 12 expanded iPS-like cell lines were obtained from 5 x 10^5 HUVECs.

Expression of hESC markers. Concomitant with the morphological changes observed, HUVEC-derived iPS cell lines exhibited changes in gene expression. Although morphologically iPS colonies were very similar to hESC, RT-PCR revealed that HUVEC-derived iPS cell lines expressed different levels of pluripotency-related genes (**Fig. 1L**). Upregulation of pluripotency-related genes (*FoxD3*, *Hesx1*, *Nanog)* was accompanied by a decrease in levels of endothelial cell-specific gene expression. We did not detect PECAM (CD31) and VE-cadherin expression in some clones, although cells in other clones expressed different levels of endothelial cell-specific genes. Immunoflourescent staining revealed expression of nuclearly localized Oct3/4 and Nanog in all clones (**Fig. 2A–C and Suppl. Table S3**). However, differences in expression of hESC surface antigens such as SSEA-4 and TRA1-60 were observed between iPS cell lines. Some iPS lines expressed these antigens in a manner similar to hESCs (**Fig. 2D and E**). CD30 expression was also detected on these iPS clones (**Suppl. Fig. S2**).[27] In contrast, the other clones exhibited heterogeneous expression of hESC surface antigens (data not shown), suggesting a mixed population of ES-like cells.

We also performed immunostaining of iPS clones with endothelial cell-specific antibodies. Despite the presence of trace amount of mRNA for PECAM in some iPS clones we were unable to detect PECAN protein (**Suppl. Fig. S2B**). CD105 expression was also lost in all iPS clones examined (**Suppl. Fig. S2C and Suppl. Table S3**). These observations indicate that HUVECs acquired not only ESC-like morphology but also expression of pluripotency markers and lost endothelial cell markers during reprogramming.

Proliferation rate and karyotype analysis of human endo-iPS cells. Proliferation and karyotype stability are important requirements for growth and expansion of iPS cells in vitro. Isolated endo-iPS clones were subdivided into two groups based on the expression of ESC markers. Two endo-iPS lines (hereafter referred to as endo-iPS 10 and endo-iPS 12) closely resembled hESCs, while other clones did not possess all features of completely reprogrammed iPS (**Suppl. Table 3**) and did not show transgenes expression (data not shown). The endo-iPS 10 and endo-iPS 12 were grown on Matrigel in mTeSR1 and exhibited similar growth kinetics than hESC lines grown in the same conditions. Population doubling time was 31–34 h for endo-iPS cells and 30–36 h for hESCs. These iPS clones were passaged at the same ratio (1:6) and frequency (every 5–6 days) as human ES cells and did not exhibit any dramatic changes in proliferation rate for 21 passages. Next we examined the karyotype of endo-iPS cells 5 and 20 weeks after derivation by standard GTG-banding techniques. Both endo-iPS cell lines demonstrated normal XX karyotype (**Fig. 1M, Suppl. Fig. S2D**).

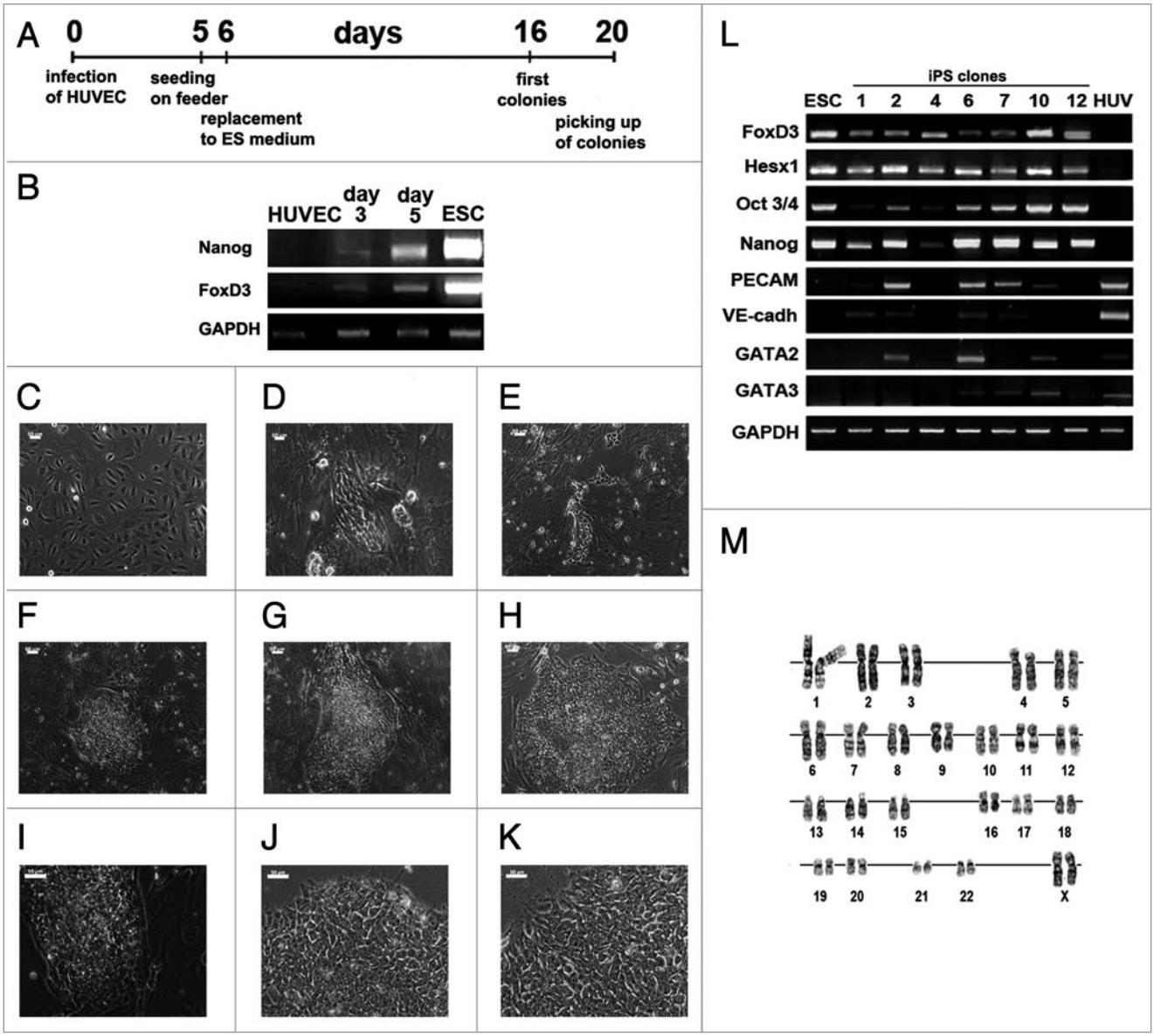

Figure 1. Generation and characterization of human endo-iPS cells. (A) Scheme of the iPS cells generation. iPS colonies were picked based on hESC-like morphology. (B) RT-PCR analysis of *Nanog* and *FoxD3* genes expression after viral infection. On the days indicated RNA was isolated for the analysis of the endogenously expressed genes. (C–K) Morphological changes during direct reprogramming. For details see Supplemental Methods. Representative colonies are shown: (C) HUVEC cells after viral infection, (D and E) after transfer on feeder majority of HUVECs died or became indistinguishable from feeder cells, first colonies of reprogrammed cells appeared on day 16, (F and G) ES-like colonies before passaging on day 20, after passaging some colonies still had distinctive from hESC morphology (H), other acquired indistinguishable from hESC morphology (clone 10 grown on feeder) (I). Examples of morphology of iPS colony (clone 12, passage 2) (J) and hESC line (hESM01) (K). grown on Matrigel are shown. (L) RT-PCR analysis of pluripotency (*FoxD3, Nanog, Oct3/4, Hesx1*) and endothelial-specific (*PECAM, VE-cadh, GATA2, GATA3*) markers expression in iPS clones, hESCs (positive control), as well as the parental HUVECs (negative control). GAPDH was used as a loading control. (M) Karyotype analysis of endo-iPS clone 12 grown on Matrigel in mTeSR1 at passage 20 is shown.

To confirm that endo-iPS clones were derived from HUVECs we performed DNA fingerprinting analyses with short tandem repeat (STR) markers (**Suppl. Table S4**). The patterns of 18 STRs were completely matched between human endo-iPS clones and the parental HUVEC line, although these patterns differed from hESC lines we used in the laboratory. Taking together our data indicate that we succeeded in generating of ES-like human endo-iPS cells that could be stably maintained in defined medium under feeder-free conditions.

Differentiation potential of human endo-iPS cells. Analyses of cell morphology and expression of molecular markers have demonstrated that lines 10 and 12 closely resemble hESCs. To determine differentiation potential of these lines we analyzed embryoid body (EB) formation in suspension culture. After 6

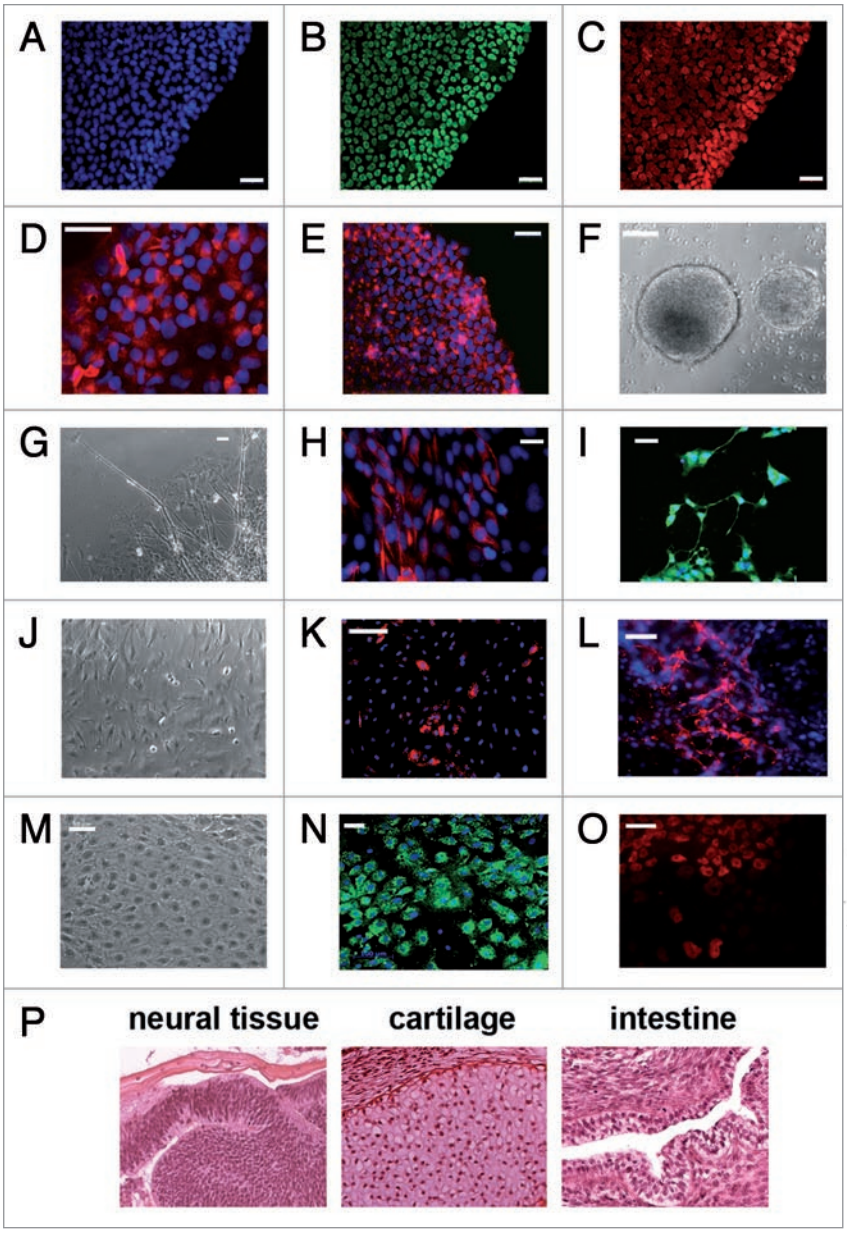

Figure 2. Immunohistochemical characterization of human endo-iPS cells and their differentiated derivatives. (A–E) Undifferentiated endo-iPS clones expressed markers common to hESCs. (A) 4,6-Diamidino-2-phenylindole (DAPI) staining indicates the total cell content per field. Immunostaining with specific antibodies for OCT3/4 (B), NANOG (C), Tra-1-81 (D), SSEA4 (E). Representative colonies of endo-iPS-10 and 12 cell lines are shown. EB differentiation of endo-iPS cells. On day 6 EB consisted of tight clusters of differentiating cells (F) later becoming cystic by day 12 (Suppl. Fig. S2E). Adherent EB showed various types of morphologies (G, J and M). Immunostaining with specific antibodies confirmed differentiation of endo-iPS cells into derivatives of three germ layers in vitro. (G–I) Ectodermal (neurons and glia) differentiation. (G) Bright field, (H) staining with antibodies against GFAP (red), (I) staining with antibodies against neuron-specific enolase. (J–L) Mesodermal differentiation. (J) Bright field image of endothelial cells, (K) endothelial cells stained for vWF, (L) endothelial cells forming cord-like structures stained with antibodies against CD31 (red). (M–O) Endodermal (primitive endoderm-like cells) differentiation. (M) Bright field, (N) staining with antibodies against alpha-fetoprotein, (O) staining with antibodies against GATA-6. Representative EBs formed by iPS-10, 12 cell lines are shown. (P) Hematoxylin and eosin staining of teratoma-like structures derived from immunodeficient mice injected with human endo-iPS cells (clone 12). Shown tissues represent all three embryonic germ layers including neural tissue (ectoderm), cartilage (mesoderm), intestinal epithelium (endoderm).

days in suspension endo-iPS cells have formed spherical EB-like structures (**Fig. 2F**). Prolonged culture resulted in the appearance of a typical cystic morphology (**Suppl. Fig. S2E**). EBs derived from both iPS clones expressed markers of the three germ layers. Immunohistological examination of EBs revealed clusters of cells positive for MOC-31, cytokeratin-7, vimentin and nestin (**Suppl. Fig. S2F–I**). Characteristic localization of these antigens within EB allowed us to distinguish an extraembryonic endoderm layer, an inner cystic epithelium, neural progenitor cells, and other indications of first steps of differentiation.

In order to determine whether iPS cell lines could differentiate into lineage-committed populations of the three germ layers, including endoderm, mesoderm and ectoderm, we transferred EBs to gelatin-coated plates and continued cultivation as described (Material and Methods). Attached cells showed a variety of cell morphologies (**Fig. 2J, G and M**). Multilineage differentiation of iPS cells into derivatives of the three germ layers was confirmed by immunostaining with respective antibodies. Ectodermal cells were stained for GFAP and neuron-specific enolase, mesodermal fibroblast-like cells were stained for prolyl 4-hydroxylase (**Fig. 2J–I and Suppl. Fig. S2K and L**). Endothelial cells generated from endo-iPS cells were stained for vWF and CD31 (**Fig. 2J–L**). CD31 positive cells, separated on magnetic beads, were subjected to the functional tests on Matrigel (**Suppl. Fig. S2M**). Endodermal differentiation was confirmed by alpha fetoprotein and GATA-6 staining (**Fig. 2M–O**).

To investigate the pluripotency of the endo-iPS cells in vivo we used well established test of the teratoma formation upon human pluripotent stem cells injection into immunodeficient mice.[14] We transplanted human endo-iPS cells into the dorsal flanks of immunodeficient (*nu/nu*) mice. Six to eight weeks after injection, we detected tumors containing differentiated tissues of all three embryonic germ layers (**Fig. 2P**). These results demonstrated the ability of human endo-iPS cells to differentiate in vitro and in vivo into the cells of all three embryonic germ layers, thus validating their pluripotent status.

Gene-specific and genome-wide DNA methylation of CpG loci is similar between iPS cells and ES cells. Reprogramming was previously shown to be associated with global changes in chromatin remodeling and DNA methylation.[25] Permanent silencing of pluripotency genes is achieved in part by DNA methylation during

differentiation from embryonic to somatic cells.[28,29] Cell type specific genes are methylated in hESCs and undergo demethylation during ESCs differentiation.[30] Based on this observation, we hypothesized that the CpG islands within promoter elements of endothelial cell-specific genes should become epigenetically silenced during reprogramming thus "locking in" reprogramming events. To test this hypothesis we performed genome-wide DNA methylation analysis using the Illumina HumanMethylation27 BeadChip assay that enables analysis of approximately 27,000 high-value methylation sites of the promoter regions or methylation hotspots of more than 14,000 genes at single-nucleotide resolution. Genomic DNA isolated from two human ESC lines, two endo-iPS cell lines, parental HUVEC and non-related human PBMC was used for bisulfite conversion and hybridization to the BeadChip.

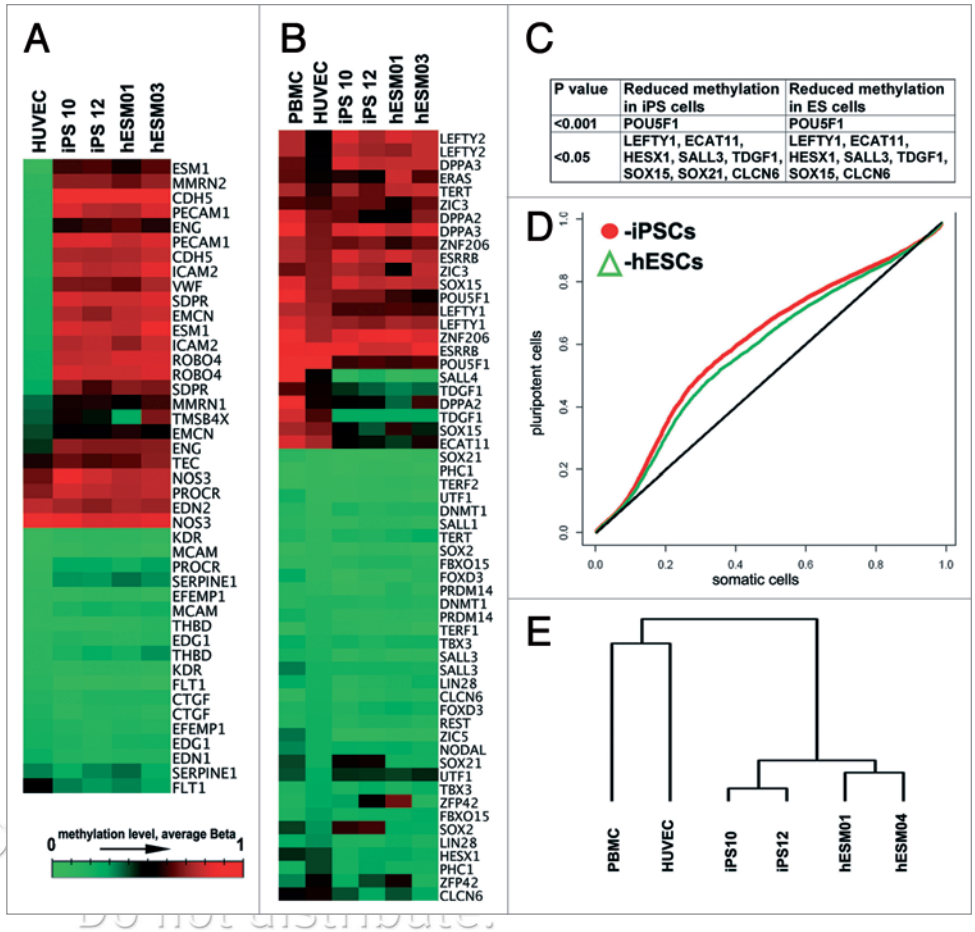

Figure 3. Genome wide and gene specific promoter CpG methylation analysis in somatic and pluripotent cells. HeatMap image of CpG methylation in 25 well-known endothelial specific genes (A) and 33 well-known pluripotency-related genes (B). (C) List of pluripotency-related genes with statistically significant reduced methylation level in pluripotent cell lines. (D) Comparison of CpG methylation level between somatic and pluripotent cells at genome scale. iPS cells are shown in red, hESCs in green. Distance from the black line indicates the level of CpG methylation in iPS and ES cells. (E) Classification of cell lines based on methylation signature. Dendrogram, as a representation of genome-wide hierarchical clustering is shown. Two groups of cell lines (somatic and pluripotent) are evidenced by clustering. For details see Supplemental Methods.

We first examined CpG methylation profiles of 25 endothelial cell-specific genes represented in the array (**Suppl. Table S5**). Promoter elements of two genes were hypermethylated in all cell lines examined (**Fig. 3A**), while ten genes were weakly methylated. Notably, approximately half of endothelial cell-specific genes underwent methylation during reprogramming to a level observed in hESCs. Therefore the majority of HUVEC lineage-specific genes examined became epigenetically silenced during reprogramming. These data are consistent with the observation that expression of PECAM, ENG (CD105) and CDH-5 (VE-cadherin) genes was silenced during reprogramming (**Fig. 1L**).

Demethylation of promoters of pluripotency genes, such as *Oct3/4* and *Nanog*, has been described to occur during reprogramming.[17,24] We therefore examined methylation levels of promoter elements of pluripotency-related genes represented by the array (**Suppl. Table S6**). Almost 60% of CpGs within promoter elements of pluripotency genes were weakly methylated, while less than 30% were highly methylated. To identify differentially methylated genes we compared methylation levels of individual CpGs of somatic cells comprising of HUVEC and PBMC, two endo-iPS cell lines, and two hESC lines (**Fig. 3B and Suppl. Table S7**). We found that differences between somatic and pluripotent cells were statistically significant for 9 out of 33 genes ($p < 0.05$) (**Fig. 3C**). Remarkably, only CpG of Oct3/4 (also know as POU5F1) met our most stringent statistical criteria ($p < 0.001$, positive B) as a differentially methylated gene between somatic and iPS cells.

Next, we evaluated genome-wide level of CpG promoter methylation, which showed that most (61%) of the genes were undermethylated (average Beta value <0.2) and only 10% were hypermethylated (average Beta value >0.8) among the whole set of analyzed cell lines. However, global methylation of both types of pluripotent cells was higher than in somatic cells, with endo-iPS cells exhibiting slightly higher methylation than ESCs as represented in the Quantile-Quantile plot (**Fig. 3D**). A close relationship between individual endo-iPS and hESC lines was demonstrated by Pearson correlation coefficient analysis and hierarchical clustering analysis (**Suppl. Table S8 and Fig. 3E**).

To establish similarities of endo-iPS and hESC lines and differences between these pluripotent cells and somatic cells, we

examined genome-wide gene-specific methylation pattern by differential analysis. We identified of a total of 2,226 genes that were differentially methylated between pluripotent and somatic cell types (8.4% of 26210 CpGs entered the analysis, $p < 0.05$) (**Suppl. Fig. S3**). Using a more stringent significance level ($p < 0.001$, positive B value), we detected 378 differentially methylated CpGs (1.4% of 26210), of which 306 CpGs were significantly differentially methylated between iPS and somatic cells, 372 between hESCs and somatic cells, and only 46 genes were differentially methylated between iPS and ESC lines. The intersection of differentially methylated CpGs of somatic/iPS and somatic/hESCs allowed us to identify 282 CpG loci different between somatic cells and both sets of pluripotent cells. Overall, our analysis confirmed a high degree of similarity between endo-iPS and hESC lines, as well as significant differences with somatic cells comprising of HUVEC and PBMC.

X chromosome reactivation in female human endo-iPS cells. X chromosome inactivation (XCI) is required for dosage compensation of X-linked genes in female cells.[31-33] It has been shown recently that in female mouse iPS cells chromatin modifications specific to inactive X (Xi) are erased and both X chromosomes are active. In turn, during differentiation of these cells reactivated X undergoes random inactivation.[25] Thus it makes mouse iPS cells very similar to mouse ES cells in respect of X chromosome status. However, no consensus on XCI status in undifferentiated human ESCs has been reached so far. Some hESC lines did not show any signs of X inactivation, in others XCI had been already achieved prior to differentiation.[34,35] We therefore examined whether reactivation of Xi occurs in human endo-iPS cells. We first studied levels of expression of *Xist* in endo-iPS and our hESCs lines. All cell lines except hESMK05 expressed moderate to high level of *Xist* (**Fig. 4A**).

In the mouse, upregulation of *Xist* expression and XCI are accompanied by trimethylation of histone H3 Lys-27 (H3me3K27) on Xi.[36] In contrast, methylation of H3 Lys-4 (H3me2K4) is generally associated with active chromatin.[37] We therefore used immunostaining to study chromatin modifications in hESCs, iPS cells and parental line HUVECs. To avoid possible influence of culture conditions pluripotent cells were grown under feeder free conditions in mTeSR1 medium. Female hESC lines showed different patterns of H3me3K27 staining. *Xist*-expressing hESC line hESM04 exhibited intense X chromosome staining of H3meK27 (**Fig. 4B**), while active chromatin marker H3me2K4 was depleted from Xi chromosome in these cells. In contrast, the hESMK05 cell line which did not express *Xist,* showed no prominent staining of H3me3K27 (**Fig. 4B and C**) on either X chromosome. To distinguish X chromosomes in this cell line we preformed FISH with an X chromosome-specific centromeric probe. However both X chromosomes exhibited abundant staining for the marker of active chromatin H3me2K4 in the hESMK05 cell line. In differentiated ESCs and in somatic cells, H3me3K27 methylation appears highly specific to Xi.[38-40] In agreement with these observations, H3me3K27 staining was detected at low to moderate levels in female HUVECs (**Fig. 4B and C**). Consistent with previously published data low level of H3me2K4 methylation was observed only as a few distinct spots on the X chromosome.[34] Methylated H3K27 in endo-iPS cells was concentrated only on one X chromosome, while no significant methylation was observed on the other. Furthermore H3meK27 staining was substantially higher than in parental HUVECs. We observed two different patterns of histone methylation along the X chromosome length. In some endo-iPS cells the staining intensity was similar between both arms while in others the p arm was markedly enriched in methylated H3K27 (**Fig. 4C**). With this exception the H3me3K27 X chromosome staining in endo-iPS cells closely resembled that of the hESM04 cell line (**Fig. 4C**). In endo-iPS cells, both X chromosomes exhibited H3me2K4 staining, although one chromosome was stained less intensely (**Fig. 4B**). Enrichment of H3me2K4 was detected over the extent of the X chromosome, with more profound staining on the distal part of p and 2 bands in q arms (**Fig. 4C**). The overall staining intensity was similar to that observed in the hESMK05 cell line where both X chromosomes were active. Thus, our findings suggest that in human female endo-iPS cells, Xi silenced in parental cells underwent significant chromatin modifications resembling a reactivated state.

XCI occurs during ESCs differentiation in vitro.[32,35] We next examined whether endo-iPS cells could change the X chromosome chromatin status during spontaneous differentiation. Immunostaining demonstrated enrichment of methylated H3me3K27 and loss of H3me2K4 staining in differentiating hESCs and endo-iPS cells (**Fig. 4D**), suggestive of XCI. Although the X chromosome inactivation status of hESC is not as clear as in mouse and appears to exhibit cell line-specific differences, endo-iPS cells clearly underwent XCI during differentiation. Taken together, our results demonstrate that transcription factor-induced conversion to pluripotent state globally resets the epigenetic state of HUVECs including X chromosome reactivation process.

Discussion

The choice of human cell types that have been successfully reprogrammed is still very limited. Our results show that human endothelial cells can be reprogrammed to pluripotency using genetic modifications. Individual HUVECs are easy to isolate and require little or no manipulation in culture, which could minimize acquisition of DNA damage. Moreover HUVECs can be collected by umbilical cord blood banks and stored for the future use. Endo-iPS cell lines were similar to hESCs in many respects, including morphology, proliferation, feeder-independence, surface markers, gene expression, DNA methylation status, in vitro and in vivo differentiation, and XCI. Although the overall efficiency of HUVEC reprogramming was relatively low (approximately 0.03%), the intrinsic sensitivity of HUVECs to ESCs culture medium provided a convenient means of iPS lines isolation without drug selection.

The dynamics of reprogramming of endo-iPS cells was similar to other iPS lines published. The first ES-like colonies were detected approximately at the same time point as iPS generated from $CD34^+$ cells and complete reprogramming was achieved within 3 weeks.[19] In endo-IPS lines, expression of the Oct4

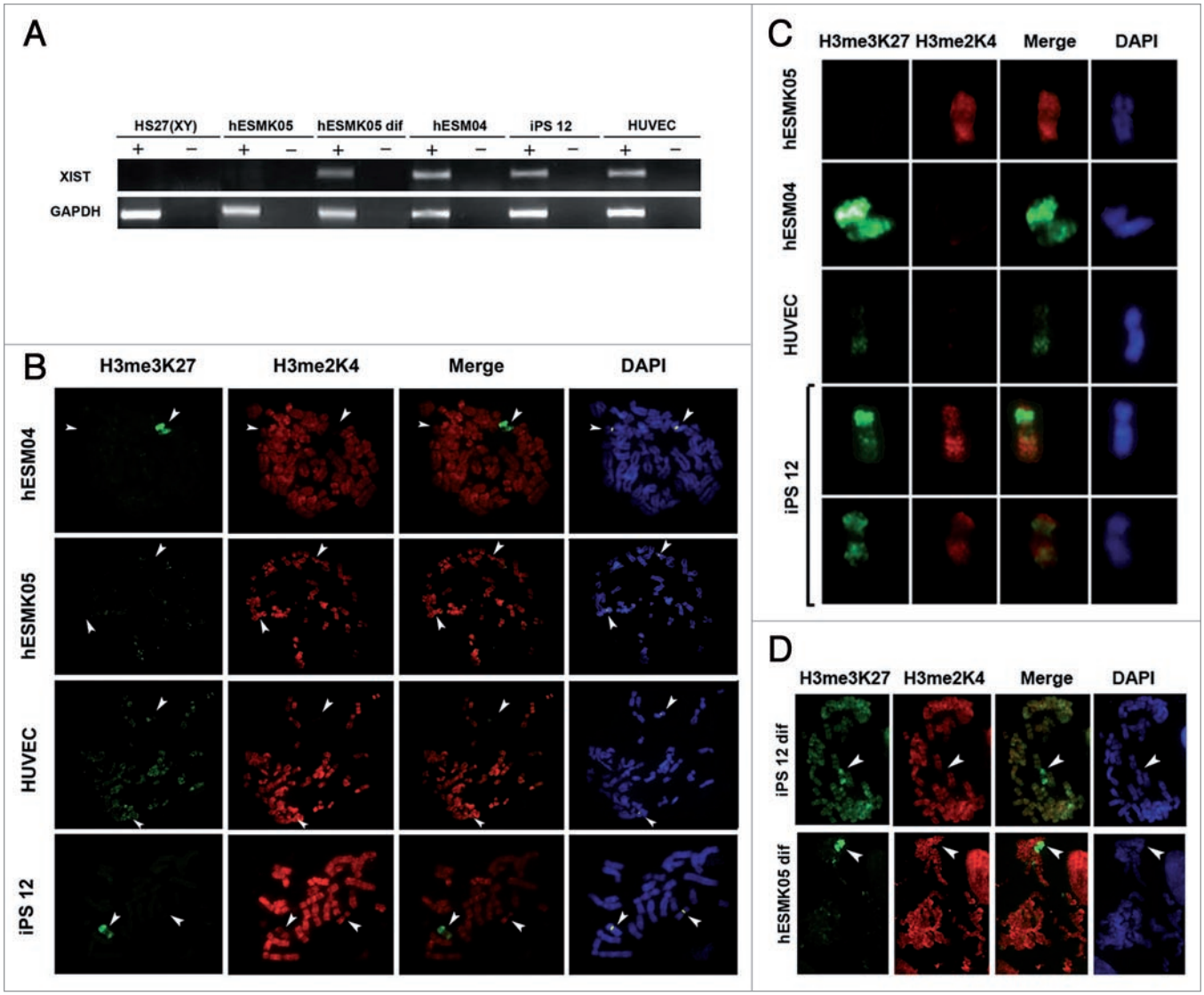

Figure 4. X chromosome reactivation in endo-iPS cells. (A) RT-PCR analysis of Xist expression in pluripotent cells. Male human fibroblasts HS27 are shown as a negative control. RNA level is normalized to GAPDH expression. (B–D) Indirect immunofluorescent analysis of H3me3K27 (green, Alexa488) and H3me2K4 (red, Alexa546) distribution on metaphase chromosomes of endo-iPS cells, HUVEC, and two female hESCs lines. Images represent typical distributions obtained from more than 20 metaphase spreads. Chromosomes were counterstained with DAPI (blue). (B) In iPS cells one X chromosome is stained for active H3me2K4 and H3me3K27 inactive chromatin markers. Metaphase spreads of undifferentiated iPS, undifferentiated hESCs, and HUVECs are shown. Arrows indicate X chromosomes. DAPI is merged with X-alpha-satellite FISH signal (yellow) to distinguish X chromosomes. (C) Higher magnification of X chromosomes shown on (B). For hESM05 cell line only one of two active X is shown. For iPS cells two different patterns of staining are shown. Overlapping signals of H3me3K27 and H3me3K4 could be observed on p and q arms of the X chromosome in iPS-12 cells. (D) During hESCs or iPS cells differentiation X chromosome looses H3me2K4 active chromatin mark. Metaphase chromosomes of differentiated iPS-12 cells (upper) and differentiated hESMK05 (lower). Inactivated X chromosome is indicated by arrow.

target Nanog appeared as early as day 3 after viral transduction, although transfer to ESC culture conditions at this time did not result in formation of stable colonies. Rather, ES-like colonies were derived more efficiently when cells were transferred to ESC culture medium on day 6. Thus although the expression of endogenous pluripotency genes was activated very early, this time period was not sufficient to establish reprogramming.

iPS cells must be epigenetically similar to ESCs, demonstrating X chromosome reactivation in female cells and similar DNA methylation pattern at least in the promoter regions of pluripotency genes.[14] In mouse female iPS both X chromosomes are active.[25] Three states of X-inactivation in cultured female hESCs: active, inactive and partially reactivated have been described.[35,41-43] In our iPS cells, we observed appearance intense immunostaining of the X chromosome with H3me3K27 and H3me2K4 in overlapping domains, indicating that at least partial reactivation of X has occurred. Some genes, including developmentally regulated, contain regions consisting of overlapping histone modifications that have opposing roles. These so-called bivalent chromatin modifications (H3me3K27 + H3me2K4) are positioned in relatively

permissive chromatin, making these loci more accessible for remodeling complexes and transcription factors.[44-47] In human iPS cells we observed painting for both repressive and activating markers in the same X chromosome regions. We hypothesize that bivalent chromatin modifications are involved in the process of reprogramming, and H3me3K27 may be a transient hallmark, indicative of further chromatin modifications to follow. This is further supported by the observation that during XCI gradual enrichment, and then loss of H3me3K27 was detected in differentiating mouse ESCs,[38,39] suggesting that abundant H3K27 histone methylation is not important for maintenance of inactivation per se.

DNA methylation is an important mechanism maintaining the epigenetic state. During ESCs differentiation, pluripotency regulatory genes undergo methylation whereas lineage-specific genes that become transcriptionally active during differentiation lose their DNA methylation.[27,29,30] We focused on functionally defined CpG sites of promoter elements throughout the genome. We showed that, during genetic reprogramming the decrease in expression of endothelial specific genes was associated with hypomethylation of their promoter regions. In hESCs promoter regions of the same genes were highly methylated, probably indicating that silencing of lineage-specific genes is very important at this developmental stage, when cells should maintain their pluripotency. Surprisingly, CpG sites in the promoters of the pluripotency-related genes were mostly undermethylated in somatic cells thus making "gene leakage" more possible. We speculate that regulation of gene expression during developmental stages is more stringent than at adulthood and it is more difficult to differentiate pluripotent cells to particular lineage than convert somatic cells to pluripotent state. Alternatively, taking into account common pathways of pluripotency and tumorigenicity, such differences may indicate that somatic cells are epigenetically predisposed for transformation.

Our study of CpG sites of more than 14,400 gene promoters distributed throughout genome demonstrated that almost half of tissue-specific genes examined underwent significant methylation during reprogramming. In general, gene promoters in iPS and ESC lines were hypermethylated on genome scale, however most significantly differentially methylated CpGs between somatic and pluripotent cells were hypomethylated in these pluripotent cell lines. These genes may provide new targets to improve or monitor reprogramming efficiency.

Recently two papers describing methylation signature of hES and iPS cells were published.[48,49] Using limited set of genes both groups showed that fibroblasts and pluripotent cells were well grouped into two different clusters. However, within pluripotent cluster hESCs were more similar in methylation profile to fibroblasts, than were iPS cells.[49] We did not observe this in our study, correlation of endo-iPS with hESCs was very high, although endo-iPS were slightly more similar to somatic than hESCs. Possibly these differences could be attributed to the cells of origin and variations in cell culture conditions. Nevertheless, the number of experimentally obtained differentially methylated regions between pluripotent and somatic cells (2226) was very close to the number extrapolated from the study of two human chromosomes (3186).[49] Thus, focusing on CpG promoter methylation on the genome-wide level is an efficient strategy to analyze effectively a large number of samples in order to confirm cell reprogramming.

In conclusion, our study defines easy accessible endothelial cells as amenable for reprogramming to pluripotency providing a valuable experimental model as well as a practical alternative of therapeutically applicable pluripotent cells. DNA methylation profiling on a genome-wide level offers new criteria for establishment of optimally efficient and safe methods of cell reprogramming.

Materials and Methods

Lentivirus production and infection. Phoenix cells were transfected with the pMXs-based retroviral vectors encoding the cDNA of OCT4, SOX2, C-MYC and KLF4 were obtained from Addgene.[16]

Cell culture. HUVEC cells were derived from umbilical vein of newborn Caucasian female which was obtained following informed consent for scientific studies approved by the Institutional Ethic Committee. Cells were isolated and cultivated according to the previously published protocol.[50]

hESC lines hESM01, hESM03, hESM04 and hESMK05 were cultivated in mTeSR1 medium on Petri dishes coated with Matrigel according to manufacturer protocol. HS-27 human foreskin fibroblasts (ATCC#CRL-1634) were grown in DMEM with 10% FBS.[28,51]

For virus transduction, HUVEC at passage 2 were seeded at a density of 3 x 10^6 cells per 100 mm dish and incubated with virus-containing supernatants (MOI 5 for each virus) on Matrigel (BD) in DMEM with 20% Fetal Bovine Serum (FBS, Hyclone), 5 ng/ml hrbFGF (Peprotech), 1% nonessential amino acids, 2 mM L-glutamine, and 50 units/ml penicillin, 50 μg/ml streptomycin (all from Invitrogen) ("HUVEC medium") for 48 hr. Then the medium was changed every other day. Five days later, cells were passaged with 0.25% trypsin to mitomycin C treated (10 μg/ml, Sigma) mouse embryonic fibroblasts (MEF) on tissue culture plates pre-coated with 0.1% gelatin (Sigma). The culture medium consisted of 80% KO DMEM, 20% KO SR, 1 mM glutamine, 1% nonessential amino acids, 50 units/ml penicillin, 50 μg/ml streptomycin (all from Invitrogen), 0.1 mM beta-mercaptoethanol (Sigma). Alternatively, cells were left on Matrigel but on day 6 HUVEC medium was replaced on mTeSR1 (StemCell Technologies). The half of medium was changed every day. Twenty days after virus transduction, iPS colonies were picked up and replated on feeder cells or Matrigel-coated 24-well plates in "ES medium" or mTeSR1, respectively. For early passages, iPS cells were propagated manually, whereas subsequent later passaging was performed with collagenase IV (1 mg/ml) or dispase (1 mg/ml) treatment. hESCs were passaged every 5–7 days by exposure to 1 mg/ml dispase (Invitrogen) for 5–10 minutes at 37°C. For doubling time calculation approximately 20,000 cells were plated on Matrigel-coated 24-well tissue culture plates. At time points 48, 72 and 96 hours after plating cells were trypsinized and counted. Each time point was evaluated in triplicate.

Metaphase chromosome preparation, immunocytochemistry and FISH. Metaphase chromosomes for immunochemistry were prepared by cytocentrifugation as described.[52] Primary antibodies were diluted 1:200 and applied overnight at 4°C. Slides were washed and visualized by incubation with Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen) and Alexa Fluor 488 goat anti-mouse IgG (Invitrogen). Slides were counterstained with DAPI. After immunocytochemistry slides were fixed in 4% PFA/PBS and FISH was performed with direct (Texas Red) labeled human X chromosome alpha satellite probe DXZ1 (Oncor) according to the manufacturer' instruction.

Embryoid bodies formation and in vitro differentiation. iPS grown on Matrigel were treated with 1 mg/ml dispase and scraped into clumps. Clumps were cultured as suspension in a medium consisting of DMEM/F12, 20% FBS, 2 mM L-glutamine, 1% MEM nonessential amino acids, and 0.1 mol/L beta-mercaptoethanol, 2 ng/ml bFGF for 10–12 days in Ultra low adhesion plates (Corning). For further differentiation EB were transferred onto gelatin-coated culture dishes in the same medium supplemented with ITS, 10 ng/ml bFGF and the attached cells were grown for 12–24 days, then morphologically distinct areas were subjected to immunohistochemical analysis. Endothelial differentiation was performed as previously described.[30]

Genome-wide DNA methylation analysis. Genomic DNA was extracted from endothelial cells, PBMC, iPS or hES cells using Genomic DNA Purification Kit (Fermentas). Bisulfide conversion was performed on 1 ug individual DNA samples using EZ DNA Methylation kit (Zymo Research) according to the manufacturer's recommendations. Hybridization onto HumanMethylation27 DNA Analysis BeadChip (Illumina) and washing were carried out using Infinium Assay kit (Illumina) according to manufacturer's recommendations. Scanning was done on the Bead Array Reader (Illumina), data was extracted and normalized with GenomeStudio Methylation Module v1.0 (Illumina). Average Beta values were computed as quantitative measure of DNA methylation ranging from zero, for completely unmethylated cytosines, to one, for completely methylated cytosines (see also Suppl. methods).

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/LagarkovaCC9-5-Sup.pdf

References

1. Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, et al. Eight calves cloned from somatic cells of a single adult. Science 1998; 282:2095-8.
2. Wakayama T, Shinkai Y, Tamashiro KL, Niida H, Blanchard DC, Blanchard RJ, et al. Cloning of mice to six generations. Nature 2000; 407:318-9.
3. Tada M, Takahama Y, Abe K, Nakatsuji N, Tada T. Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr Biol 2001; 11:1553-8.
4. Matveeva NM, Shilov AG, Kaftanovskaya EM, Maximovsky LP, Zhelezova AI, Golubitsa AN, et al. In vitro and in vivo study of pluripotency in intraspecific hybrid cells obtained by fusion of murine embryonic stem cells with splenocytes. Mol Reprod Dev 1998; 50:128-38.
5. Davis RL, Weintraub H, Lassar AB. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 1987; 51:987-1000.
6. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006; 126:663-76.
7. Maherali N, Ahfeldt T, Rigamonti A, Utikal J, Cowan C, Hochedlinger K. A high-efficiency system for the generation and study of human induced pluripotent stem cells. Cell Stem Cell 2008a; 3:340-5.
8. Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. Science 2008; 322:949-53.
9. Meissner A, Wernig M, Jaenisch R. Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. Nat Biotechnol 2007; 25:1177-81.
10. Blelloch R, Venere M, Yen J, Ramalho-Santos M. Generation of induced pluripotent stem cells in the absence of drug selection. Cell Stem Cell 2007; 1:245-7.
11. Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, et al. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 2008; 26:101-6.
12. Kim JB, Zaehres H, Wu G, Gentile L, Ko K, Sebastiano V, et al. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature 2008; 454:646-50.
13. Kim JB, Sebastiano V, Wu G, Araúzo-Bravo MJ, Sasse P, Gentile L, et al. Oct4-induced pluripotency in adult neural stem cells. Cell 2009; 136:411-9.
14. Maherali N, Hochedlinger K. Guidelines and techniques for the generation of induced pluripotent stem cells. Cell Stem Cell 2008b; 3:595-605.
15. Zhou H, Wu S, Joo J, Zhu S, Han D, Lin T, et al. Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins. Cell Stem Cell 2009; 4:381-4.
16. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007; 131:861-72.
17. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007; 318:1917-20.
18. Aasen T, Raya A, Barrero MJ, Garreta E, Consiglio A, Gonzalez F, et al. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat Biotechnol 2008; 26:1276-84.
19. Loh YH, Agarwal S, Park IH, Urbach A, Huo H, Heffner GC, et al. Generation of induced pluripotent stem cells from human blood. Blood 2009; 113:5476-9.
20. Nagler A, Korenstein-Ilan A, Amiel A, Avivi L. Granulocyte colony-stimulating factor generates epigenetic and genetic alterations in lymphocytes of normal volunteer donors of stem cells. Exp Hematol 2004; 32:122-30.
21. Tigue CC, McKoy JM, Evens AM, Trifilio SM, Tallman MS, Bennett CL. Granulocyte-colony stimulating factor administration to healthy individuals and persons with chronic neutropenia or cancer: an overview of safety considerations from the Research on Adverse Drug Events and Reports project. Bone Marrow Transplant 2007; 40:185-92.
22. Brambrink T, Foreman R, Welstead GG, Lengner CJ, Wernig M, Suh H, Jaenisch R. Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. Cell Stem Cell 2008; 2:151-9.
23. Stadtfeld M, Maherali N, Breault DT, Hochedlinger K. Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. Cell Stem Cell 2008; 2:230-40.
24. Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, et al. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 2007; 448:318-24.
25. Maherali N, Sridharan R, Xie W, Utikal J, Eminli S, Arnold K, et al. Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell 2007; 1:55-70.
26. Kisselbach L, Merges M, Bossie A, Boyd A. CD90 expression on primary cells and elimination of contaminating fibroblasts from cell cultures. Cytotechnology 2009; 59:31-44.
27. Lagarkova MA, Volchkov PY, Philonenko ES, Pfannkuche K, Prokhorovich MA, Zabotina T, et al. CD30 is a marker of undifferentiated human embryonic stem cells rather than a biomarker of transformed hESCs. Cell Cycle 2008; 7:3610-2.
28. Yeo S, Jeong S, Kim J, Han JS, Han YM, Kang YK. Characterization of DNA methylation change in stem cell marker genes during differentiation of human embryonic stem cells. Biochem Biophys Res Commun 2007; 359:536-42.

29. Lagarkova MA, Volchkov PY, Lyakisheva AV, Philonenko ES, Kiselev SL. Diverse epigenetic profile of novel human embryonic stem cell lines. Cell Cycle 2006; 5:416-20.
30. Lagarkova MA, Volchkov PY, Philonenko ES, Kiselev SL. Efficient differentiation of hESCs into endothelial cells in vitro is secured by epigenetic changes. Cell Cycle 2008b; 7:2929-35.
31. Heard E. Recent advances in X-chromosome inactivation. Curr Opin Cell Biol 2004; 16:247-55.
32. Valley CM, Willard HF. Genomic and epigenomic approaches to the study of X chromosome inactivation. Curr Opin Genet Dev 2006; 16:240-5.
33. Payer B, Lee JT. X chromosome dosage compensation: how mammals keep the balance. Annu Rev Genet 2008; 42:733-72.
34. Hoffman LM, Hall L, Batten JL, Young H, Pardasani D, Baetge EE, et al. X-inactivation status varies in human embryonic stem cell lines. Stem Cells 2005; 23:1468-78.
35. Silva SS, Rowntree RK, Mekhoubad S, Lee JT. X-chromosome inactivation and epigenetic fluidity in human embryonic stem cells. Proc Natl Acad Sci USA 2008; 105:4820-5.
36. Sidhu SK, Minks J, Chang SC, Cotton AM, Brown CJ. X chromosome inactivation: heterogeneity of heterochromatin. Biochem Cell Biol 2008; 86:370-9.
37. Boggs BA, Cheung P, Heard E, Spector DL, Chinault AC, Allis CD. Differentially methylated forms of histone H3 show unique association patterns with inactive human X chromosomes. Nat Genet 2002; 30:73-6.
38. Plath K, Fang J, Mlynarczyk-Evans SK, Cao R, Worringer KA, Wang H, et al. Role of Histone H3 Lysine 27 Methylation in X Inactivation. Science 2003; 300:131-5.
39. Rougeulle C, Chaumeil J, Sarma K, Allis CD, Reinberg D, Avner P, Heard E. Differential histone H3 Lys-9 and Lys-27 methylation profiles on the X chromosome. Mol Cell Biol 2004; 24:5475-84.
40. Chadwick BP, Willard HF. Multiple spatially distinct types of facultative heterochromatin on the human inactive X chromosome. Proc Natl Acad Sci USA 2004; 101:17450-5.
41. Shen Y, Matsuno Y, Fouse SD, Rao N, Root S, Xu R, et al. X-inactivation in female human embryonic stem cells is in a nonrandom pattern and prone to epigenetic alterations. Proc Natl Acad Sci USA 2008; 105:4709-14.
42. Hall LL, Byron M, Butler J, Becker KA, Nelson A, Amit M, et al. X-inactivation reveals epigenetic anomalies in most hESC but identifies sublines that initiate as expected. J Cell Physiol 2008; 216:445-52.
43. Dvash T, Fan G. Epigenetic regulation of X-inactivation in human embryonic stem cells. Epigenetics 2009; 4:19-22.
44. Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 2006; 125:315-26.
45. Zhao XD, Han X, Chew JL, Liu J, Chiu KP, Choo A, et al. Whole-genome mapping of histone H3 Lys4 and 27 trimethylations reveals distinct genomic compartments in human embryonic stem cells. Cell Stem Cell 2007; 1:286-98.
46. Gan Q, Yoshida T, McDonald OG, Owens GK. Concise review: epigenetic mechanisms contribute to pluripotency and cell lineage determination of embryonic stem cells. Stem Cells 2007; 25:2-9.
47. Pan G, Tian S, Nie J, Yang Ch, Ruotti V, Wei, et al. Whole-genome analysis of histone H3 lysine 4 and lysine 27 methylation in human embryonic stem cells. Cell Stem Cell 2007; 1:299-312.
48. Ball MP, Li JB, Gao Y, Lee JH, LeProust EM, Park IH, et al. Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. Nat Biotechnol 2009; 27:361-8.
49. Deng J, Shoemaker R, Xie B, Gore A, LeProust EM, Antosiewicz-Bourget J, et al. Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming. Nat Biotechnol 2009; 27:353-60.
50. Baudin B, Bruneel A, Bosselut N, Vaubourdolle M. A protocol for isolation and culture of human umbilical vein endothelial cells. Nat Protoc 2007; 2:481-5.
51. Eremeev AV, Svetlakov AV, Polstianoy AM, Bogomazova AN, Philonenko ES, Sheina Yu I, et al. Derivation of a novel human embryonic stem cell line under serum-free and feeder-free conditions. Doklady Biological Sciences 2009; 426:270-2.
52. Jeppesen P. Immunofluorescence in cytogenetic analysis: method and applications. Genet Mol Biol 2000; 23:1107-14.