Cell Stem Cell
Article

A Mesenchymal-to-Epithelial Transition Initiates and Is Required for the Nuclear Reprogramming of Mouse Fibroblasts

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DOI 10.1016/j.stem.2010.04.014

SUMMARY

Epithelial-to-mesenchymal transition (EMT) is a developmental process important for cell fate determination. Fibroblasts, a product of EMT, can be reset into induced pluripotent stem cells (iPSCs) via exogenous transcription factors but the underlying mechanism is unclear. Here we show that the generation of iPSCs from mouse fibroblasts requires a mesenchymal-to-epithelial transition (MET) orchestrated by suppressing pro-EMT signals from the culture medium and activating an epithelial program inside the cells. At the transcriptional level, Sox2/Oct4 suppress the EMT mediator Snail, c-Myc downregulates TGF-β1 and TGF-β receptor 2, and Klf4 induces epithelial genes including E-cadherin. Blocking MET impairs the reprogramming of fibroblasts whereas preventing EMT in epithelial cells cultured with serum can produce iPSCs without Klf4 and c-Myc. Our work not only establishes MET as a key cellular mechanism toward induced pluripotency, but also demonstrates iPSC generation as a cooperative process between the defined factors and the extracellular milieu.

INTRODUCTION

The direct reprogramming of somatic cells to an embryonic stem cell (ESC)-like state by defined factors is an approach commonly known as iPS (Takahashi and Yamanaka, 2006). This technology is devoid of ethical concerns and can produce pluripotent stem cells from an individual that are compatible with his/her own immune system. Compared to somatic cell nuclear transfer (SCNT) (Wilmut et al., 1997), iPS not only has solved a technical hurdle but also offers a unique experimental system to investigate key questions regarding cell fate determination and epigenetic regulation. iPSC generation is viewed as a multiple-step course of action mediated by transcription factors that progressively induce the expression of ESC-like genes and suppress the somatic cell genetic program (Brambrink et al., 2008). Because the appearance of iPSCs takes at least 12–24 days and is very inefficient, nuclear reprogramming is considered a stochastic process in which successive barriers must be overcome to reach a state toward pluripotency (Hanna et al., 2009b; Yamanaka, 2009), but the nature of these barriers is poorly understood. Remarkably, a series of compounds including TGF-β (transforming growth factor β) inhibitors have been identified that accelerate or improve the reprogramming (Esteban et al., 2010; Huangfu et al., 2008; Ichida et al., 2009; Maherali and Hochedlinger, 2009; Mikkelsen et al., 2008), and this may shed light into the putative reprogramming roadblocks.

One of the first noticeable changes during the reprogramming of fibroblasts is their transformation into tightly packed clusters of rounded cells in a process that resembles a MET (Qin et al., 2007; Takahashi and Yamanaka, 2006), the opposite of EMT. During development, EMT events occur as early as gastrulation and are frequent afterwards, for example in the delamination of the neural crest (Thiery et al., 2009). EMT consists of coordinated changes in cell-cell and cell-matrix interactions that lead to loss of epithelial features and acquisition of mesenchymal characteristics. A previous analysis has estimated that EMT changes the expression of about 4000 genes (~10% of the human genome) (Zavadil et al., 2001), with two genes reproducibly changed in all forms of EMT: E-cadherin (downregulated) and Snail (upregulated). Snail is a basic helix-loop-helix transcription factor that binds to specific cognate sequences termed E-boxes and represses the transcription of E-cadherin and other key epithelial regulators (Batlle et al., 2000; Cano et al., 2000; Nieto, 2002). E-cadherin is a transmembrane constituent of intercellular adherens junctions responsible for maintaining epithelial cohesion (Cavallaro and Christofori, 2004) and has also been linked to the control of ESC pluripotency (Chou et al., 2008; Soncin et al., 2009). Here we demonstrate that the exogenous reprogramming factors activate an epithelial program and shut down key mesenchymal genes to overcome the EMT epigenetic barrier of fibroblasts and allow their successful reprogramming into pluripotent stem cells.

RESULTS

The Reprogramming of Fibroblasts Starts with a MET

Morphological differences between elongated fibroblasts and mouse ESCs are profound (Figure 1A). During reprogramming,

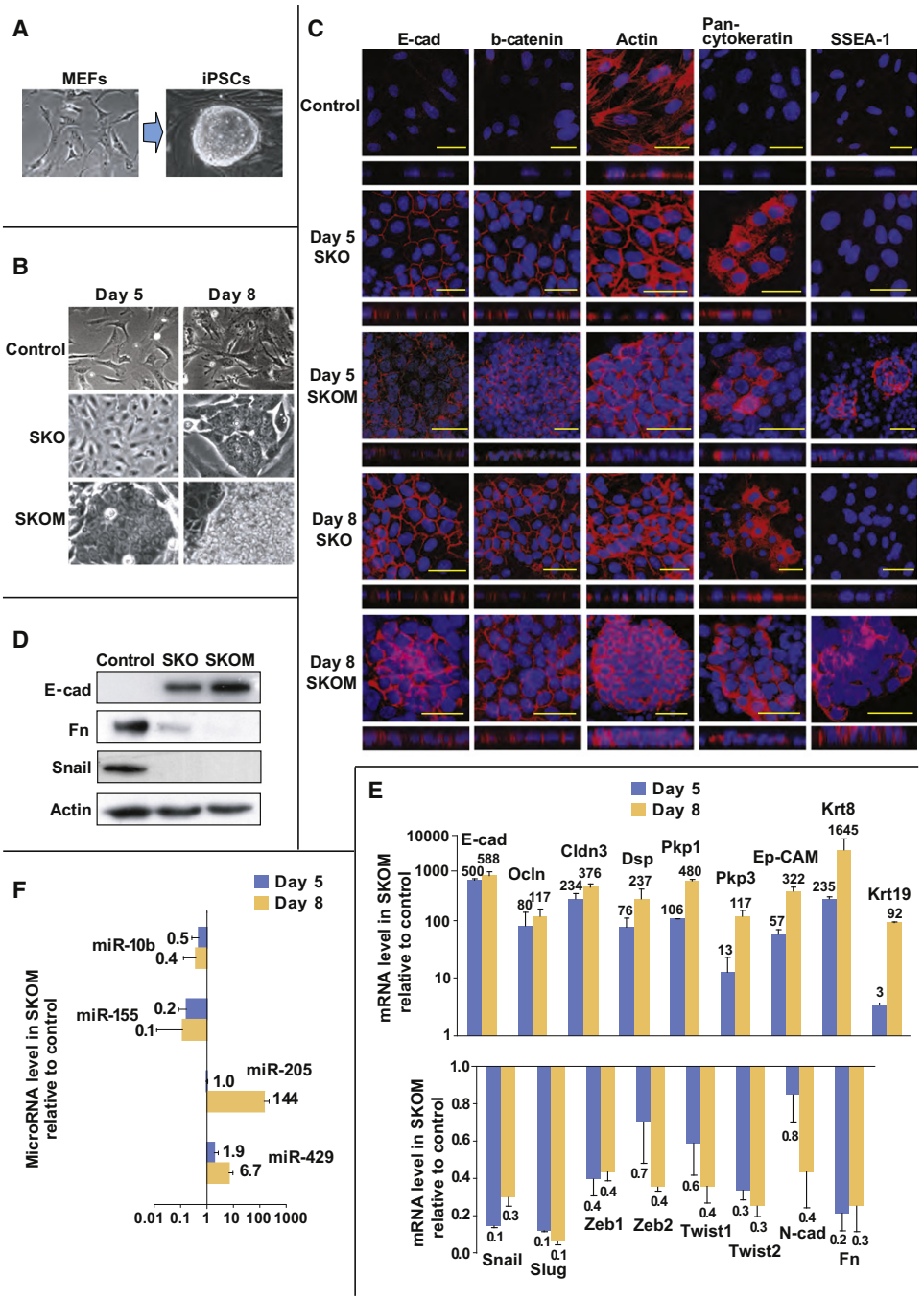


Figure 1. MET Is an Early Event during the Reprogramming of Mouse Fibroblasts

(A) Phase-contrast photographs of nontransduced MEFs and an iPSC line generated from the same MEFs.

(B) Phase-contrast photographs of MEFs transduced with empty vector, and SKOM or SKO combinations at days 5 and 8.

(C) Immunofluorescence microscopy for the indicated markers in a similar experiment as in (B); the nuclei are stained in blue with DAPI. Vertical computer reconstructions are shown below each item. A representative experiment (this applies hereafter unless otherwise indicated) is shown. E-cad stands for E-cadherin (also hereafter); scale bars indicate 50 μm (also hereafter).

(D) Western blotting for the indicated proteins of lysates corresponding to a similar experiment; actin is the loading control.

(E) qPCR for the indicated genes of three independent SKOM time-course experiments. Samples were measured in triplicate and the mean values + standard deviation (SD) are shown (this applies hereafter to any graph containing error bars).

(F) qPCR for the indicated microRNAs of a time course experiment with SKOM-transduced MEFs; values are referred to MEFs.

Related to Figure S1.

we observed that mouse embryonic fibroblasts (MEFs) undergo epithelial-like morphological changes around day 5. This prompted us to characterize at the molecular level the early events that accompany the generation of iPSCs from mouse fibroblasts, aiming to demonstrate the existence of a MET (Figure S1A available online). MEFs containing a transgenic *Oct4* promoter that expresses GFP (green fluorescent protein) (Silva et al., 2008) were transduced with combined retroviruses producing SKOM (Sox2, Klf4, Oct4, and c-Myc) or SKO (same without c-Myc) transcription factors. We took phase-contrast photographs at days 5 and 8 postinfection (Figure 1B) and performed immunofluorescence microscopy for E-cadherin, β-catenin, pan-cytokeratin (cytokeratins are highly expressed in epithelia), and actin (Figure 1C). For comparisons, immunofluorescence of uninfected mammary epithelial cells (MECs) and MEFs, iPSCs, and R1 mouse ESCs is provided in Figure S1B. MEFs undergoing reprogramming became rounded and aggregated with quicker kinetics in SKOM than SKO (Figure 1B). They also formed well-defined epithelial-like intercellular junctions and displayed increased cytokeratin starting at day 5 postinfection (Figure 1C). These epithelial-like clusters appeared before the ESC antigen SSEA-1 could be detected, as shown by the comparison between SKOM and SKO (Figure 1C). Western blot analysis validated the increase in E-cadherin and showed a concomitant reduction of the mesenchymal-like marker fibronectin (Fn) and the transcriptional repressor Snail (Figure 1D). Real-time RT-PCR analysis (qPCR) of three independent SKOM time-course experiments showed increased mRNA for multiple cell-cell adhesion proteins (E-cadherin, epithelial-cell adhesion molecule or Ep-CAM, and diverse desmosomal and tight junction constituents) and cytokeratins, and reduced mRNA for mesenchymal markers (Fn and N-cadherin), a panel of transcriptional repressors including Snail and Slug, and cell-matrix adhesion genes (Figure 1E; Figure S1C). SKOM factors also induced rapid downregulation of the microRNAs miR-155 and miR-10b, which have been associated with EMT (Kong et al., 2008; Ma et al., 2007), and upregulation (albeit more delayed) of miR-205 and -429 (Figure 1F; Gregory et al., 2008; Park et al., 2008), which have the opposite role. These microRNAs were studied on the basis of high levels of expression in MECs, iPSCs, and mouse ESCs, compared to MEFs (Figure S1D). These comprehensive phenotypic changes in MEFs undergoing reprogramming had functional repercussions as well, as demonstrated by incomplete gap closure after wound healing and reduced invasion of Matrigel-coated transwell filters (Figures S1E and S1F). Comparable changes in cell morphology and in the expression of selected EMT/MET target genes were observed after transduction of adult mammary fibroblasts (data not shown). Therefore, the reprogramming of mouse fibroblasts initiates with a bona fide process of MET that occurs before the acquisition of ESC-like characteristics.

TGF-β Prevents MET and Blunts the Reprogramming

We next studied whether the observed MET is a consequence or is required for the formation of iPSCs. TGF-β was recently described to block nuclear reprogramming (Maherali and Hochedlinger, 2009) and is a well-known EMT inducer that acts at least in part through activation of Snail (Peinado et al., 2003). We added TGF-β1 to MEFs undergoing reprogramming and observed that it inhibited the upregulation of epithelial markers and the downregulation of Snail (but not Slug) as measured by qPCR at day 6 postinfection (Figure 2A); another TGF-β family constituent, Activin A, did not have any effect. TGF-β1 and overexpression of Snail also prevented the assembly of intercellular junctions as analyzed at day 6 (Figure 2B; data not shown) and inhibited the formation of alkaline phosphatase-positive (AP^+) colonies by ~40% to 70% measured at days 14 (SKOM) and 25 (SKO) (Figure 2C, upper panel). Besides, the remaining AP^+ colonies were fibroblastic rather than ESC-like (Figure 2D). TGF-β2 (Maherali and Hochedlinger, 2009) and TGF-β3 blunted AP^+ colony formation as well, but overexpression of Slug did not have any effect (Figure 2C). The latter indicates specificity and is consistent with Snail and Slug sharing functions in some settings but not in others (Côme et al., 2006). Although AP staining is a not-stringent-enough criterion to indicate pluripotency (Brambrink et al., 2008; Silva et al., 2008), these experiments show that TGF-β and Snail mostly abort the reprogramming at an early stage. In SKO-infected MEFs, we also observed that on average 6–12 colonies became GFP^+ (indicative of full reprogramming) by day 25 compared to few or none in cells treated with TGF-β cytokines or overexpressing Snail (Figure 2C, lower panel and Figure 3F). Appearance of GFP^+ colonies in SKOM-infected MEFs required overgrowth of cells beyond day 20, or secondary splitting, and the frequency was similar to other studies (0.1% of the original population [Nakagawa et al., 2008]). However, colonies produced with SKOM plus TGF-β or Snail remained mostly GFP^- after multiple passages (data not shown). We observed reduced cell number in SKOM- (at day 6) or SKO- (at day 9) infected MEFs treated with TGF-β cytokines, and it is now known that proliferation is needed for the acquisition of stochastic epigenetic changes during reprogramming (Hanna et al., 2009b). But reduced proliferation in the presence of TGF-β may, if anything, only partially explain the effect on iPSC colony formation, as for example Snail increased rather than decreased the cell number (Figure S2). We also performed DNA microarray analysis of reprogramming MEFs (at day 10) cultured with TGF-β1 or coexpressing Snail compared to the control, which showed profound transcriptomic changes (Table S1). The set of genes differentially modulated by TGF-β1 and Snail mostly distributed into KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways related to cell-cell adhesion and the interaction with the extracellular matrix (Table S1). Taken together our experiments suggest that MET is needed for the reprogramming of fibroblasts into iPSCs.

The Yamanaka Factors Suppress EMT by Blocking TGF-β Signaling

One of the main concerns over iPSC technology is that the reprogramming efficiency is very low (Silva et al., 2008). We speculated that high levels of pro-EMT cytokines such as TGF-β contained in serum or secreted by fibroblasts might impair the reprogramming by counteracting MET. To assess this, we quantified TGF-β cytokines in our iPSC culture system by using ELISA and found that two different batches of ESC-defined fetal bovine serum (FBS) contained significant amounts of TGF-β1 and 2 (Figure 3A) but no TGF-β3 (data not shown). We also noticed that the starting MEFs and the feeder cells produced abundant TGF-β1, very low TGF-β2 (Figure 3A), and no TGF-β3 (data not

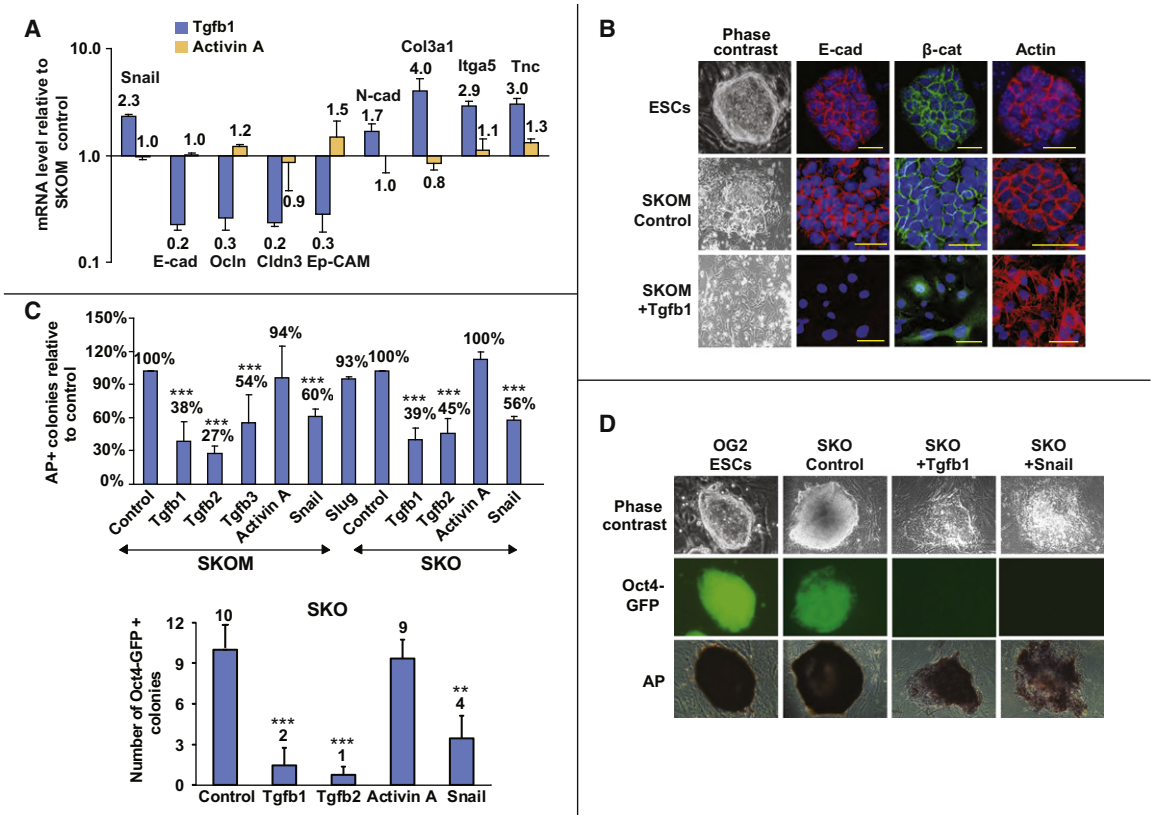


Figure 2. Preventing MET Impairs iPSC Generation

(A) qPCR analysis for the indicated genes of SKOM-infected MEFs (day 6) treated with TGF-β1 or Activin A.

(B) Immunofluorescence (double for E-cadherin and β-catenin, and single for actin) of SKOM-infected MEFs control or treated with TGF-β1; R1 ESCs are also included as positive control.

(C) Top: quantification via ImagePro 5.0 of AP^+ colonies in SKOM- (at day 14) or SKO- (at day 25) infected MEFs. Mean values for n experiments (n = 9 for SKOM + TGF-β1; n = 9 for SKOM + Snail; n = 6 for SKO + TGF-β1; n = 4 for SKO + Snail) is provided. Asterisks indicate statistical significance: * < 0.05, ** < 0.01, *** < 0.001; this also applies hereafter. Bottom: quantification of GFP^+ colonies in SKO-infected MEFs treated with TGF-β cytokines or overexpressing Snail.

(D) Phase-contrast photographs of cell morphology and AP staining, and Oct4-GFP immunofluorescence of an SKO experiment with or without TGF-β1 or coexpressing Snail; mouse ESCs obtained from OG2 mice were used as a control.

Related to Figure S2 and Table S1.

shown). Interestingly, the reprogramming factors progressively reduced the secretion of TGF-β1 in the conditioned medium of SKOM-transduced MEFs, and TGF-β2 increased but levels were extremely low (Figure 3B). Supporting the ELISA data, TGF-β1 mRNA (and TGF-β3) diminished in SKOM since early time points postinfection, while TGF-β2 mRNA was increased (Figure 3C). TGF-β cytokines bind to TGF-β receptor 2 (TGF-βR2), which then recruits TGF-βR1 and this triggers autophosphorylation (Shi and Massagué, 2003) and a signaling cascade that ultimately activates Snail; TGF-β receptor 3 (TGF-βR3) acts as a coreceptor. TGF-βR2 and TGF-βR3 mRNAs were quickly reduced by the exogenous factors but TGF-βR1 remained relatively unchanged (Figure 3C). TGF-β1, TGF-β3, TGF-βR2, and TGF-βR3 were very low in R1 ESCs and iPSCs compared to MEFs, possibly explaining why prolonged culture in the presence of serum or added TGF-β1 cannot induce their differentiation (Figures S3A–S3C).

Next, we analyzed the contribution of each reprogramming factor to the above-mentioned transcriptional alterations of TGF-β signaling constituents and EMT/MET-related genes, for which we expressed the four Yamanaka factors individually and compared their effect with the SKOM and SKO cocktails. Sox2 and Oct4 suppressed TGF-βR3, Oct4 and Klf4 suppressed TGF-β3, and c-Myc suppressed TGF-β1 and TGF-βR2 (Figure 3D), TGF-β2 mRNA was modestly upregulated by Sox2 and Klf4, and none of the factors could independently downregulate TGF-βR1 (data not shown). These data suggest that SKO factors are less effective in producing iPSCs than SKOM because without c-Myc the TGF-β pathway is not efficiently shut down. Supporting this, coexpression of TGF-β receptors 1-3 in SKOM-infected MEFs reduced the number of AP^+ colonies as efficiently as a constitutively active form of TGF-βR1 (T204D) (Figure 3E) and picked colonies did not turn GFP^+ after multiple passages compared to the control (data not shown). This is consistent with whole-genome ChIP-on-Chip analysis in the study by Sridharan et al. (2009) that shows a pattern of histone modifications for the TGF-βR2 promoter indicative of sustained active transcription in pre-iPSCs compared to iPSCs.

We also detected that incompletely reprogrammed GFP$^-$ pre-iPSC clones retain high mRNA levels of TGF-β1 and TGF-βR2, high levels of Snail and low of E-cadherin, compared to iPSCs (Figure S3D). On the other hand, use of exogenous (TGF-β receptor inhibitors or Alk5i) or endogenous (Smad7) (Nakao et al., 1997) inhibitors of TGF-β signaling improved reprogramming efficiency (based on the number of GFP$^+$ colonies) with SKO factors to ~2% of the original population (Figure 3F). The effect of Alk5i was less remarkable in SKOM (data not shown), in agreement with the role of c-Myc in suppressing TGF-β signaling. Alk5i or Smad7 only moderately increased cell number at day 9 (Figure S3E), which points to a direct effect on the reprogramming rather than an indirect effect caused by stochastic events during cell proliferation. As described in recent reports (Ichida et al., 2009; Maherali and Hochedlinger, 2009), we could generate chimera-competent iPSCs from MEFs by using Alk5i and otherwise nonproductive KOM or KO cocktails (data not shown), and we saw the same coexpressing Smad7 (Figures 3G and 3H). No colonies were produced when Klf4 was excluded from the retroviral cocktail. In summary, the combined action of Sox2, Oct4, Klf4, and c-Myc in the recipient fibroblasts downregulates TGF-β1 secretion and signaling to prevent the self-perpetuation of EMT, and this is important for the reprogramming.

E-cadherin Is Required for Reprogramming

Overexpressing the exogenous factors individually, we also observed that Sox2 and Oct4 downregulate Snail mRNA but not Slug (Figure 4A), thereby providing an additional way to shut down EMT during reprogramming. Interestingly, Klf4 potently induced E-cadherin mRNA (more than 200-fold on average) and other epithelial markers as well (Figure 4B). Upregulation of E-cadherin by Klf4 was verified with western blot, and Sox2, Oct4, and Klf4 reduced Snail with the same assay (Figure 4C). This latter effect of Klf4 possibly reflects modulation of Snail protein stability (Zhou et al., 2004). Immunofluorescence microscopy further confirmed the increase in E-cadherin triggered by Klf4 (Figure 4D), while bona fide Klf4 binding sites in the E-cadherin promoter were demonstrated with chromatin immunoprecipitation (ChIP) and semiquantitative RT-PCR (Figure 4E). Given that E-cadherin is an important regulator of epithelial homeostasis (Cavallaro and Christofori, 2004) and has been linked to mouse ESC pluripotency (Chou et al., 2008; Soncin et al., 2009), we next studied whether knocking down E-cadherin expression would blunt iPS by preventing MET. For this we used three different sets of retroviruses producing specific shRNA hairpins (Figure 5A). The reprogramming was performed in the presence of vitamin C in order to increase the number of GFP$^+$ colonies and hence magnify differences (Esteban et al., 2010). The formation of AP$^+$ colonies did not change after knocking down E-cadherin, but the resulting clones were mostly GFP$^-$ and fibroblastic (Figures 5B and 5C) and resembled those produced with TGF-β1 or Snail (see Figure 2). The few remaining GFP$^+$ colonies (hereafter named shECAD colonies) displayed a more loose 2D morphology than the control, and three colonies of each group were picked and expanded for further characterization (Figures 5D and 5E). shECAD colonies were SSEA-1 positive, expressed similar levels of endogenous Oct4 and Nanog as the controls (Figures 5E and 5F), and had silenced the transgenes (data not shown). Moreover, they displayed increased levels of N-cadherin and reduced expression of the pluripotent genes Lefty1, Sox2, and Klf4 (Figure 5F). The differentiation markers Cer1, Sox17, and Fgf5, which are expressed in epiblast stem cells (EpiSCs) and the more recently described FAB-SCs (Chou et al., 2008), were also increased in shECAD cells compared to the controls (Figure 5F). FAB-SCs were derived from mouse blastocysts via bFGF, Activin A, and the GSK3β inhibitor BIO, and share many characteristics of EpiSCs but are not the same entity. Notably, their phenotype is at least in part due to reduced E-cadherin expression. The ability of shECAD cell lines 4 and 8 to form chimeras was strikingly reduced compared to the controls (Figure 5G). shECAD 9 cells expressed higher residual levels of E-cadherin (mRNA and protein) (Figure 5D; Table S2C) and could still produce chimeras with high frequency but without germline contribution (Figure 5G). Differences in E-cadherin expression between shECAD cell lines are probably reflection of different number of shRNA retroviral insertions into the genome and/or their specific location. Nevertheless, DNA microarrays showed significant changes in gene expression in all three shECAD cell lines relative to the controls, including reduced expression of germline-related genes like Dazl (Figure S4 and Tables S2A and S2B; Lin et al., 2008). Stella (Dppa3), a germline-related gene used to differentiate mouse ESCs from EpiSCs (Bao et al., 2009), was not changed (Figure 5F). Differentially expressed genes between shECAD cell lines and the controls also displayed an enriched distribution in pathways related to focal adhesions, actin cytoskeleton, the extracellular matrix, and the TGF-β pathway (Table S2C). The latter suggests that shECAD cell lines may retain the ability to respond to pro-EMT signals triggered by TGF-β in the culture medium. In summary, Klf4 induces an epithelial program during the reprogramming of mouse fibroblasts, and in the absence of adequate levels of E-cadherin, a stable ground condition of "near pluripotency" is established that differs from EpiSCs and is more similar to FAB-SC cells.

Inducing MET Enhances Reprogramming

Next, we hypothesized that using epithelial cells as a starting population would improve iPSC generation by circumventing the need to switch off the EMT state characteristic of fibroblasts. Indeed, it has been reported that human epithelial cells such as keratinocytes or hair follicle cells reprogram with higher efficiency than fibroblasts (Aasen et al., 2008), for which the authors used a low-serum protocol in order to prevent cell differentiation. We used epithelial cells from the mammary gland (MECs), a widely used model for studying EMT (Mani et al., 2008). MECs had to be cultured in low serum to prevent EMT (Figure 6A; Eirew et al., 2008), and this culture condition produced significantly more GFP$^+$ colonies than using fibroblasts and a full serum protocol (Figure 6B). Addition of Alk5i to MECs cultured with serum also prevented EMT and generated GFP$^+$ iPSC colonies with high efficiency (Figures 6A and 6B). Interestingly, we observed that MECs express high endogenous levels of the complementary factor Klf4, which quickly diminished in the presence of serum unless the cells were treated with Alk5i (Figure 6C). Consistent with a major role of Klf4 during the reprogramming being the induction of MET, and because epithelial cells don't need such transition, we could successfully

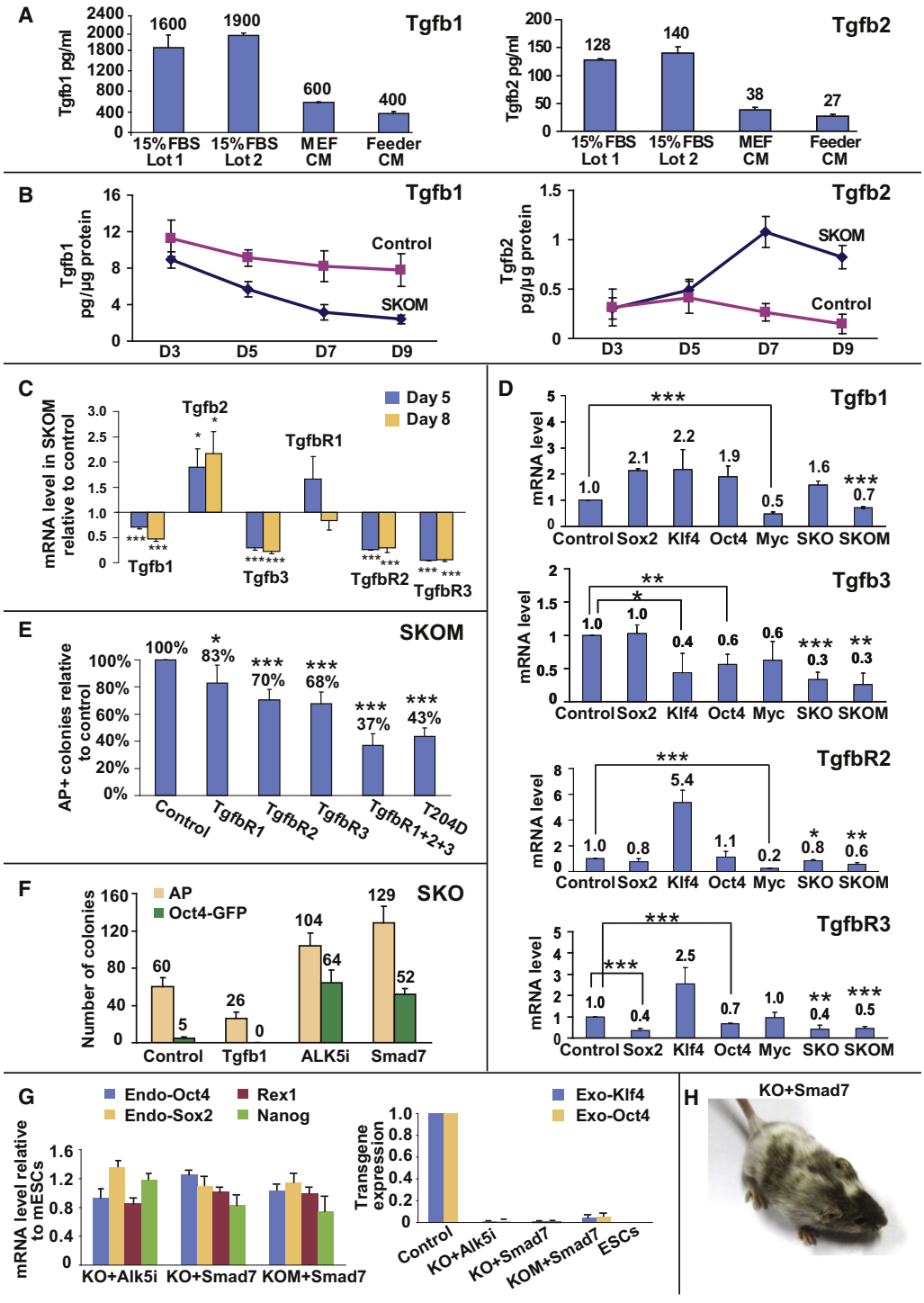


Figure 3. Sox2, Oct4, and c-Myc Suppress TGF-β Signaling

(A) Measurement via ELISA of TGF-β1 and TGF-β2 in mouse ESC-tested FBS (lot 1 number 709778, lot 2 number 737443) and conditioned medium (CM) from uninfected MEFs and feeder layers. For collecting supernatants, cells (1.8×10^5 MEFs and 2×10^5 feeders) were split on 6-well dishes and cultured with Optimem + 0.5% FBS for 48 hr.

(B) ELISA for TGF-β1 and TGF-β2 in the conditioned supernatant of control and SKOM-infected MEFs. Values were normalized to the amount of protein contained in lysates from the same wells.

(C) qPCR of three independent SKOM time-course experiments for the indicated genes.

(D) qPCR for the indicated genes (three independent experiments) at day 6 after transducing MEFs with the exogenous factors individually. Infections with SKO and SKOM were performed in parallel as controls.

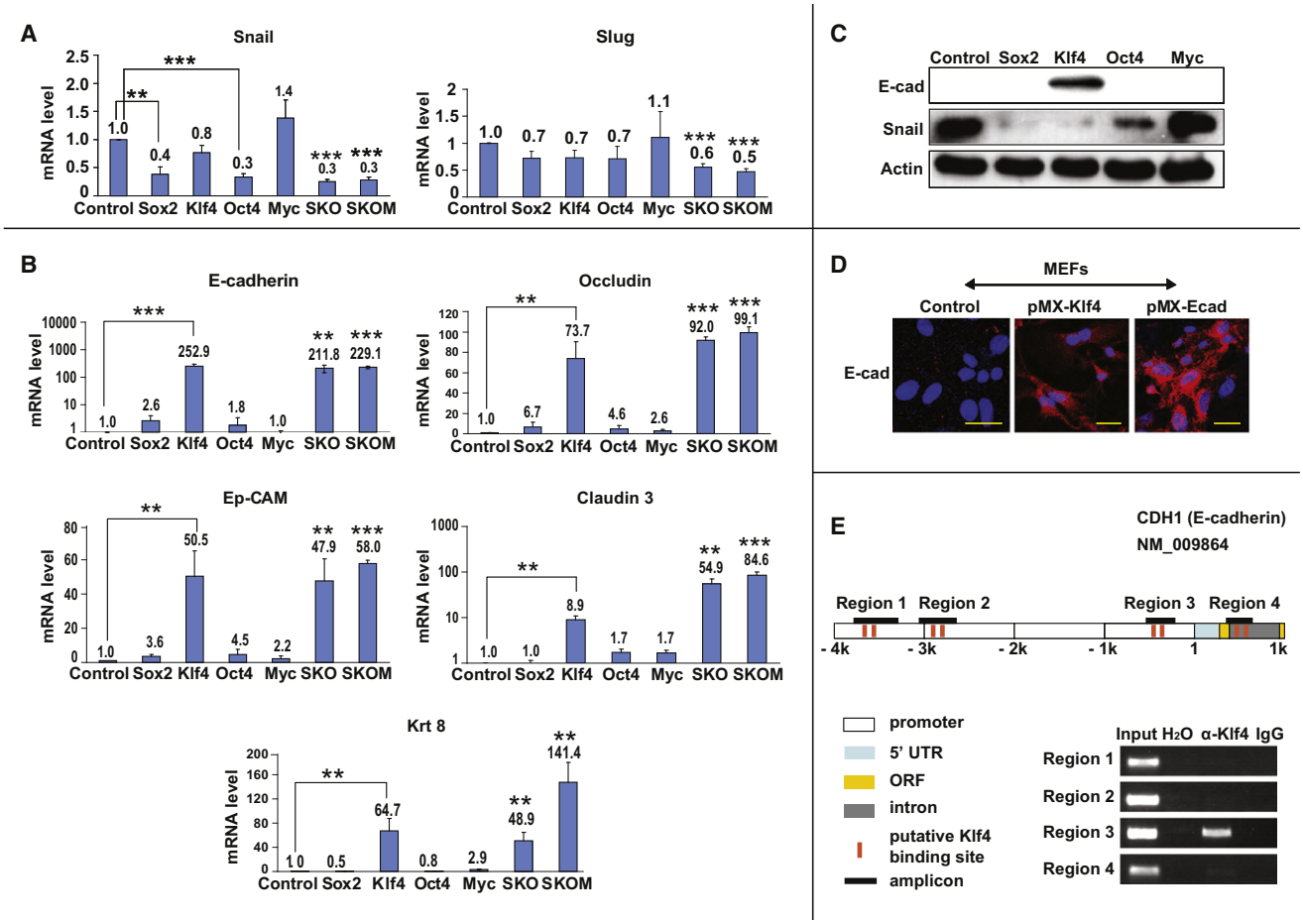


Figure 4. Klf4 Activates an Epithelial Gene Expression Program

(A and B) qPCR for the indicated genes at day 6 after transducing MEFs with the exogenous factors individually; infections with SKO and SKOM were used as controls.

(C) Western blot for E-cadherin and Snail in MEFs overexpressing the exogenous factors individually.

(D) Immunofluorescence microscopy of MEFs overexpressing Klf4; empty vector and a retroviral plasmid encoding E-cadherin are the controls.

(E) Schematic representation of the E-cadherin promoter and ChIP with anti-Klf4. Positions for the respective putative binding sites (identified with Matt Inspector) are: for region 1, −3615 and −3499; region 2, −2915 and −2853; region 3, −473 and −351; and region 4, +477 and +515.

reprogram MECs cultured with Alk5i with only Sox2 and Oct4 (SO). Semiquantitative RT-PCR of genomic DNA was used to detect specific transgene integration and exclude the possibility of plasmid contamination (Figure 6D). These iPSC clones, and also those produced as in Figure 6B, exhibited ESC characteristics and were pluripotent (Figures 6E–6J; Figure S5). Therefore, the maintenance of an epithelial phenotype facilitates reprogramming and Klf4 can be omitted from the reprogramming cocktail when epithelial cells are used as donor cells.

DISCUSSION

We have identified a key cellular process, MET, as an early requisite event during the reprogramming of mouse fibroblasts into iPSCs by exogenous factors. Our work also shows that somatic cell reprogramming is not merely a nuclear event but rather that it is determined by the fine crosstalk between nuclear engines and the extracellular milieu. The machinery triggered by the exogenous factors initiates the MET program to power

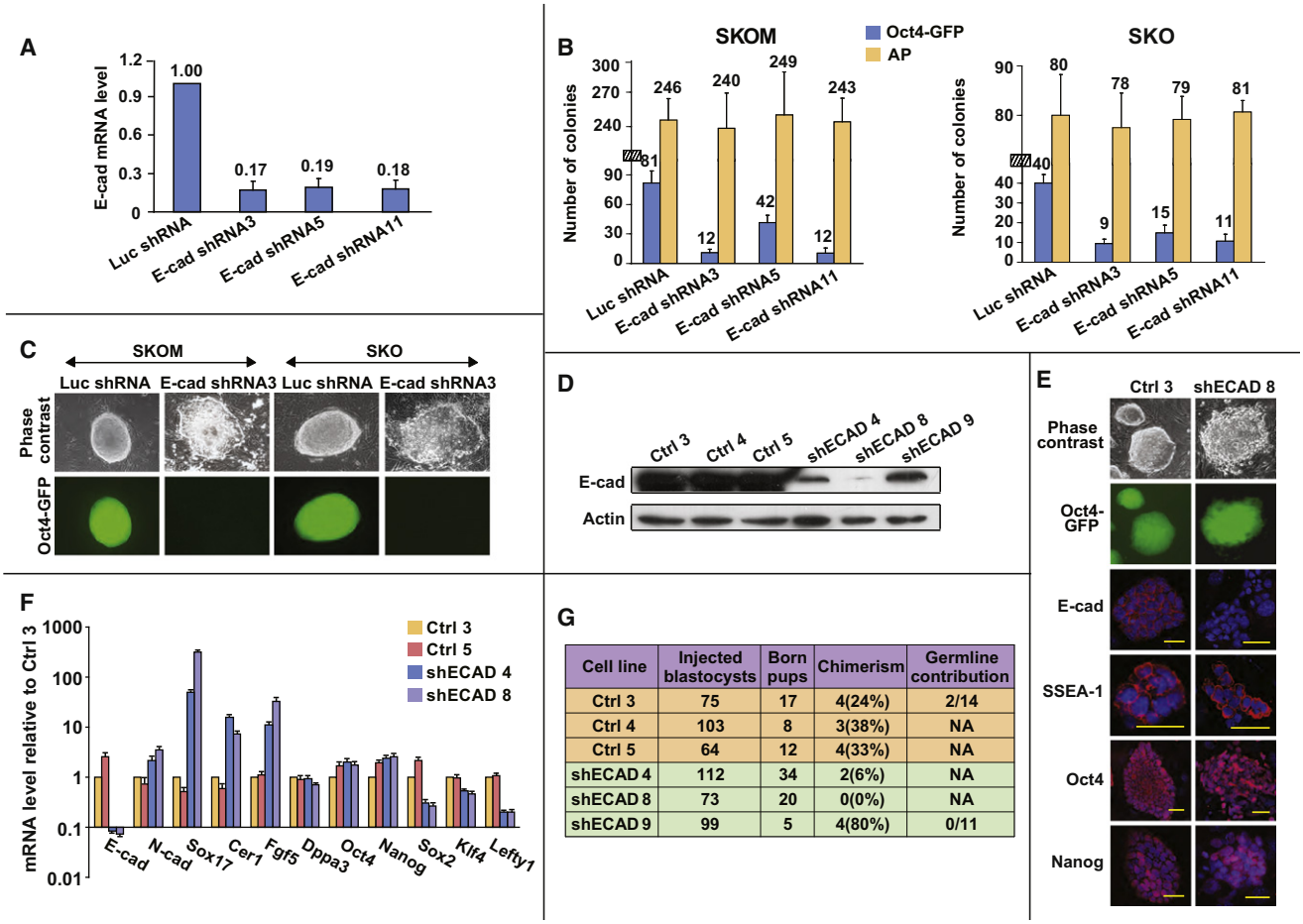
(E) Effect on the number of AP^+ colonies of coexpressing the TGF-β receptors (either individually or in combination) together with SKOM factors; T204D was used as a positive control.

(F) Quantification of AP^+ and GFP^+ colonies in SKO-infected MEFs treated with Alk5i or coexpressing Smad7; control TGF-β1 treatment is included.

(G) qPCR for measuring the expression of ESC genes (left) and the exogenous transgenes (right) in KO + Smad7 and KOM + Smad7 iPSCs generated from MEFs; KO colonies produced with Alk5i were also included. For the transgenes, RNA from SKOM-transduced cells extracted at day 6 and R1 ESCs were used as positive and negative controls, respectively.

(H) Photographs of a chimeric mouse produced with iPSC clones derived from MEFs transduced with KO + Smad7.

Related to Figure S3.



Cell line	Injected blastocysts	Born pups	Chimerism	Germline contribution
Ctrl 3	75	17	4(24%)	2/14
Ctrl 4	103	8	3(38%)	NA
Ctrl 5	64	12	4(33%)	NA
shECAD 4	112	34	2(6%)	NA
shECAD 8	73	20	0(0%)	NA
shECAD 9	99	5	4(80%)	0/11

Figure 5. E-cadherin Is Needed for the Reprogramming of MEFs

(A) qPCR for E-cadherin in MEFs transduced with SKOM and the indicated shRNA vectors; Luc-shRNA indicates control shRNA.

(B) Effect of knocking down E-cadherin on the appearance of AP$^+$ and GFP$^+$ colonies in SKOM- and SKO-infected MEFs.

(C) Representative GFP$^+$ colonies produced with control shRNA and non-ESC-like GFP$^-$ colonies representative of most colonies produced with shRNA vectors for E-cadherin.

(D) Western blot for E-cadherin in three expanded GFP$^+$ shRNA control clones (hereafter labeled as ctrl) or E-cadherin shRNA.

(E) Immunofluorescence microscopy for the indicated markers of a control clone and an shECAD clone.

(F) qPCR for the indicated genes in two shECAD and two control cell lines.

(G) Table summarizing the injection of control iPSCs and shECAD cell lines into heterologous blastocysts for chimeric mice generation.

Related to Figure S4 and Table S2.

fibroblasts through an epigenetic barrier established throughout development, very probably during EMT processes involved in early differentiation decisions. EMT and MET are subject to regulation not only at the transcriptional level but also by the extracellular environment (Peinado et al., 2003). Hence, pro-EMT cytokines in the culture medium can create an EMT-compatible situation that sustains the fibroblast-like identity, prevents MET, and results in abortive reprogramming despite maintained expression of the exogenous factors inside the nuclei of infected cells. To bypass this, the exogenous factors must suppress an intrinsic barrier represented by fibroblast-enriched transcription factors (e.g., Snail) that preserve the mesenchymal phenotype, and another one extrinsic typified by TGF-β1 (and possibly other cytokines as well) contained in the culture medium. While the nuclear engines try to eliminate the first hurdle (e.g., suppressing Snail and inducing E-cadherin), the extracellular milieu can still derail the process by providing positive feedback on Snail, unless a TGF-β inhibitor is added. In this regard, it was reported recently that Alk5i can help maintain rat iPSCs dedifferentiated (Li et al., 2009) and improves mouse (Ichida et al., 2009; Maherali and Hochedlinger, 2009) and human (Lin et al., 2009) iPSC generation. These articles suggested that blockade of EMT is important for reprogramming, but no experimental proof or mechanistic insight (apart from the involvement of Nanog [Ichida et al., 2009]) was provided. Here we have shown that Sox2/Oct4 and c-Myc suppress Snail and TGF-β1/TGF-βR2, respectively, and Klf4 upregulates E-cadherin and other epithelial genes (Figure 7). In this context it is straightforwardly understood that Alk5i or Smad7 can substitute Sox2 and c-Myc when iPSCs are generated from fibroblasts, and that Klf4 is rescindable from the reprogramming cocktail in epithelial cells. By dissecting this network, we demonstrate that rational approaches based on

understanding iPSC in a cell-specific and tissue culture-specific context may lead to better practice. Additional exploration of these pathways may as well provide novel ways to improve the quality of iPSCs and/or the generation efficiency and perhaps facilitate the use of nonintegrating approaches.

Regarding how TGF-β and Snail ultimately exerts their effects, this may involve several targets but a critical step is downregulation of E-cadherin. Snail is needed for EMT during gastrulation (Carver et al., 2001; Murray and Gridley, 2006) and its overexpression induces ESC differentiation (Ivanova et al., 2006). On the other hand, E-cadherin knockout mice do not proceed beyond the epiblast stage (Larue et al., 1994) and a number of reports have linked this molecule to pluripotency (Chou et al., 2008; Soncin et al., 2009). For example, FAB-SCs (Chou et al., 2008) and E-cadherin$^{-/-}$ mouse ESCs (Soncin et al., 2009) are trapped in a semipluripotent state that shares similarities with EpiSCs (Bao et al., 2009) including the failure to form chimeric mice. It is therefore not surprising that mouse ESC differentiation and somatic cell reprogramming converge in the same crossroad represented by E-cadherin. As for how E-cadherin knockdown blocks reprogramming, this does not seem to involve β-catenin signaling (Clevers, 2006) because this molecule is well confined to intercellular junctions in shECAD iPSC clones (see Figure 5E) possibly through interaction with N-cadherin. Moreover, we did not see any effect when β-catenin was coexpressed with SKO or SKOM in MEFs (data not shown), and in the original description by Yamanaka (Takahashi and Yamanaka, 2006), β-catenin moderately enhanced iPSC generation. Instead, E-cadherin may regulate pluripotency by ensuring that fully compacted cells have access to putative critical autocrine signals or by promoting cell-cell exchange of signals perhaps through gap junctions (Houghton, 2005). Because E-cadherin has also been linked to pluripotency in human ESCs (Li et al., 2010), it is likely that its knockdown will influence as well human somatic cell reprogramming. But human ESCs rely on TGF-β signaling to maintain their characteristics (James et al., 2005) and it remains to be seen whether the epigenetic remodeling of human iPSCs generated with or without Alk5i are equivalent. Importantly, E-cadherin is not the only mechanism by which Klf4 induces reprogramming, as shown by the fact that substitution of this transcription factor by E-cadherin could not produce iPSC clones in MEFs (data not shown). Coexpression of E-cadherin did not increase either the reprogramming efficiency of SKOM- or SKO-infected MEFs (data not shown), though this could be due to quick E-cadherin upregulation after transduction with the exogenous factors. It is possible that other Klf4-regulated adhesion molecules play as well a role during reprogramming, but redundancy may exist and for example knockout mice for tight junction molecules like Occludin or the Claudins show a milder phenotype (Furuse, 2009) compared to E-cadherin. Likewise, shRNA for Snail did not increase the efficiency of reprogramming in SKOM- or SKO-infected MEFs (data not shown), which could imply that other transcriptional repressors (see Figure 1C) participate too or that rapid Snail downregulation during reprogramming does not allow further improvement. Our DNA arrays also suggest that the effects of TGF-β1 and Snail involve the actin cytoskeleton and extracellular matrix, but this could be a consequence and not the cause (see Table S1). Further study of how E-cadherin knockdown blocks iPSCs and a more detailed comparison between shECAD cell line EpiSCs and FAB-SCs will be relevant to understand the reprogramming process and the metastable character of ESCs (Guo et al., 2009; Hanna et al., 2009a).

Interestingly, EMT has been shown to induce stem cell properties in mammary epithelial cells (Mani et al., 2008), which contrasts with our results presented here but probably reflects that pluripotency and stemness are not the same. In any case, knowledge diverted from EMT in this or other contexts may allow better understanding of reprogramming, and the reverse situation can be envisaged too. For example, TGF-βR2 is reduced in liver tumors overexpressing c-Myc (Amicone et al., 2002; Factor et al., 1997), and it is well known that epithelial cells express high levels of Klf4 (De Craene et al., 2005). Yet, a direct link between TGF-βR2 and c-Myc or a dominant role for Klf4 in controlling epithelial characteristics had not yet been described. Interestingly, Klf4 is repressed by Snail during EMT of MECs (De Craene et al., 2005) and it is tempting to speculate that TGF-β/Snail may block nuclear reprogramming by directly preventing the activation of endogenous Klf4 and perhaps other pluripotency regulators as well.

EXPERIMENTAL PROCEDURES

Cell Culture

MEFs and MECs from OG2 mice (Silva et al., 2008) were isolated as described (Mani et al., 2008). ESCs from OG2 mice were also isolated as described (Ying et al., 2008) and where indicated we used R1 mouse ESCs (purchased from ATCC). MEFs and MECs were transduced at passage 2 or 3 with pMX-based retroviruses in 6-well culture dishes as in previous reports (Esteban et al., 2010). For SKOM, 2,000 cells were split on feeders at day 6, for SO 20,000 at day 9, and for other factor combinations 5,000 cells at day 9. MEF medium (high-glucose DMEM [HyClone], nonessential amino acids [GIBCO], penicillin/streptomycin, L-glutamine, and 10% FBS [HyClone]) or MEC medium (DMEM/F12 [Invitrogen] supplemented with 2% FBS [GIBCO; 15% in the full serum condition], 10 mmol/L HEPES, 10 ng/mL epidermal growth factor [Invitrogen], 10 μg/mL insulin [Invitrogen], 5% bovine serum albumin, and penicillin/streptomycin) was changed at day 2 postinfection to standard mouse ESC culture medium (high-glucose DMEM, nonessential amino acids, penicillin/streptomycin, L-glutamine, leukemia inhibitory factor [Millipore], beta-mercaptoethanol, sodium pyruvate, and 15% FBS [GIBCO]). TGF-β1-3 and Activin A were from R&D Systems and used at 2 ng/ml and 10 ng/ml, respectively. A-83-01 (0.5 μM) was purchased from Tocris and added from day 3 to the end of the experiment except for MECs (added since moment of derivation). Vitamin C was purchased from Sigma and used at 50 mg/ml from day 3 until the end of the indicated experiments. Established iPSCs used throughout as controls were produced from MEFs via SKO factors and proved to be chimera competent.

Plasmids and ChIP

pMX-Snail and pMX-E-cadherin were subcloned from plasmids provided by Dr. Antonio Garcia de Herreros and Dr. Masatoshi Takeichi, respectively; pMX-Slug, TGF-βR1-3, T204D (Wieser et al., 1995), and Smad7 were prepared by ourselves with cDNA from MEFs as a template and site-directed mutagenesis (T204D). All cDNAs correspond to mouse and were verified by sequencing. ELISA kits for TGF-β1-3 were from R&D Systems. pRetroSuper vectors containing shRNA sequences for E-cadherin or the control firefly luciferase gene were also made by us; after infection, cells were selected in puromycin for 3 days. The target sequences for Ecad-shRNA3 were GGAG ATGCAGAATAATTAT (Chou et al., 2008), Ecad-shRNA5 GCTGGAATCTTTG TCCATGTA, and Ecad-shRNA11 GCAGGAAGAGAACATTCTA. After infection with shRNA vectors, MEFs were treated with puromycin from day 3 to 5, split on feeder at day 6, and then maintained without the antibiotic. ChIP was performed with a Klfh-specific kit purchased from R&D Systems.

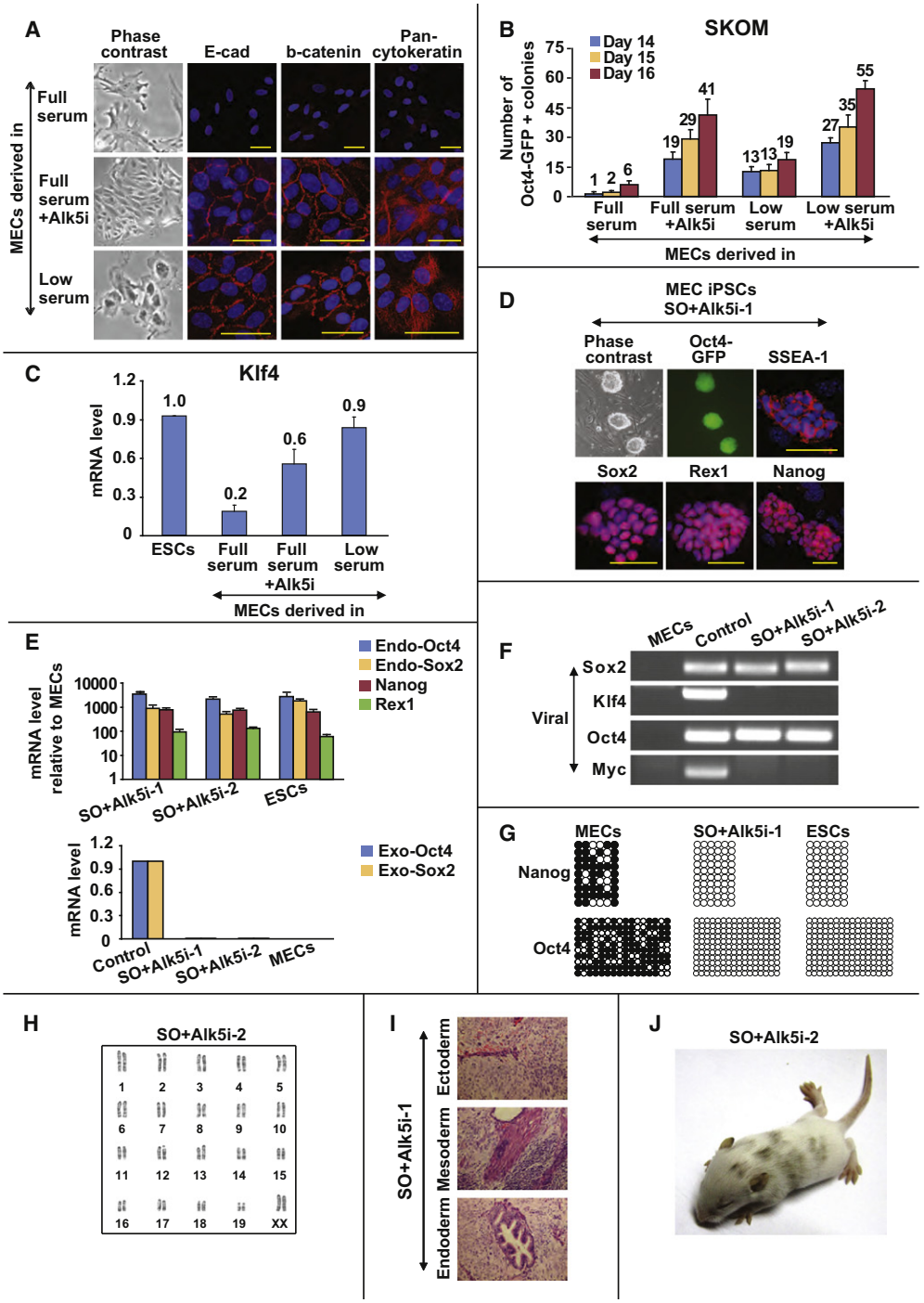


Figure 6. Maintenance of Epithelial Characteristics Is Needed for the Efficient Reprogramming of MECs

(A) Phase-contrast photographs and immunofluorescence microscopy of MECs derived in the indicated culture conditions.

(B) Time-course quantification of GFP$^+$ colonies in MECs derived in the indicated conditions and transduced with SKOM factors.

(C) qPCR of endogenous Klf4 in MECs cultured for 5 days as indicated, R1 ESCs were used as positive control.

(D) Phase-contrast photographs and immunofluorescence microscopy for Oct4-GFP or selected ESC markers in SO (four clones were produced out of 4×10^4 transduced cells) iPSCs produced from MECs treated with Alk5i (A-83-01).

(E) Top: qPCR for measuring the expression of ESC genes in SO iPSCs from MECs, R1 ESCs were used as a positive control. Bottom: qPCR for the exogenous transgenes in the same SO iPSCs; RNA from SKOM-transduced cells extracted at day 6 and uninfected MECs were used as positive and negative controls, respectively.

(F) Semiquantitative RT-PCR (MEFs infected at day 6 were the positive control) to detect exogenous transgene integration.

(G) DNA methylation profile of the Oct4 and Nanog proximal promoters in uninfected MECs, R1 ESCs, and SO iPSCs derived from MECs.

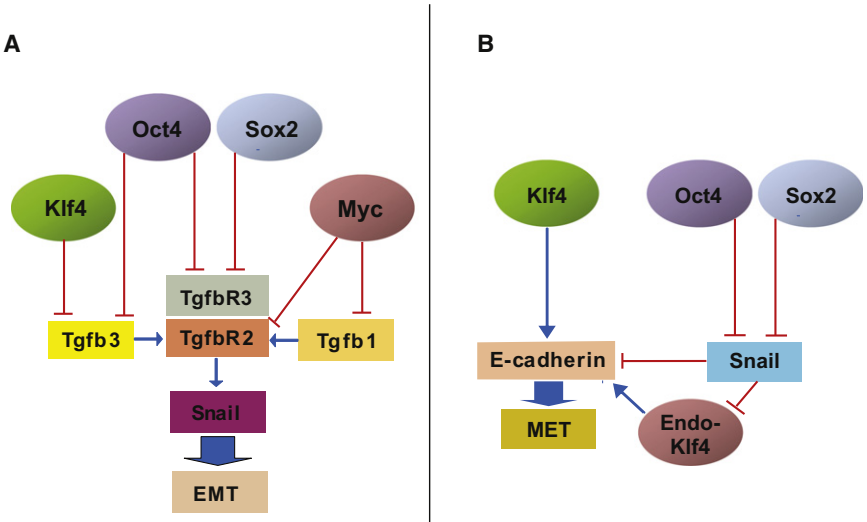


Figure 7. Molecular Network for the Suppression of EMT and Activation of MET by the Exogenous Factors

(A) Schematic representation of how the exogenous factors suppress TGF-β signaling to prevent the self-perpetuation of EMT in the reprogramming fibroblasts.

(B) Schematic representation of the suppression of Snail by Oct4 and Sox2 and the activation of an epithelial program by Klf4 during the reprogramming of fibroblasts.

Cell Line Characterization

Antibodies were purchased from Cell Signal (E-cadherin), BD Bioscience (β-catenin), Abcam (pan-cytokeratin and Snail), Sigma (Fn and actin), Invitrogen (SSEA-1), and R&D systems (Nanog); Sox2/Oct4 and Rex1 were self made. Rodamine-phalloidin was used to stain actin and we purchased it from Invitrogen. Cells were fixed with ice-cold methanol for 10 min at −20 degrees, except for rhodamine-phalloidin staining that required paraformaldehyde, and washed with PBS. A Leica TCS SP2 Spectral confocal microscope was used for all immunoflorescence studies. qPCR was performed with SYBR green (Takara), and samples were analyzed in triplicate and normalized on the basis of β-actin values. Sequences for these and other primers are available upon request. MicroRNAs were measured by qPCR with SYBR green as reported by others (Chen et al., 2005). Western blotting detection was performed with ECL$^+$ (Amersham). Transwell chambers were purchased from Millipore and stained according to the instructions by the manufacturer. Karyotyping, bisulfate sequencing, semiquantitative RT-PCR for detecting transgene integration, AP staining, teratomas, and injection of iPSCs into chimeric mice were performed like in our previous reports (Esteban et al., 2010). DNA microarrays were performed with GeneChip Mouse Genome 430 2.0 Array (for Figure S3B) and MoGene 1.0 ST chip (the others) from Affymetrix. A Zeiss SteREO Lumar V12 fluorescence microscope was used to observe GFP$^+$ germ cells in the genital ridge of embryos extracted at 13 and a half days. Statistical significance was evaluated where convenient with Student's t test. All experiments involving animals were approved by the Guangzhou Institutes of Biomedicine and Health review board.

ACCESSION NUMBERS

GEO database accession numbers are GSE15267, GSE21062, GSE21064, and GSE21067.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and two tables and can be found with this article online at doi:10.1016/j.stem.2010.04.014.

ACKNOWLEDGMENTS

This work is supported by the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-YW-R-48, KSCX2-YW-R-221), Chinese Academy of Sciences/SAFEA International Partnership Program for Creative Research Teams, Chinese Academy of Sciences (KSCX2-YW-R-48, KSCX2-YW-R-244), National Natural Science Foundation of China (30725012, 230725012, 90813033, 30630039), Ministry of Science and Technology 973 program (2006CB701504, 2006CB943600, 2007CB948002, 2007CB947804, 2007CB947900, 2009CB941102, 2009CB940902, 2010CB944800), Ministry of Science and Technology International Technology Cooperation Program (2010DFB30430), National High Technology Research and Development Program of China (2006AA02A103, 2007G-P034, 2007Z3-C7031), and Bureau of Science and Technology of Guangzhou Municipality (2008A1-E4011).

Received: February 7, 2010
Revised: March 26, 2010
Accepted: April 21, 2010
Published online: June 17, 2010

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(H) Karyotype for the same SO iPSCs.
(I and J) Hematoxylin/eosin-stained sections of teratomas composed of tissues derived from the three germ layers and chimeric mouse produced with SO iPSCs from MECs.
Related to Figure S6.

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