E-cadherin is crucial for ESC pluripotency and can replace Oct4 during somatic cell reprogramming

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Running Title: E-cadherin in pluripotency and reprogramming

Character count: 29,825

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Abstract

We report novel functions of the cell adhesion molecule E-cadherin in murine pluripotent cells. E-cadherin was highly expressed in mouse embryonic stem cells, and interference with E-cadherin caused differentiation. During cellular reprogramming of mouse fibroblasts by Oct4, Sox2, Klf4 and c-Myc, fully reprogrammed cells were exclusively observed in the E-cadherin positive cell population, and could not be obtained in the absence of E-cadherin. Moreover, reprogrammed cells could be established by viral E-cadherin in the absence of exogenous Oct4. Thus, reprogramming requires spatial cues that crosstalk with essential transcription factors. The cell adhesion molecule E-cadherin has important functions in pluripotency and reprogramming.

Keywords: Pluripotency, Somatic Cell Reprogramming, E-cadherin, Oct4

Introduction

The regulation of pluripotency in mouse embryonic stem cells (mESCs) is a fascinating biological question with important implications both for basic science and for the therapeutic potential that is foreseen for such cells. The same holds true for induced pluripotent stem (iPS) cells of somatic origin. Both pluripotency and reprogramming are influenced by a number of cellular processes, such as regulation of transcription, signal transduction, regulation of miRNAs and epigenetic changes (reviewed in Jaenisch & Young, 2008). The concept of somatic cell reprogramming was initiated by the seminal finding that mouse embryonic fibroblasts (MEFs) can be converted to iPS cells by retroviral expression of four transcription factors, i.e. Oct4, Sox2, Klf4, and c-Myc (Takahashi & Yamanaka, 2006).

The cadherins are membrane-spanning proteins of adherens junctions that play critical roles in cell-cell contact formation. A switch from epithelial components, such as E-cadherin (Cadherin-1) expression, to mesenchymal can occur, termed epithelial-mesenchymal transitions (EMT), which promotes the migration of cells in the development of many organs and in the metastasis of tumors (Heuberger & Birchmeier, 2010). Opposite mechanisms, mesenchymal-epithelial transitions (MET) are important in strengthening tissue integrity following migratory processes in embryonic development. The function of E-cadherin in cell-cell adhesion is also connected to various signaling pathways that relay information about cell interactions to the cytoplasm and the nucleus (Stepniak et al, 2009). In early mammalian development, E-cadherin-based cell-cell contacts are required for the integrity of the embryonic blastocyst, and deletion of the E-cadherin gene results in failure of cell compaction at this stage (Larue et al, 1994).

In the present study, we show that E-cadherin is essential for the maintenance of pluripotency of mESCs, and that disturbance or loss of E-cadherin induces EMT. During reprogramming of MEFs to iPS cells, E-cadherin production is always

associated with the reprogramming process and, remarkably, exogenous expression of E-cadherin can replace the requirement of Oct4 during reprogramming.

Results and Discussion

E-cadherin is required to maintain pluripotency of mESCs

We compared the expression of E-cadherin in undifferentiated 129/Sv3 mESCs grown in the presence of leukemia inhibitory factor (LIF) and in differentiating cells. Quantitative RT-PCR showed significant downregulation of E-cadherin and the pluripotency genes Oct4 and Nanog five days after LIF withdrawal (Fig. 1A). Conversely, N-cadherin mRNA was upregulated. The protein levels of E-cadherin and Oct4 decreased significantly in differentiating mESCs, while N-cadherin increased as shown by Western analysis and immunofluoresence (Fig. S1A, B). Moreover, cells changed their morphology from compact colonies to scattered cells after LIF withdrawal (Fig. S1B). Thus, high E-cadherin expression associates with stem cell-like morphology of undifferentiated mESCs, whereas N-cadherin expression associates with reduced cell-cell contacts and differentiation.

We next analyzed whether E-cadherin is functionally required for the maintenance of pluripotency. Indeed, downregulation of E-cadherin in mESCs by a retrovirus that expressed an E-cadherin shRNA (shEcad) led to changes of cell morphology from compact colonies to scattered cells in the presence of LIF (Fig. 1B) accompanied by a strong downregulation of stem cell-associated genes Oct4, Nanog, Dppa5 and Nr5a2 (Fig. 1C). We tested the effect of blocking E-cadherin by a function-interfering antibody, DECMA-1 (Fig. S1B). Reduced staining for Oct4 and strong staining for N-cadherin was observed, and cells displayed a scattered morphology. Protein levels of E-cadherin, Oct4 and Nanog were downregulated in shEcad-transfected mESCs (Fig. S1C). Our results thus demonstrate that E-cadherin is required to maintain the undifferentiated state of mESCs and that mESCs undergo an EMT upon differentiation.

E-cadherin is induced during reprogramming

Reprogramming of MEFs through viral expression of Oct4, Sox2, Klf4, and c-Myc proceeds in a stepwise manner that gives rise not only to iPS cells, but also to intermediate cell states. We used this procedure to generate iPS cells and sorted for cells that expressed the stem cell marker SSEA-1 by FACS analysis (Fig. 2A). 12.5% SSEA-1 positive cells were obtained eight days after retroviral transduction, and these cells maintained SSEA-1 expression (Fig. S2A). Intriguingly, SSEA-1 positive cell clones expressed E-cadherin but not N-cadherin, as detected by immunofluoresence and Western blotting, whereas SSEA-1 negative cell clones were low in E-cadherin but high in N-cadherin (Fig. 2B, Fig. S2B). Nanog was expressed only in the SSEA-1 positive cells, suggesting that these cells had undergone complete reprogramming. In line with the immunostaining, qRT-PCR analysis revealed that SSEA-1 positive cell clones expressed high levels of E-cadherin ($Ecad^{high}$) and low levels of N-cadherin. SSEA-1 negative cell clones displayed low levels for E-cadherin mRNA ($Ecad^{low}$), but high N-cadherin (Fig. 2C). The $Ecad^{low}$ cell clones exhibited residual expression of viral Oct4, Sox2, Klf4 and c-Myc and low levels of the endogenous genes, showing that the retroviral expression was not silenced (Fig. 2C, Fig. S2C). High levels of Oct4 were detected in all cell clones, whereas the pluripotency factors Nanog and Dppa5 were present only in $Ecad^{high}$ clones.

We then tested $Ecad^{high}$ and $Ecad^{low}$ clones for their differentiation capacity (Fig. 2D). E-cadherin, Oct4, and Nanog mRNA levels were downregulated in $Ecad^{high}$ clones upon LIF withdrawal, and the mRNAs for N-cadherin and Msx2, a transcription factor that is upregulated early upon differentiation, were induced. $Ecad^{low}$ cells showed no changes of expression of these genes upon LIF withdrawal. Protein levels of cadherins and pluripotency factors and cell morphologies in the presence and absence of LIF were confirmed (Fig. S2D and E). We then examined the capability of our cells to form embryoid bodies (EB) that are characteristic for pluripotent stem cells (Fig. S2E). Only $Ecad^{high}$ clones could form EBs. These results

demonstrate that E-cadherin-positive cells exhibit mESC-like properties, consistent with reprogramming and pluripotency, whereas Ecad^{low} cells are not reprogrammed.

Ecad^{high} cell clones formed teratomas and participated in embryonic development

To analyze the differentiation potential of Ecad^{high} and Ecad^{low} cells *in vivo,* we tested their capacity to form teratomas following subcutaneous injection in NOD/SCID mice. Ecad^{high} cells formed large, differentiated tumors by day 12 (0.17 +/- 0.04 cm^3; Fig. 2E) that exhibited epithelial, neuronal and mesenchymal (muscular) structures, comparable to the teratomas formed by mESCs (0.18 +/- 0.07 cm^3). Expression profiles of these tumors were similar to those of mESC-derived teratomas (Fig. S2F). In contrast, Ecad^{low} cells produced very small tumors (0.04 +/- 0.04 cm^3) without differentiated structures (Fig. 2E). Ecad^{low} tumors also did not express tissue-specific markers, which indicated that the specific tissues were not formed (Fig. S2F).

Next we marked Ecad^{high} and Ecad^{low} cells with a GFP-expression construct to allow detection during mouse development. When Ecad^{high} cells were injected into mouse blastocysts, chimerism was observed in different organs of newborn pups, as shown by the expression of GFP (Fig. 2F). Ecad^{low} cells did not participate in embryonic development. Taken together, our data demonstrate that high expression of E-cadherin and absence of N-cadherin are hallmarks of fully reprogrammed iPS cells, and that only these cells form teratomas and contribute to embryonic development.

Conditional deletion of E-cadherin in Ecad$^{flox/flox}$ MEFs prevents reprogramming

We isolated MEFs from mouse embryos that harbored two floxed E-cadherin alleles, Ecad$^{flox/flox}$ (Boussadia et al, 2002). Cre-mediated deletion of E-cadherin was achieved by the treatment of cultured cells with His-tat-NLS (HTN)-cre, a membrane-penetrable Cre recombinase, which resulted in a strong reduction of E-cadherin-positive cells (Fig. 3A). We then attempted to induce reprogramming of these cells

into iPS cells by viral transduction of OSKM and followed colonies by morphological inspection. An 80% reduction of colony numbers was found (Fig 3B). These MEFs were also analyzed for expression of pluripotency genes (Fig. 3C). Both Oct4 and Nanog were expressed at low levels (40% and 15%, respectively) in the HTN-cre-treated MEFs. We conclude that reprogramming is severely impaired in the absence of E-cadherin and that an MET with an induced expression of E-cadherin is required for somatic nuclear reprogramming.

Forced expression of E-cadherin in MEFs can overcome the requirement of Oct4 for reprogramming

We examined systematically, whether the expression of exogenous E-cadherin influences the overall efficiency of reprogramming and may even replace one of the OSKM factors during cellular reprogramming. Transduction of MEFs with the E-cadherin-expressing retrovirus, pMXs-Ecad, led to a strong increase of E-cadherin expression, as observed for the other pluripotency factors (Fig. S3A). The analysis of the reprogramming efficiency with different combinations of pluripotency factors and E-cadherin revealed that E-cadherin did not induce a general increase in reprogramming efficiency, as measured by SSEA-1 expression or alkaline phosphatase (AP) activity (Fig. S3B, C).

We also tested single cell clones that were produced by different factor combinations and scored them for the generation of those with iPS cell-like morphology (Fig. 4A). Intriguingly, the pMXs-Ecad retrovirus in combination with Sox2, Klf4 and c-Myc (ESKM), i.e. without Oct4, produced cells with the typical morphology of pluripotent iPS cells (Fig. 4B), although at a lower frequency than with OSKM (Fig. S4A). When E-cadherin was omitted in the SKM combination, cell clones of this morphology were rarely found (Fig. 4B). Cell clones from ESKM and SKM combinations were cultivated for several passages and characterized.

Remarkably, ESKM cell clones were positive for endogenous Oct4 (Fig. 4C), Nanog and SSEA-1, which was not observed in SKM cells (Fig. S4B).

Independent ESKM clones (#1,2,3) indeed showed elevated mRNA expression for E-cadherin and for the pluripotency genes Oct4, Nanog, Dppa5, and Nr5a2, while expression of N-cadherin was not observed (Fig. 4D). Viral E-cadherin expression was present two days after the infection, but was subsequently silenced. E-cadherin and Oct4 proteins were produced in ESKM clones, while N-cadherin protein was absent (Fig. S4C). ESKM-derived cell clones were able to form EBs (Fig. S4D) and showed expression of genes for endodermal (FoxA2), neuronal (Nestin) and mesodermal (α-SMA) lineages (Fig. S4E). Moreover, ESKM-reprogrammed iPS cells formed teratomas in NOD/SCID mice with epithelial, neuronal and muscular structures (Fig. 4E, Fig. S4F). SKM clones exhibited only small cell lumps without significant growth or specific tissue structure formation. These data demonstrate that iPS cells can be derived in the presence of E-cadherin instead of Oct4 and showed all characteristics of pluripotent, reprogrammed cells.

Our analysis is focused on understanding the function of E-cadherin, both in EMT of embryonic stem cells during differentiation, and in MET of fibroblasts during iPS cell reprogramming. First, we demonstrate that interference with E-cadherin in mESCs caused EMT and differentiation. Another study also shows that E-cadherin is a critical determinant in the conversion of mouse epiblast stem cells to pluripotent mESCs (Chou et al, 2008). These data prove that E-cadherin plays a crucial role in the maintenance of ESC pluripotency and the compact shape of the colonies. Second, in reprogramming of mouse embryonic fibroblasts by the four Yamanaka factors (OSKM), we found that iPS cells were exclusively E-cadherin positive, and could not be obtained in $Ecad^{flox/flox}$ cells following Cre-mediated deletion of E-cadherin. This latter finding is particularly important, since it is based on genetic evidence and not on overexpression of factors or treatment with inhibitors. For instance, shRNA viruses directed against E-cadherin could prevent iPS cell reprogramming and chemical

intervention leading to upregulation of E-cadherin promoted somatic cell reprogramming (Chen et al, 2010). Recently, E-cadherin has also been found to be upregulated during reprogramming in global expression analyses (Samavarchi-Tehrani et al, 2010) and in a screen for cell adhesion molecules (Li et al, 2010). In our reprogramming system, overexpression of E-cadherin alone could not enhance the efficiency of iPS cell production; such an effect was recently reported (Chen et al, 2010). Reprogramming might thus also depend on the delivery system and the cellular context used. Taken together, these findings add entirely novel aspects to the processes of nuclear reprogramming: next to transcriptional networks (Jaenisch & Young, 2008), epigenetic changes (Hochedlinger & Plath, 2009) and changes in miRNA expression (Judson et al, 2009), apparently also spatial cues based on the function of E-cadherin are of importance.

Third, our finding that the expression of E-cadherin is able to replace the exogenous transcription factor Oct4, adds an attractive new layer to our understanding of reprogramming. It shows that iPS cell reprogramming is not just a nuclear event, but that spatial cues also participate, and that these crosstalk with essential transcription factors in a novel fashion. Specific cell-cell contacts of pluripotent cells appear to tie directly into the pluripotent transcription factor circuit, which lastly influences the master transcription factor, Oct4. Signaling events downstream of E-cadherin may provide a competent stimulus to upregulate endogenous Oct4. It is tempting to speculate that E-cadherin could control Oct4 through binding and sequestering β-catenin, i.e., by modulation of canonical Wnt signaling. It has been shown previously that inhibition of GSK3 enhances the pluripotency during somatic reprogramming (Silva et al, 2008), and that both β-catenin and E-cadherin are targets of GSK3, which may regulate the stability of cell-cell adhesion (Lickert et al, 2000). Alternately, the effects of E-cadherin on induced pluripotency may also require the involvement of Rho-family GTPases (Fukata & Kaibuchi, 2001). Cadherins modulate the activities of RhoA family of GTPases and their downstream kinases such as Rock.

It has been shown that inhibition of Rock has a positive role on pluripotency of human embryonic stem cells (Watanabe et al, 2007). The elucidation of the molecular mechanisms that establishes the crosstalk between the cell surface and nuclear machinery, especially the regulation of Oct4, awaits further analysis. With these three parts of our investigation, we have discovered entirely new functions for the cell adhesion molecule E-cadherin in pluripotency and reprogramming.

Material and Methods

Plasmids

Annealed small hairpin-forming oligos with the E-cadherin targeting sequence: 5'-AGCTTAAAAAAGAACCTGGTTCAGATCAAATTCTCTTGAAATTTGATCTG AACAGGTTCTGGG-3' (top) and 5'-GATCCCCAGAACCTGGTTCAGATCAAATTTCAAGAGAATTTGATCTGAAC CAGGTTCTTTTTTA-3' (bottom) were cloned into pMSCV (Clontech). For overexpression of E-cadherin, the coding sequence of mouse E-cadherin was introduced in the pMXs vector before.

Cell culture

129/Sv3 derived mESCs (from S. Noggle, NYSCF, New York) and established stable iPS cell lines generated were cultured on mitomycin C-treated (2mg/ml) mouse embryonic fibroblasts (MEF). MEFs were isolated according to standard procedures either from embryos of the mouse strain B6.129/Sv3-*Cdh1*^{tm2Kem}/J (Ecad$^{flox/flox}$), or from the outbreed strain Cf1 (Takahashi & Yamanaka, 2006).

Generation of iPS cells

Generation of induced pluripotent stem cells was performed as described previously (Takahashi & Yamanaka, 2006). In brief, 5 x 10^6 Plat-E cells/10cm dish were seeded one day before transfection with pMXs-based retroviral vectors (Oct4, Sox2, Klf4 and c-Myc; all from Addgene) using calcium phosphate precipitation in Plat-E medium supplemented with 25 μM chloroquine as previously described.

Cell-based assays

Immunofluoresence; Cells were fixed for 15 min with 100% ice-cold methanol, washed for 15 min at room temperature with PBS, 0.1% Triton-X-100 and blocked for 15 min in PBS with 2% BSA and 2% horse serum (blocking solution). The cells

were incubated with primary antibodies (as indicated) in blocking solution washed with PBS, 0.1% Triton-X-100 and incubated for 40 min with respective secondary antibodies. DAPI nuclear counter stain was used as described by the manufacturer (Sigma, D-9542). Stained cells were imaged following standard procedures on a Leica LAS AF 6000 Deconvolution.

Quantitative real-time PCR and Western analysis; These assays were performed as described before (Diecke et al, 2008). Antibodies used were against E-cadherin (BD and DECMA-1), N-cadherin (BD), Oct4 (Santa Cruz), Nanog (abcam), α-Tubulin (Sigma) and SSEA-1 (R&D). Secondary antibodies were used (Zymed and Jackson Laboratories).

Flow cytometry and FACS; Flow cytometric analysis of cells was performed using antibodies indicated. For flow cytometry and fluorescence-activated cell sorting (FACS), cells were washed once with ice cold PBS, harvested and centrifuged. The cell pellets were resuspended in wash puffer (20 % FBS/Hanks balanced salt solution, 1 mM $CaCl_2$, Mg^{2+} and Ca^{2+} free). For labeling 2.5 x 10^5 cells were diluted in a total volume of 200 µl and primary antibodies (15 min, 4°C) were added, followed by secondary antibodies (5 min, 4°C). Labeled cells were analyzed using FASCalibur or sorted using FACSAriaII (both BD).

Cre-recombinase treatment; Cells were treated with 3 µM purified, membrane-penetrable His-tat-NLS (HTN)-cre for 24 hours as described previously (Peitz et al, 2002).

Animal-based assays

Teratoma formation and histological analysis; 1 x 10^6 Cells were injected subcutaneously in NOD/SCID mice for teratoma formation as described (Takahashi & Yamanaka, 2006). Tumors were grown for 21 days and surgically dissected,

tumors were stained with haematoxylin and eosin (H&E) and biopsies were taken for RT-PCR analysis.

Blastocyst injection; For blastocyst injection, iPS cells (10-12 cells) stably transfected with the GFP expressing plasmid MP71-gfp were injected either at blastocyst stage of developing embryo. Blastocysts were transferred in uteri of surrogate mothers. Pups were analyzed for GFP positive spots 2 days after birth.

Supplementary information is available.

Acknowledgments

The authors thank A. Kirschner and M. Mühlbauer for technical assistance and A. Klaus, H.-P. Rahn, B. von Eyss, K. Eckert and I. Fichtner and B. Jerchow and K. Becker for technical help. We express our appreciation to R. Hodge, U. Ziebold, I. Ibanez-Tallon and M. Gossen for critical reading of the manuscript.

A BMBF grant in the joined project START-MSC (01GN0941) and an EC Marie Curie IRG (FP6-021407) supported this work.

The authors have no conflicting financial interests.

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Figure Legends

Figure 1. E-cadherin expression is required for maintenance of pluripotency

(A) Quantitative real-time RT-PCR for E-cadherin (Ecad), Oct4 and Nanog and N-cadherin in 129/Sv3 mESCs, undifferentiated control (+LIF Ctl) or differentiated in the absence of LIF (as indicated in days (d)). The mRNA expression levels were normalized to β-actin, and values for undifferentiated mESCs were set to 1. Values are presented in duplicates with median values indicated by bars and higher values indicated by error bars. (B) Immunofluorescence of 129/Sv3 cells for Ecad, Oct4 and Nanog, 4 days after viral transduction with a pMSCV vector (empty) or a vector constitutively expressing shRNA against E-cadherin (shEcad). Nuclei were stained with DAPI. Magnification 400x, bars 50 μm. (C) Real-time RT-PCR of 129/Sv3 mESCs in LIF not transduced (mock) or transduced as in (B). mRNA levels for Ecad, Oct4, Nanog, Dppa5 and Nr5a2 are shown as in (A).

Figure 2. SSEA-1 positive iPS cells express endogenous pluripotency genes, form teratomas and contribute to blastocysts forming chimeras

(A) MEFs were retrovirally transduced with the four factors Oct4, Sox2, Klf4 and c-Myc (OSKM). Sorting for SSEA-1 was performed. Mesenchymal-to-epithelial transition, MET. (B) Characterization of established cell clones derived from SSEA-1 positive ($Ecad^{high}$) cells and SSEA-1 negative ($Ecad^{low}$) cells, in comparison to 129/Sv3 mESC. Immunofluorescence for Ecad (cyan), SSEA-1 (red) and Nanog (green) are shown. Nuclei were stained with DAPI (blue). Ncad (red) was analyzed in independent clones. Magnification 400x, bars 50 μm. (C) Agarose gel electrophoresis of RT-PCR products for marker expression of three stable $Ecad^{high}$ iPS cell clones (#4, #6 and #11) in comparison to cells of an $Ecad^{low}$ clone (#1, $Ecad^{low}$), 129/Sv3 and MEFs. SSEA-1 expression was determined by flow cytometry and is indicated (+/–). Expression of Ecad, Ncad, viral (v) and total (t) Oct4, Nanog and Dppa5 was analyzed. β-Actin was the loading control. (D) Quantitative real-time RT-PCR

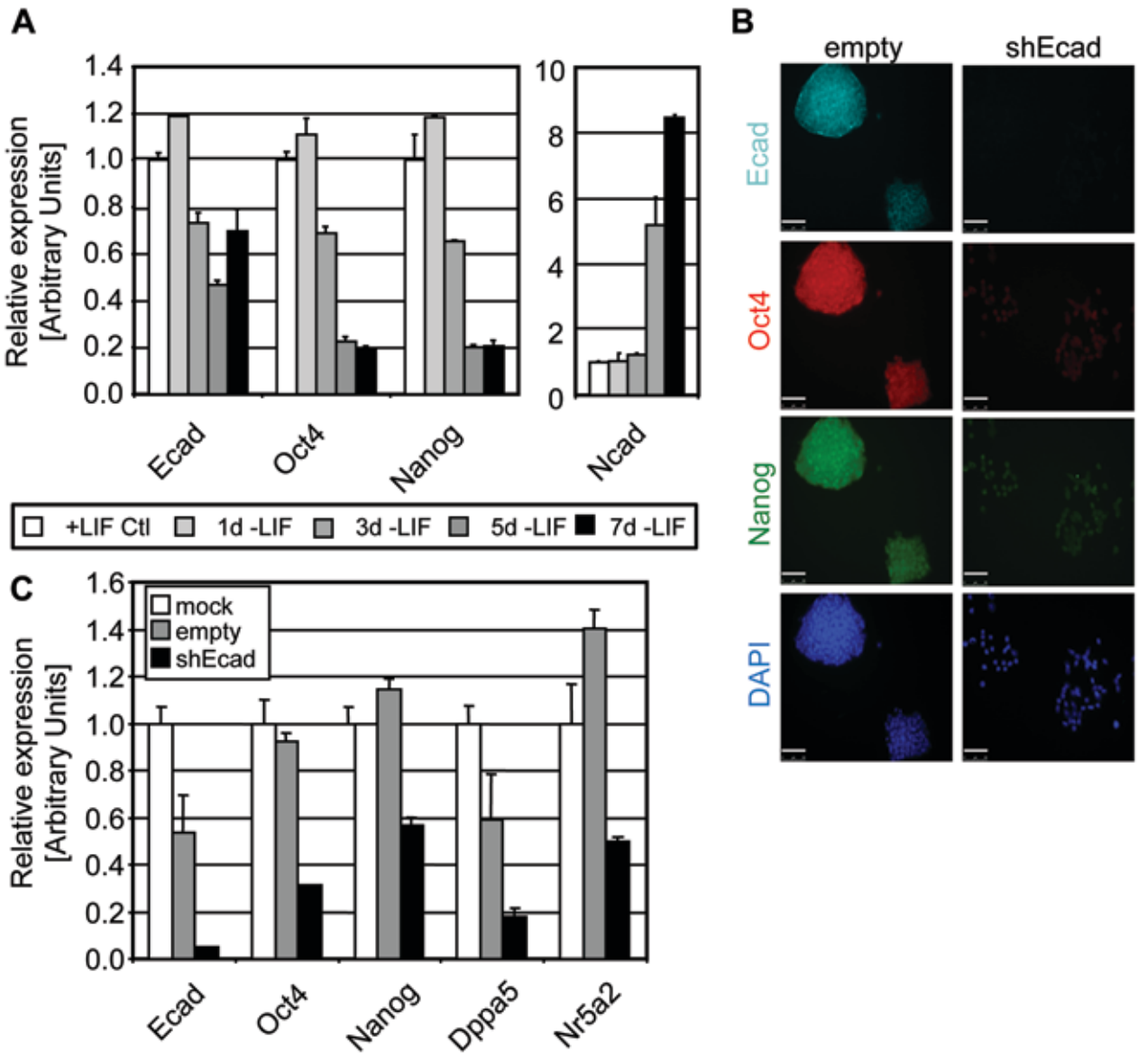
analysis of spontaneously differentiated Ecad^{high} iPS (Ecad^{high}) cells, Ecad^{low} cells and 129/Sv3 cells grown for 4 days in presence or absence of LIF. mRNA levels for Ecad, Oct4 and Nanog (left panel) and Ncad and Msx2 (right panel) are presented as in Fig. 1A and related to Ecad^{high} iPS cells in LIF set to 1. (E) Morphology of tumors derived from Ecad^{high}#4 cells (1) or Ecad^{low} cells (5) in NOD/SCID mice (n=3/cell clone). Histological analysis of tissue sections by haematoxylin and eosin (H&E) staining of differentiated tumors (Ecad^{high}#4) comprising epithelial (2), neuronal (3) and muscle derived (4) structures, or of a undifferentiated tumor (Ecad^{low}#1; 6). Magnification 25x (H&E staining), bars 500μm. (F) Chimera formation following injection of GFP-labeled Ecad^{high}#11 cells in mouse blastocysts (n=3/cell clone) and analysis of newborn pups (1) for GFP positive areas by fluorescence (2). GFP-labeled organs like the gut (3) were detected by fluorescence (4). No GFP-labeled Ecad^{low} cells were visible (5 and 6).

Figure 3. Loss of E-cadherin expression reveals its necessity during the induced pluripotency process in murine fibroblasts

(A) Treatment of Ecad$^{flox/flox}$ MEFs or wild-type (wt) MEFs with HTN-cre for 24 hours and transduction of OSKM. Ecad protein levels were detected in whole cell lysate by Western blotting. (B) Number of colonies observed following no treatment (white bars) or HTN-cre treatment (black bars) of cells treated as in (A) and viral transduction of the factors OSKM. The number of colonies is shown in % to untreated controls. (C) Quantitative real-time RT-PCR analysis of expression of Ecad, Ncad, Oct4, Nanog and Dppa5 in Ecad$^{flox/flox}$ MEFs treated as in (A) after induction of pluripotency by OSKM. Expression levels for untreated, virally transduced cells are set to 1.

Figure 4. E-cadherin is capable of replacing Oct4 in the induction of stem cells in somatic cell reprogramming

(A) Derivation of ESKM-iPS cell clones following viral transduction of MEFs in presence of Ecad but absence of Oct4. Transduced MEFs were seeded and ES-cell like colonies were visible after 12-14 days. Primary clones were isolated without sorting and propagated. (B) Morphology of two independent primary clones photographed 12 days after viral transduction of MEFs with ESKM (upper panel) or SKM (lower panel). Primary clones were isolated and cultured for 4 days on feeder cells. Morphology of ESKM or SKM cells is shown in the right panels. (C) Immunofluorescence analysis of established ESKM (#2, #3) and SKM cells for expression of Oct4. Nuclei were stained with DAPI. Magnification 400x, bars 50 μm. (D) Agarose gel electrophoresis of RT-PCR products was performed with ESKM clones (#1, #2 and #3), ESKM induced MEFs 2 days after infection (2d pi), SKM subcloned cells (SKM), SKM infected MEFs (2d pi), OSKM-derived iPS cells and OSKM induced MEFs (2d pi). Expression levels of viral (v) and total (t) Ecad, Ncad, viral (v) and total (t) Oct4, Nanog, Dppa5 and Nr5a2 of cells were determined. β-Actin was the loading control. (E) Whole morphology of tumors derived from ESKM-derived iPS cells (ESKM#2; 1) or SKM-derived cells (5; n=3/cell clone). Histological analysis of tissue sections by H&E staining of differentiated tumors (ESKM#2) comprising epithelial (2), neuronal (3) and muscle derived (4) structures or of a undifferentiated tumor (SKM; 6). Magnification 25x (H&E staining), bars, 500μm.



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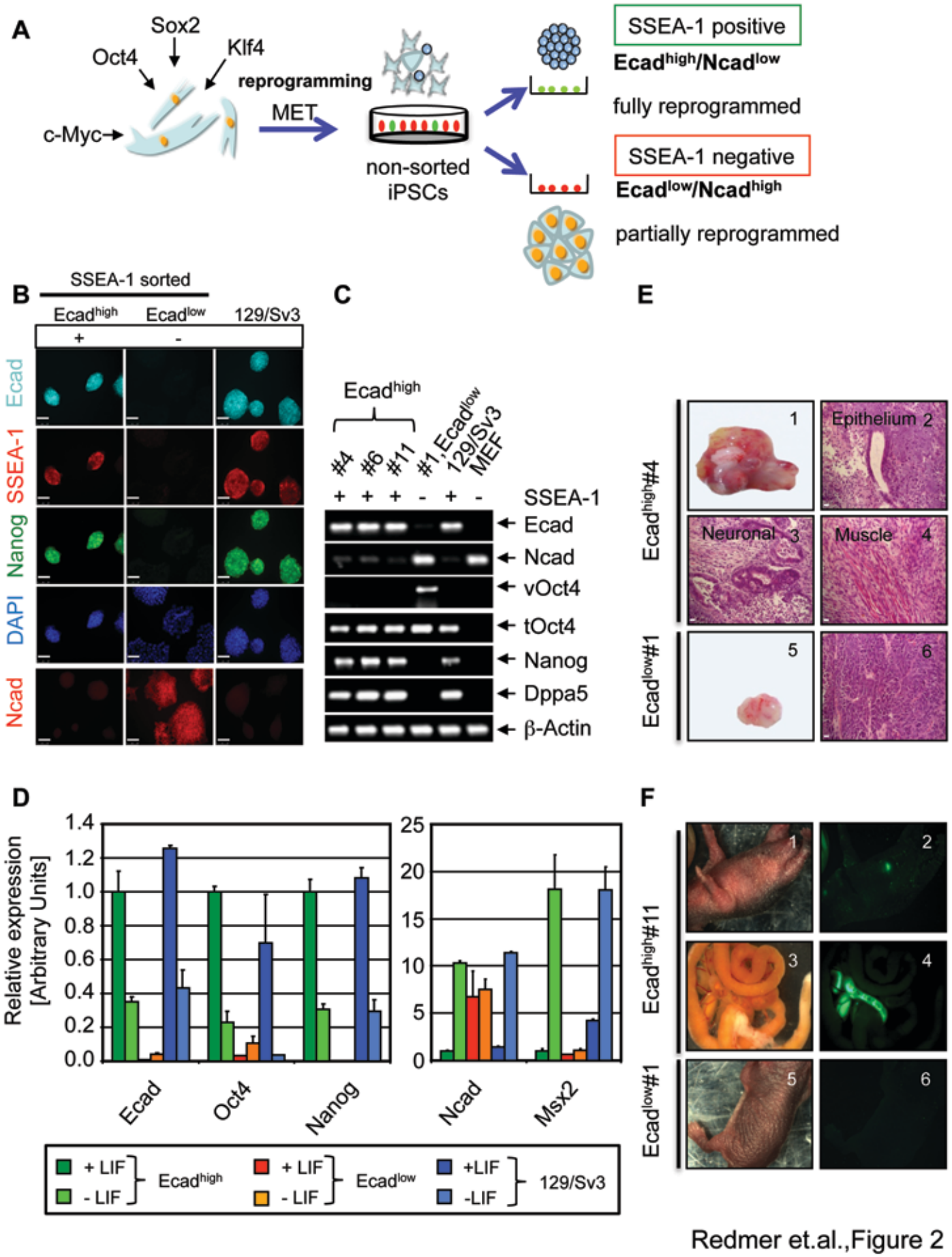

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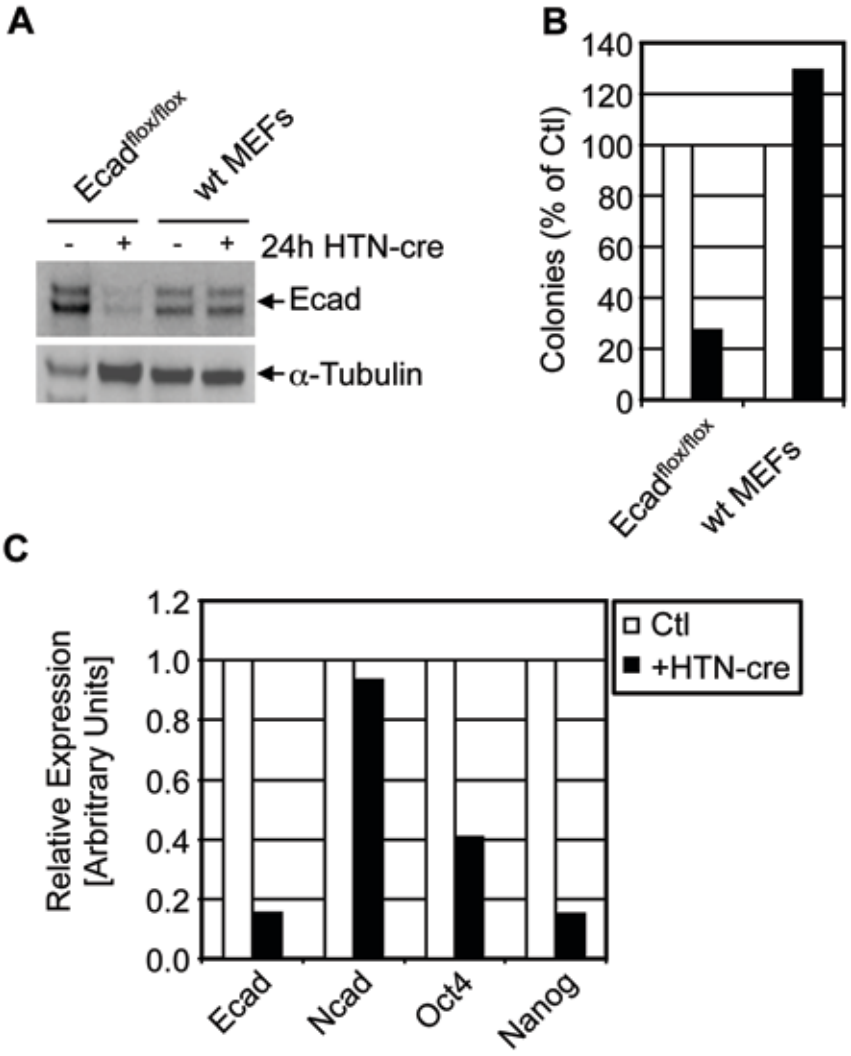
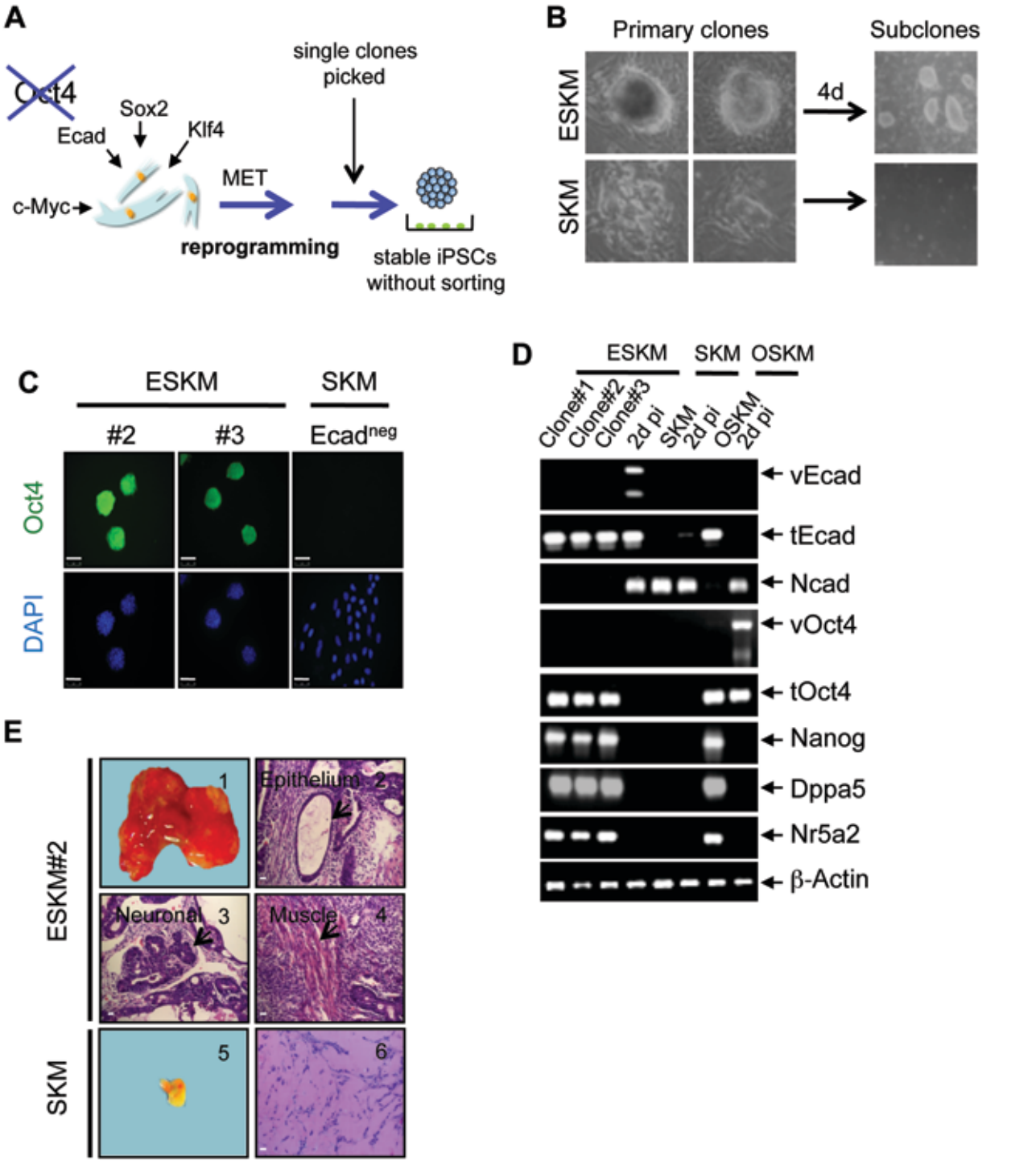


Redmer et al.,Figure 3



Redmer et al.,Figure 4

Supplementary Material:

Table for primer sequences used in quantitative real-time PCR:

Gene	*Accession No.*	*Sequence*
Ecad-total-s	NM_009864	TTGAGGCCAAGCAGCAATACATCC
Ecad-total-as		AGATGTGATTTCCTGACCCACACC
Ecad-endo-s	NM_009864	CTCAGTGTTTGCTCGGCGTCTGC
Ecad-endo-as		CAGGACATGGCCTCTCTCCAGGT
Ecad-viral-s		ACGGCGGTGGTGAGGACGACTAG
Ecad-viral-as		ATCCTGTTTGGCCCATATTCAGCT
Ncad-s	NM_007664	TTGCTTCTGACAATGGAATCCCGC
Ncad-as		AAGGAAAGATCAAACGCGAACGGC
Klf4, total-s	NM_010637	CACCATGGACCCGGGCGTGGCTGCCAGAAA
Klf4, total-as		TTAGGCTGTTCTTTTCCGGGGCCACGA
Klf4, endo-s	NM_010637	GCGAACTCACACAGGCGAGAAACC
Klf4, endo-as		TCGCTTCCTCTTCCTCCGACACA
c-Myc, total-s	NM_010849	CAGAGGAGGAACGAGCTGAAGCGC
c-Myc, total-as		TTATGCACCAGAGTTTCGAAGCTGTTCG
c-Myc, endo-s	NM_010849	GACCTAACTCGAGGAGGAGCTGGAATC
c-Myc, endo-as		AAGTTTGAGGCAGTTAAAATTATGGCTGAAGC
Oct4, total-s	NM_002701	CTGAGGGCCAGGCAGGAGCACGAG
Oct4, total-as		CTGTAGGGAGGGCTTCGGGCACTT
Oct4, endo-s	NM_002701	TCTTTCCACCAGGCCCCCGGCTC
Oct4, endo-as		TGCGGGCGGACATGGGGAGATCC
Oct4, viral-s		AAGAACCTAGAACCTCGCTGGAAAGG
Oct4, viral-as		CTGTAGGGAGGGCTTCGGGCACTT
Sox2, total-s	NM_011443	GGTTACCTCTTCCTCCCACTCCAG
Sox2, total-as		TCACATGTGCGACAGGGGCAG
Sox2, endo-s	NM_011443	TAGAGCTAGACTCCGGGCGATGA
Sox2, endo-as		TTGCCTTAAACAAGACCACGAAA
Afp-s	NM_007423	AATGACTAGCGATGTGTTGGCTGC
Afp-as		TTCATGTGCTTTGCAACTCTCGGC
Brachyury (T)-s	NM_009309	ACCACCGCTGGAAATATGTGAACG
Brachyury (T)-as		AACTCTCACGATGTGAATCCGAGG
β-Actin-s	NM_007393	TCGTGCGTGACATCAAAGAGAAGC
β-Actin-as		ATGGATGCCACAGGATTCCATACC
Dppa5-s	NM_025274	GAAATATCTGTTTGGCCCACAGGG
Dppa5-as		GCCATGGACTGAAGCATCCATTTAGC
GATA4-s	NM_008092	TCACAAGATGAACGGCATCAACCG
GATA4-as		GCAGGCATTACATACAGGCTCACC
Isl-1-s	NM_021459	TGATTTCCCTGTGTGTTGGTTGCG
Isl-1-as		ATCACGAAGTCGTTCTTGCTGAAGCC
Krt8-s	NM_031170	AGCATTCATACGAAGACCACCAGC
Krt8-as		TTGGACACGACATCAGAAGACTCG
Mef2c-s	NM_025282	AGCAGCAGCACCTACATAACATGC
Mef2c-as		ATGCGCTTGACTGAAGGACTTTCC
MMP2-s	NM_008610	AGATGCAGAAGTTCTTTGGGCTGC
MMP2-as		AAAGCATCATCCACGGTTTCAGGG

Msx2-s	NM_013601	AAATCTGGTTCCAGAACCGAAGGG
Msx2-as		CATGGTAGATGCCATATCCAACCG
Myl7-s	NM_022879	TTCAGCTGCATTGACCAGAACAGG
Myl7-as		TGAGGAAGACGGTGAAGTTGATGG
Nanog-s	NM_028016	AACCAAAGGATGAAGTGCAAGCGG
Nanog-as		TCTGGTTGTTCCAAGTTGGGTTGG
Nestin-s	NM_016701	AACAGAGCAAGATGAGGACAGAGC
Nestin-as		TCTTTGCCTTCACACTTTCCTCCC
Nr5a2-s	NM_030676	ACACAGAAGTCGCGTTCAACAACC
Nr5a2-as		TAGTTGCAAACCGTGTAGTCCAGC
Sox1-s	NM_009233	ATGCACAACTCGGAGATCAGCAAGC
Sox1-as		AGTACTTGTCCTTCTTGAGCAGCG
Sox17-s	NM_011441	ATGTAAAGGTGAAAGGCGAGGTGG
Sox17-as		ATCTTGCTTAGCTCTGCGTTGTGC
Tbx5-s	NM_011537	CAAATGGTCCGTAACTGGCAAAGC
Tbx5-as		ACGCAGTGTTCTTTGAACCGAACC
TropT2-s	NM_001130174	TCCAACATGATGCACTTTGGAGGG
TropT2-as		AGGTTGTGAATACTCTGCCACAGC

PCR primers were designed with http://eu.idtdna.com/Scitools/Applications/Primerquest/ using the whole coding sequence of the target genes (NCBI). Primers were designed as follows: Primer size 20 – 27 bp; T_M 58 – 62 °C; GC% 35 – 65; Product size 150 – 250 bp; GC Clamp 2.

Supplementary Figures and Results

Supplementary Figure 1. Reduction of E-cadherin results in loss of pluripotency marker expression

(A) Analysis of protein extracts from 129/Sv3 cells undifferentiated or differentiated for 3, 5 or 7 days (d) with antibodies against Ecad, Ncad, Oct4 and as loading control α-Tubulin.

Ecad and Oct4 protein levels are downregulated during differentiation while the level for Ncad strongly increased after 5 days of differentiation.

(B) Immunofluorescence of 129/Sv3 mESCs undifferentiated (+LIF) and differentiated in absence of LIF for 7 days (-LIF) or transfected with siRNA against Ecad (Dharmacon, siEcad) for 3 days or a neutralizing antibody DECMA-1 for 3 days both in presence of LIF with antibodies against Ecad (light green fluorescence) and Ncad (red fluorescence) and Oct4 (dark green fluorescence) as indicated. DAPI nuclear staining (blue) was performed to visualize cell nuclei. Pictures were taken with a magnification of 400x. Bars, 50 μm.

(C) Analysis of Ecad, Oct4 and Nanog protein levels by Western blotting of 129/Sv3 mESCs in LIF not transduced (mock) or transduced with a pMSCV vector (empty) or a vector constitutively expressing shRNA against E-cadherin (shEcad).

Undifferentiated and untreated 129/Sv3 cells reveal a strong Ecad and Oct4 staining but low staining for Ncad by immunofluoresence experiments. When cells were treated either with a neutralizing antibody DECMA-1 or siRNA against Ecad (siEcad) or kept in absence of LIF for 7 days (-LIF, 7d) a cadherin-switch was observed where mESCs start to express Ncad while expression of Ecad and Oct4 were lost.

Supplementary Figure 2. SSEA-1 expression correlates with E-cadherin levels and pluripotency

(A) Sorting of MEFs (10.000 cells) eight days after viral transduction (OSKM) using SSEA-1 (CD15) as cell surface marker into SSEA-1 positive (12.5% of total cells) and negative cells (87.5%) (left panel). Flow cytometry (right panel) of SSEA-1 positive and negative cells cultured further on feeder cells for 8 days to established stable cell clones confirmed the presence and absence of SSEA-1 on SSEA-1^{pos} cells and on SSEA-1^{neg} cells, respectively.

(B) High Ecad and Oct4 protein expression in stable SSEA-1 positive iPS cell clones (#4, #6 and #11) and SSEA-1 negative cells (#1).

Ecad was highly expressed in SSEA-1^{pos} cells but nearly absent in SSEA-1^{neg} cells, whereas expression of Oct4 was visible in all cells even though Oct4 expression level was higher in SSEA-1^{neg} cells due to expression of viral Oct4.

(C) Analysis of mRNA expression levels of $Ecad^{high}$ and $Ecad^{low}$ expressing cells, 129/Sv3 mESCs and MEFs for viral, endogenous and total levels of Oct4, Sox2, Klf4 and c-Myc. mRNA expression levels were normalized to the respective β-actin expression and values for MEFs were set to 1, the experiment was carried out in duplicate with the median values indicated by bars and the higher values indicated by an error bar.

Levels of endogenous expressed Oct4, Sox2, Klf4 and c-Myc of $Ecad^{high}$ iPS cells were as high as in 129/Sv3 cells, whereas $Ecad^{low}$ cells displayed low endogenous Sox2 levels and nearly no expression of endogenous Klf4 and c-Myc. $Ecad^{low}$ cells showed high expression of viral genes since no silencing had occurred.

(D) Protein levels of Ecad, Ncad, SSEA-1 and Oct4 in MEFs, 129/Sv3 mESCs, $Ecad^{low}$ cells and $Ecad^{high}$ iPS cells cultured in presence or absence of LIF. Equal amounts of total protein were verified by α-Tubulin.

Differentiation of Ecad^{high} cells is similar to 129/Sv3 mESCs. In presence of LIF, Ncad is not expressed, whereas Ecad is expressed on high levels. When LIF is withdrawn, Ecad level is reduced in both Ecad^{high} and 129/ Sv3 cells but Ncad becomes upregulated. In addition, Oct4 and SSEA-1 level were also decreased in both cell lines. Interestingly, Ecad^{low} cells, bearing still high Ncad levels resulting from an inefficient MET process, are unable to undergo spontaneous differentiation. Ncad and Oct4 levels in Ecad^{low} cells were not changed upon LIF withdrawal. The high Oct4 proteins levels in Ecad^{low} cells were from the virally expressed Oct4 gene.

(E) Morphological changes of Ecad^{high} iPS cells (1 and 4) and 129/Sv3 mESCs (3 and 6) of cells grown in presence of LIF (1 and 3) and following spontaneous differentiation in absence of LIF for 4 days (4 and 6). Morphology of Ecad^{low} cells (2 and 5) did not change under differentiating conditions when compared to growth in presence of LIF. EB formation of Ecad^{high} cells (7) and 129/Sv3 cells (9) was observable, whereas Ecad^{low} cells did not form EBs (8). The magnification is 100x. Bars, 100 μm.

Ecad^{high} and 129/Sv3 cells display a round shaped morphology in presence of LIF whereas Ecad^{low} cells form loose flattened colonies. Upon spontaneous differentiation, round shaped colonies of Ecad^{high} and 129/Sv3 cells form scattered colonies with cells migrating out from the edge of colonies. The morphology of Ecad^{low} cells did not change. Further, when Ecad^{high} and 129/Sv3 cells were plated for EB formation, well shaped spheres become visible upon differentiation in suspension. When Ecad^{low} cells did not show formation of EBs, instead they form loose cell clumps and single floating cells.

(F) Expression of differentiation marker genes of teratoma analyzed by agarose gel electrophoresis of RT-PCR products comprising representative markers of all three germ layers. Expression levels of E-cadherin (Ecad, epithelial), N-cadherin (Ncad, mesenchymal/neuroectoderm), Islet-1 (endoderm, neuroectoderm), Sox1

(neuroectoderm), Sox2 (neuroectoderm), Msx2 (mesenchymal/neuroectoderm), Cytokeratin 8 (Krt8, non-neural ectoderm), α-Fetoprotein (Afp, visceral and definitive endoderm), MMP-2 (mesoderm), Brachyury (T, pan-mesoderm), Mef2c (myogenic), Tbx5 (cardiomyogenic), GATA4 (definitive endoderm), Troponin T2 (cardiomyogenic) and Myl7 (Myosin-light-chain 7, myogenic).

This experiment revealed the presence of derivatives of endoderm (Ecad, Islet-1, Krt8, and Afp), mesoderm (MMP-2, T, Mef2c, GATA4, TropT2 and Myl7) but also ectoderm derived cells (Sox1, Sox2, Msx2 and Ncad) in $Ecad^{high}$ and 129/Sv3 derived tumors indicating that $Ecad^{high}$ cells were able to develop well differentiated tumors while $Ecad^{low}$ cells were not.

Supplementary Figure 3. E-cadherin overexpression during iPS cell induction did not increase efficiency

(A) Western blot analysis (upper panel) and real-time PCR analysis (lower panel) of MEFs viral transduced with single pMXs-vectors carrying sequences of either Ecad, Oct4, Klf4 (Antibody: R&D, AF3158) Sox2 (R&D, AF2018), or c-Myc (Sigma, C3956).

Transduced MEFs were analyzed for expression of indicated proteins and mRNAs, 2 days after viral transduction. This analysis confirmed that all five factors were expressed in MEFs after viral transduction.

(B) Flow cytometric analysis for SSEA-1 expression of MEFs following 8 days after viral transduction with different combinations of OSKM in presence or absence of pMXs-Ecad. SSEA-1 positive cells are shown in % of 10,000 analyzed cells. (C) Alkaline phosphatase assay of cells treated like in (B). For determination of alkaline phosphatase activity, cells were washed once with PBS and fixed with PBS/4% PFA. Cells were washed and alkaline phosphatase activity was visualized using both substrates NBT and BCIP.

To clarify whether Ecad overexpression during iPS cell-induction could improve efficiency of reprogramming in dependence of factor combinations, we performed flow cytometric analysis of induced MEFs transduced with different combinations (10) of four factors in absence or presence of Ecad. Number of SSEA-1 positive cells was decreased under most factor combinations. In addition, no significant increase in alkaline phosphatase positive cells colonies was observed in the presence of Ecad compared to colonies observed in the absence of Ecad.

Supplementary Figure 4. Characterization of ESKM-iPS cell clones

(A) Number of colonies of two independent transduction series and re-seeding in duplicates to 2 x 10^5 cells per 10 cm dish. Numbers were shown from duplicate experiments with the averages display as bars and the higher values displayed as error bars.

Yields of iPS cell-colonies received from re-plated ESKM induced MEFs were much lower than those arising from OSKM. ESKM colonies were stable, expandable and morphologically indistinguishable from OSKM colonies. SKM-derived colonies were rare and immediately differentiated following re-plating.

(B) Immunofluorescence analysis of established ESKM (#2, #3) and SKM cells for expression of Ecad, SSEA-1 and Nanog, as indicated.

(C) Characterization of three established ESKM-clones in comparison to ESKM iPS cells, SKM-induced cells, 129/Sv3 cells and MEFs, respectively. Expression of Ecad, Ncad and Oct4 was determined by Western blotting as indicated, α-Tubulin was used as loading control.

ESKM clones (#1,#2,#3) display high expression of Ecad and Oct4 as 129/Sv3 mESCs, but no expression of Ncad indicating that these cells underwent proper MET and reprogramming, whereas Ecad and Oct4 are not expressed in SKM cells.

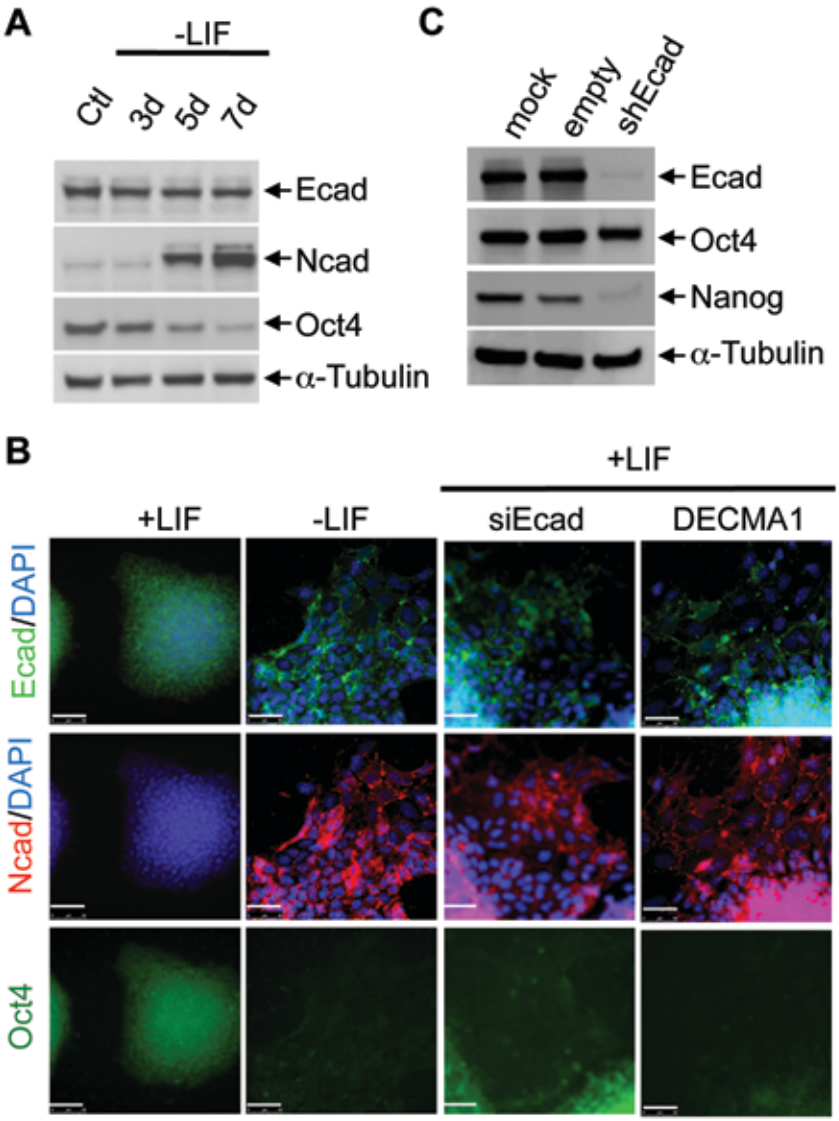
(D) Pictures of EB formed by ESKM clones (#2 and #3). No EBs were detected for the SKM clones.

(E) Outgrowth of EBs derived from ESKM clones (#2 and #3, Fig. S4D) was analyzed for marker genes of all three germ layers. Using immunofluorescence, expression of FoxA2 (endoderm; antibody from Abcam, AB40874), Nestin (ectoderm; antibody was a gift from C. Birchmeier) and α-smooth-muscle actin (α-SMA; antibody from abcam, ab5694) was detectable in outgrown EBs of both iPS cell clones. DAPI was used for nuclear staining, the magnification is 400x. Bars, 50 μm.

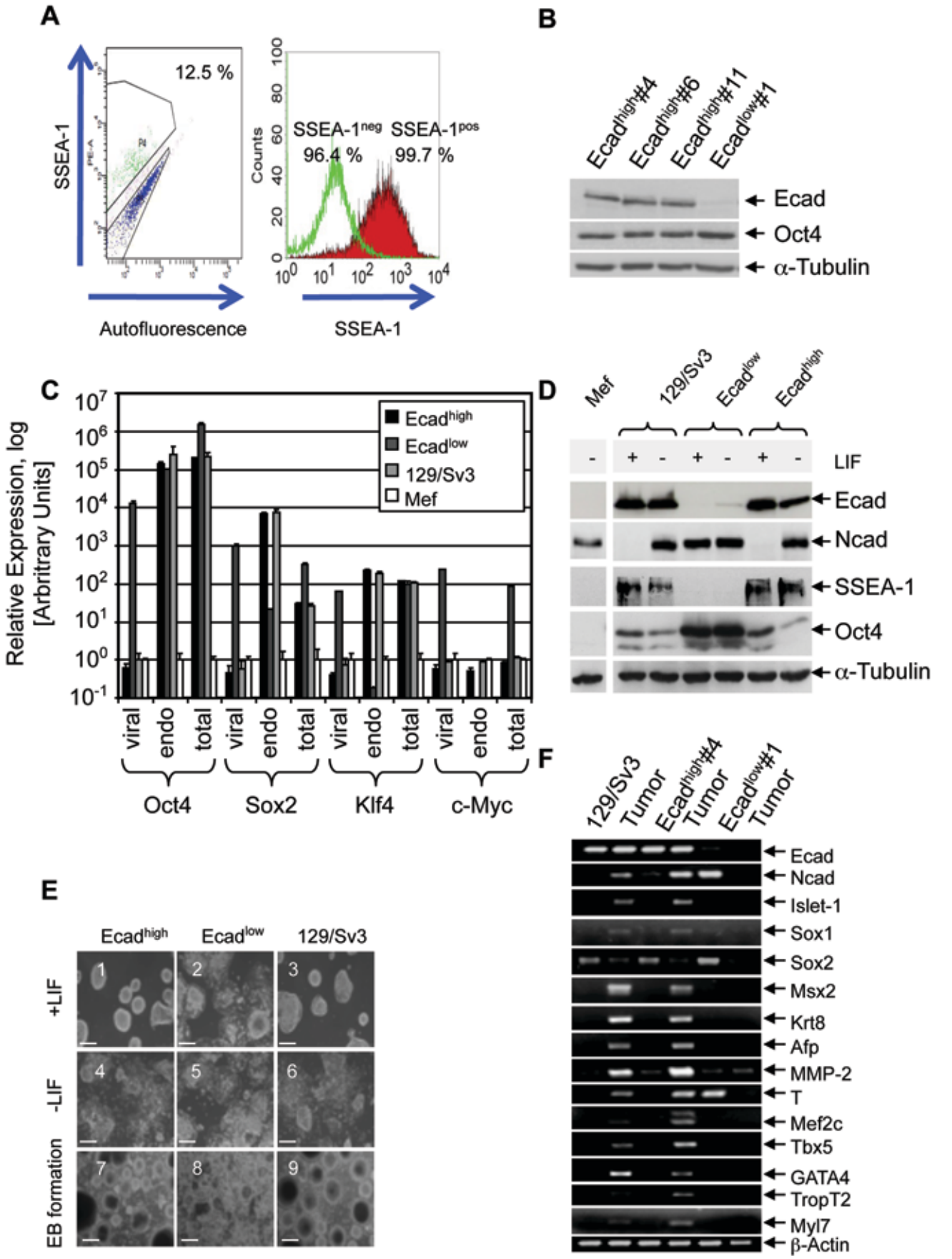
ESKM cells of clones #2 and #3 have the potential to form derivatives of all three germ layers when differentiated via EB formation indicating that these cells reached the pluripotent stage.

(F) Teratomas grown after injection of ESKM-iPS cells (clone#3, 1) in NOD/SCID mice. Sections were stained with H&E and typical epithelial/luminal structures were indicative for derivatives of endoderm (2).

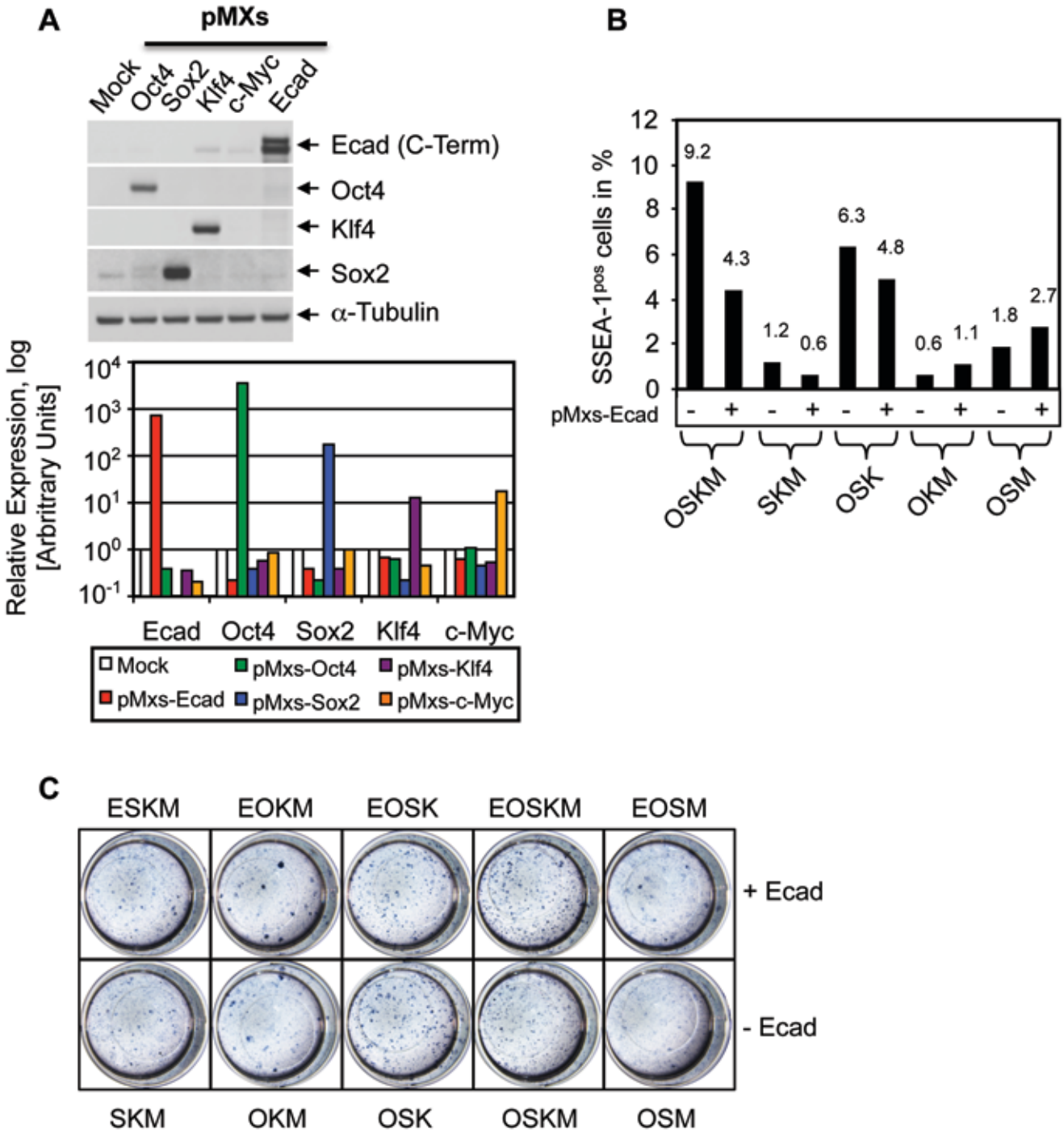
Formation of teratoma as described in Fig. 4, but with an additional clone ESKM#3.



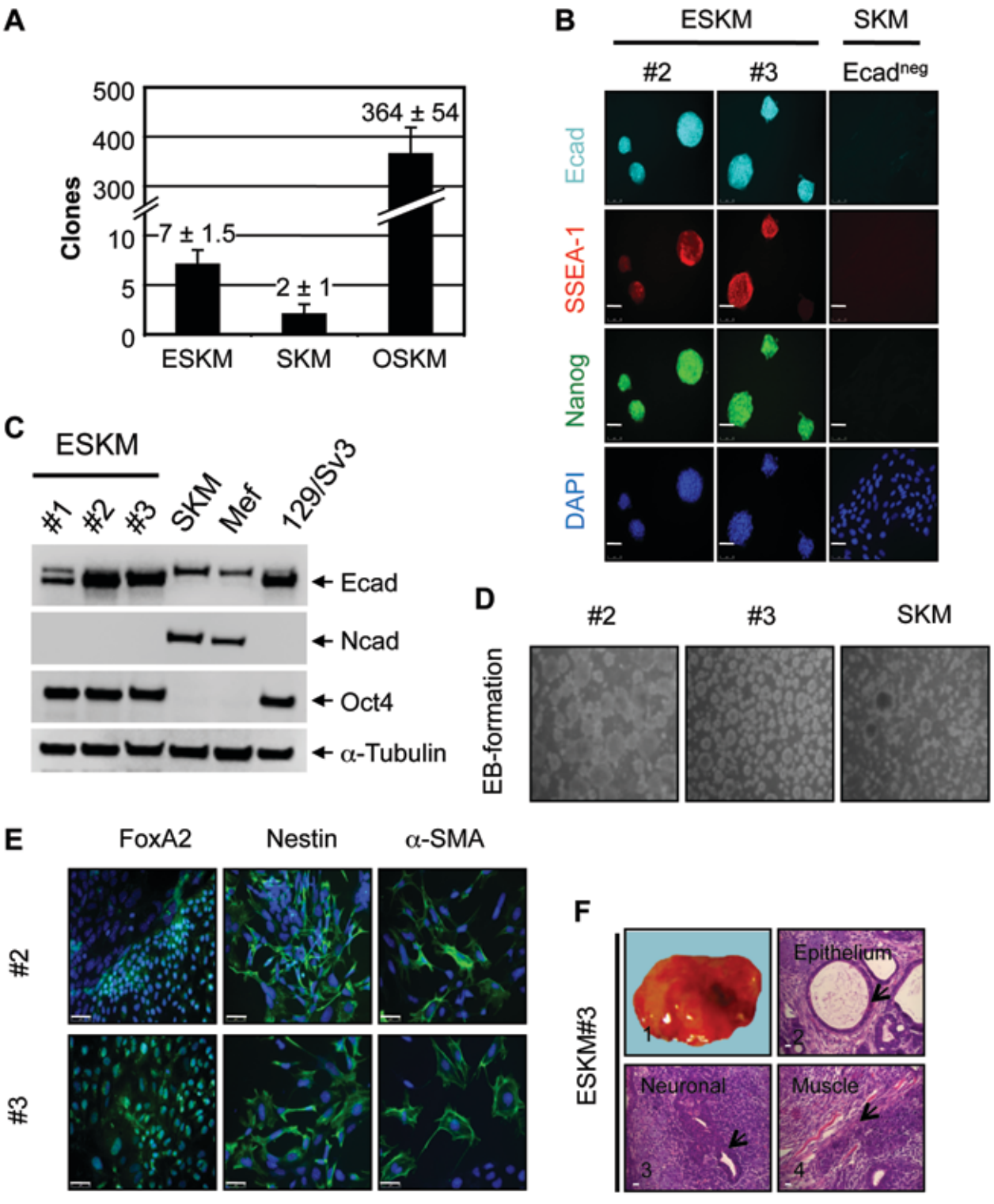
Redmer et.al., Supplementary Figure 1



Redmer et.al., Supplementary Figure 2



Redmer et.al., Supplementary Figure 3



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