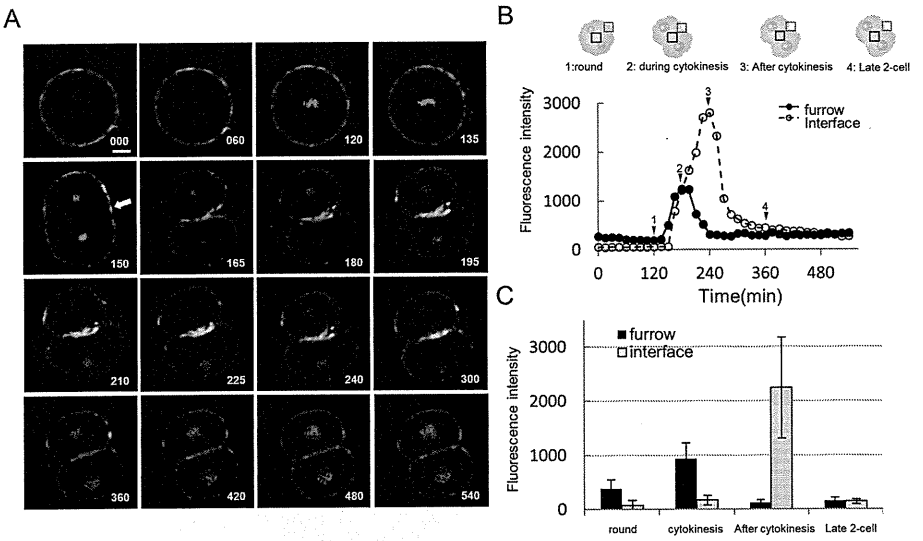

Figure 2 MSGb5Cer accumulates at the interface during cytokinesis in preimplantation embryos. (A) Frames from a series of confocal time-lapse movies of live cytokinesis-stage cells stained with 6E2 for MSGb5cer (green) and with DAPI for nuclei (red). Frames are shown at 60-minute intervals from 0 to 120 minutes, 15-minute intervals from 120 minutes to 240 minutes, and 60-minute intervals from 240 minutes to 540 minutes. Time is expressed in minutes after the start of culture. The arrow at 150 minutes points to the site of a future furrow in the spherical zygote. Scale bar represents 20 μm. The images shown are representative of nine sample zygotes. (B) Temporal changes in fluorescence intensity at the furrow and the interface region during cytokinesis. The fluorescence intensity in pixel values was measured in boxes of equal size located at the furrow (red square) and the interface (black square) of the frames taken every 15 minutes from a confocal time-lapse movie of the zygote of Figure 2A. The fluorescence intensity values at the furrows (closed circles) and interface (open circles) were plotted against time in minutes. Schematic embryos at the round stage (1), during cytokinesis (2), after cytokinesis (3), and the late 2-cell stage (4) were cultured for 105 minutes, 165 minutes, 240 minutes, and 405 minutes, respectively. This data shown is representative of nine sample zygotes. (C) The fluorescence intensity of MSGb5Cer at the furrow (closed column) and interface (dotted column) in round, cytokinesis, post-cytokinesis and late 2-cell stage embryos. Mean values and s.d. were calculated from the results obtained from the time-lapse movies of 6 embryos shown in Figure 2B.

by 48 hours of culture. These results suggest that MSGb5Cer is more sensitive than GM1 to raft integrity, and that lipid raft is prerequisite to cell adhesion and normal development of preimplantation embryos.

Extensive accumulation of MSGb5Cer to interfaces is related to delay and suppression of normal development

As shown in Figure 5A, incubation for 1 hour in the presence of 100 μg/ml of anti- MSGb5Cer Mab 6E2, but not of the isotype-matched control Mab 15B2, led to extensive accumulation of MSGb5Cer at the interfaces in the embryos, and a large aggregate of BODIPY®FL-MSGb5Cer was found.

Prolonged culture of 2-cell embryos in the presence of 6E2 caused a decrease in survival rate, and more than 90% of the embryos died before developing into blastocysts (Figure 5B). 80% of embryos were died after 12 hour culture in 6E2, whereas all of embryos cultured in control Mabs were alive. After 24 hour-culture, embryos cultured in 6E2 cleaved abnormally, and their cleavage rate was delayed as compared with those cultured in control Mabs. The morula embryo, which was able to avoid being injured, seemed to be compacted (arrow in 48 hour culture in 6E2). The anti-MSGb5Cer Mab 6E2 we used in this study is not toxic and do not have non-specific effects on preimplantation embryos, because 100 μg/ml of 6E2 antibody does not affect viability of blastocyst stage embryos that no longer express MSGb5Cer (see Figure S1 in additional file 2). In addition, when either the isotype-matched control Mab 15B2 or anti-E-cadherin Mab ECCD-2 was similarly tested, they did not affect viability of embryos (Figure 5).

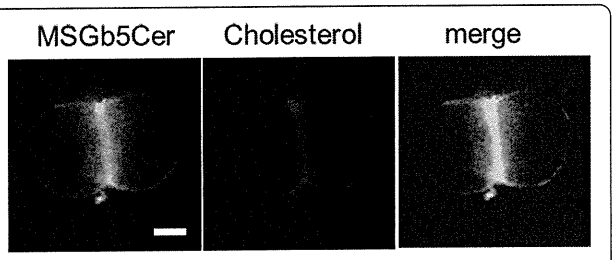

Figure 3 MSGb5Cer is overlapped by cholesterol at the interface in a 2-cell embryo. A two-cell stage embryo was stained with 6E2 for MSGb5cer (green) and then filipin III for cholesterol (blue) as described in Materials and Methods. Scale bar: 20 μm. Ten sample 2-cell stage embryos were used for this experiment represented in images.

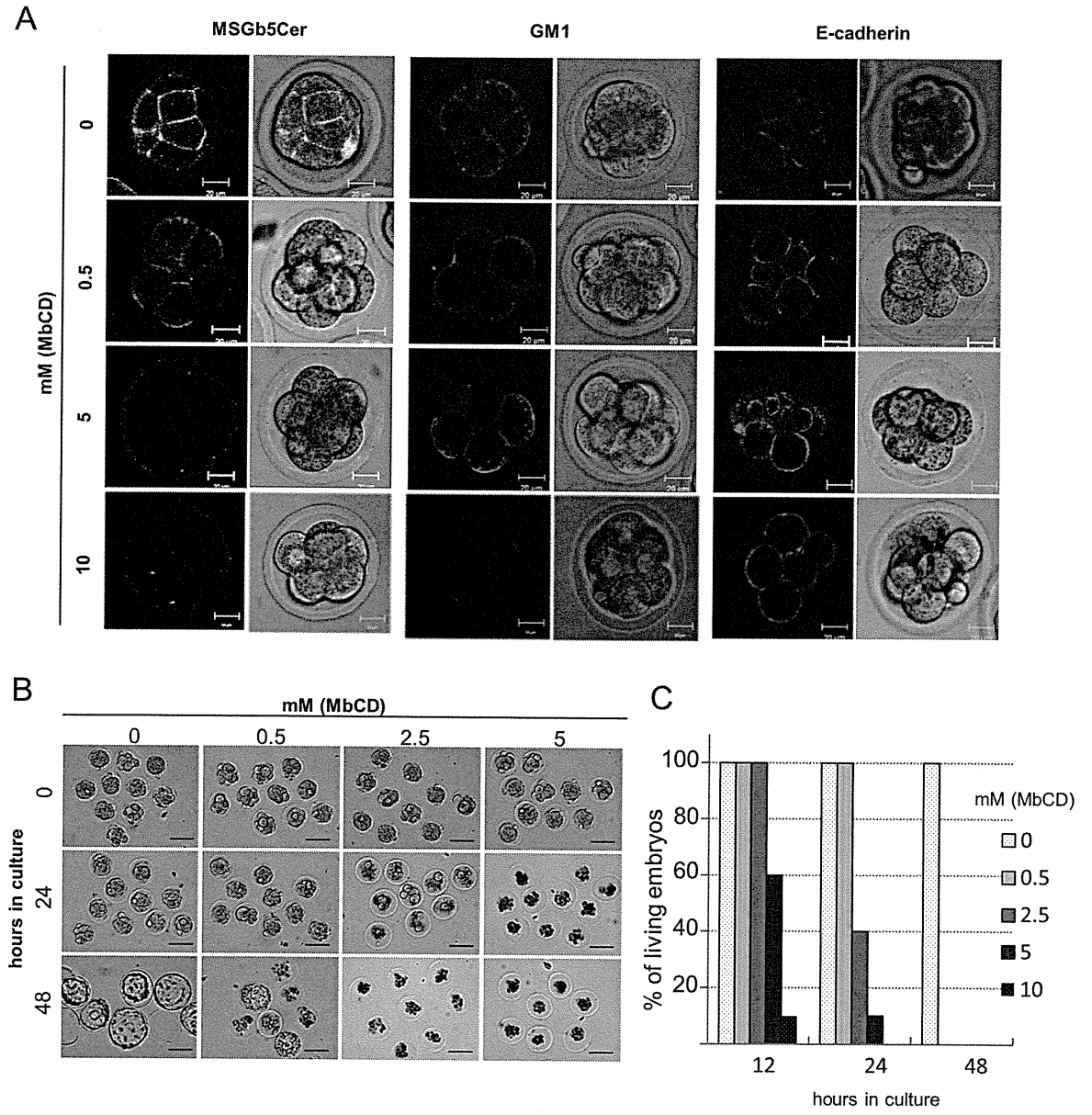

Figure 4 Pretreatment with MbCD inhibits cytokinesis and development of mouse preimplantation embryos. (A) Effect of MbCD on the expression of MSGb5Cer (green), GM1 (red) and E-cadherin (red) on 8-cell embryos. The left column and the right column of each panel represent a 2D image and the overlay of a 2D image and a differential interference contrast (DIC) micrograph, respectively. Thirty 8-cell embryos were used for the experiment represented in the images. (B) Representative DIC images of embryos cultured for 0, 24 and 48 hours in M16 medium after preincubation with 0-5 mM MbCD. Scale bars: 100 μm. Twenty 8-cell embryos were used for the experiment represented each images. (C) The percentages of living embryos cultured for 12, 24 and 48 hours after preincubation with 0-5 mM MbCD. Twenty 8-cell embryos were cultured for each concentration of MbCD.

In the 2-cell embryos cultured for 2 hours with 6E2, a large amount of F-actin accumulated at the interfaces, whereas no E-cadherin was detected at the interfaces, and it almost localized on the outer surface of blastomeres (Figure 5C). The entry of E-cadherin into the interfaces in living embryos was inhibited after treatment with Mab 6E2 in a dose-dependent manner (Figure 5D).

MSGb5Cer did not accumulate at the interfaces in actin-depolymerized 2-cell embryos

Actin filaments are thought to generate a mechanical force that drives membrane molecules or domains during cytokinesis. We investigated the effect of disruption of actin filaments with CCD on the localization of MSGb5Cer. As shown in Figure 6, when 2-cell stage embryos preincubated with 0, 0.2, and 2 μg/ml of CCD

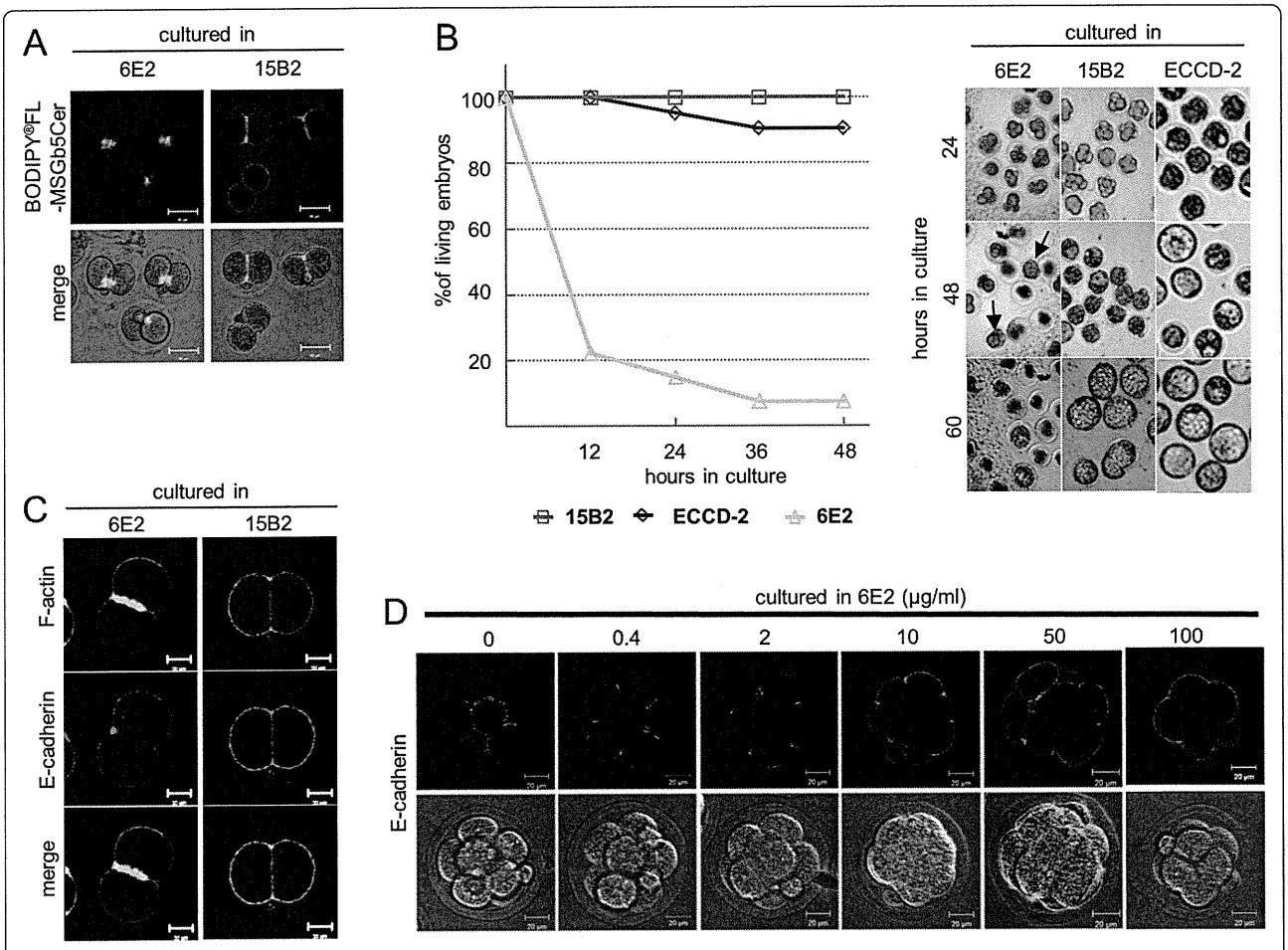

Figure 5 Extensive cross-linking of MSGb5Cer with 6E2 inhibits cytokinesis and development of mouse preimplantation embryos. (A) Deposition of BODIPY®FL-MSGb5Cer at the interfaces of 2-cell stage embryos cultured in the presence of 6E2. Embryos labeled with BODIPY®FL-MSGb5Cer (green) were cultured for 1 hour in M16 medium containing 100 μg/ml of 6E2 or 15B2 antibodies. A fluorescence 2D image of BODIPY®FL-MSGb5Cer and the overlay of a 2D image and DIC micrograph of cultured embryos are shown. These images shown are representative of twelve 2-cell embryos. (B) Effect of cross-linking of MSGb5Cer with 6E2 on cytokinesis and the development of mouse preimplantation embryos. Two-cell embryos were cultured for 60 hours in M16 medium containing 100 μg/ml of 15B2 (red squares), ECCD-2 (blue diamonds), and 6E2 (green triangles), and the living embryos were counted and their DIC images were taken at the times indicated. Arrows indicate compacted embryos. Twenty of 2-cell stage embryos were cultured for each experiment groups. (C) Localization of F-actin and E-cadherin in MSGb5Cer-cross-linked 2-cell embryos. Two-cell embryos treated for 2 hours with 6E2 (left column) or 15B2 (right column) were fixed and stained with Alexa Fluor® 633 phalloidin and anti E-cadherin/Alexa Fluor® 546-conjugated rabbit anti-rat IgG. These images shown are representative of twelve 2-cell embryos. (D) Inhibitory effect of cross-linking of MSGb5Cer with 6E2 on the localization of E-cadherin at interfaces. Embryos were immunostained with ECCD-2/Alexa Fluor® 546-conjugated Rabbit Anti-rat IgG (red) in the presence of various concentrations of 6E2. The upper row and the bottom row of each panel represent a 2D image and the overlay of a 2D image and DIC micrograph, respectively. These images shown are representative of thirty morula embryos.

were immunostained with anti-MSGb5Cer Mab 6E2, accumulation of MSGb5Cer at the interface was suppressed in a dose-dependent manner. These results suggest that inhibition of F-actin polymerization by CCD prevents the localization of MSGb5Cer at the interface.

Absence of E-cadherin causes heterotopic localization of MSGb5Cer on the blastomere surface

To investigate the involvement of E-cadherin in the localization of MSGb5Cer at the interface, we generated embryos lacking maternal E-cadherin, and examined them. In control 2-cell embryos (Genotype; Floxed/+), MSGb5Cer and E-cadherin exhibited a similar distribution pattern and accumulated at the interface (Figure 7 upper row). In E-cadherin null mutant 2-cell embryos (Genotype; Floxed del/+), adhesion between the blastomeres was weaker and the area of the interface plane was greatly reduced (Figure 7 middle and bottom row). In these embryos, MSGb5Cer formed heterotopic aggregates (indicated by the arrow) or assembled at the outer

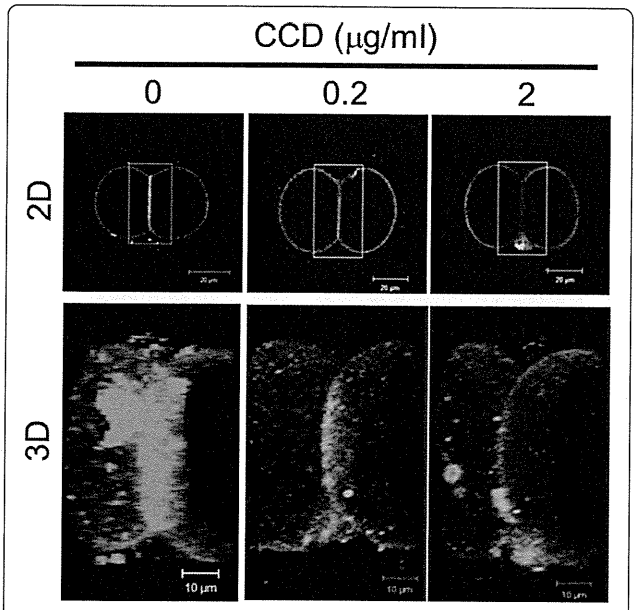

Figure 6 Disruption of actin filaments suppresses localization of MSGb5Cer at the interface. Two-cell embryos pretreated with 0, 0.2, and 2 μg/ml of CCD were immunostained with Alexa Fluor® 488-conjugated 6E2 (green). The 3D images were constructed by stacking optical slice images of the area enclosed by the square. Scale bar: 20 μm (2D), 10 μm (3D). These images shown are representative of thirty 2-cell stage embryos.

surface membranes of the blastomeres (indicated by the arrowhead). These results suggest that E-cadherin is required not for assembly, but for localization of MSGb5Cer at the interface of blastomeres.

Discussion and Conclusions

In this study, we investigated the involvement of MSGb5Cer in the development of mouse preimplantation embryos. As presented in Figure 2, MSGb5Cer moved to the future site of the cleavage furrow and accumulated at the interfaces between blastomeres as cytokinesis proceeds during embryonic development of mouse preimplantation embryos. Once, however, cytokinesis is complete the MSGb5Cer have accumulated at the interfaces evenly redistribute themselves over the cell surface (Figure 2).

MSGb5Cer is thought to be involved in the formation of raft membrane domains. Indeed, we observed overlapped localization of MSGb5Cer and cholesterol, an important constituent of lipid rafts (Figure 3). The finding that disruption of the integrity of lipid rafts by removal of cholesterol from the cell membrane with MbCD caused the release of MSGb5Cer into the perivitelline space (Figure 4) should further support above notion. Interestingly, as we presented in this study (Figure 1), GM1 and MSGb5Cer localize differently, suggesting that they may belong to different lipid rafts that possess distinct functions as described in several studies [16-18].

Since GM1 is easily detected with CTX-B, it is considered one of the most important marker GSLs for lipid rafts. Comiskey and Warner reported observation that GM1 visualized with biotinylated CTX-B were enriched at the cleavage furrow in mouse 2-cell and 4-cell embryos which actively undergo cytokinesis [10]. Burgess DR and his co-workers also reported that plasma membrane domains enriched in GM1 contain signalling machinery that contributes to cytokinesis and accumulate in the equatorial plasma membrane at mid-anaphase in sea urchin eggs [9], and further revealed that cells are polarized upon insertion of distinct basolateral membrane at the first division using the apical marker GM1 and the aPKC-PAR6 complex [19]. Although GM1 is constantly expressed throughout the cleavage stage in preimplantation embryos, MSGb5Cer is expressed only in the early cleavage stage from the unfertilized egg stage to morula stage embryos and more abundantly enriched at the cleavage furrow than GM1 during the course of cytokinesis (Figure 1). MSGb5Cer should therefore play a more specific role as a constituent of lipid rafts at this stage.

As described above, MbCD-mediated disruption of the integrity of lipid rafts caused the release of MSGb5Cer into the perivitelline space and decompaction of compacted 8-cell embryos and suppressed cell division. Comiskey and Warner also showed that cholesterol depletion by treatment of zygotes with MbCD inhibits preimplantation development from zygotes to blastocysts stage in culture in a dose-dependent manner [10]. On the other hand, heavy cross-linking of MSGb5Cer with MSGb5Cer-specific Mab induces extensive aggregation of MSGb5Cer (Figure 5) and suppressed cytokinesis. As a consequence, normal embryonic development would be terminated in both cases. Therefore, lipid rafts enriched in MSGb5Cer should play an important role in cytokinesis as well as in embryogenesis.

During cytokinesis, the cortical actin network form a scaffold for membrane proteins and thereby transfer them toward the cleavage furrow [3]. E-cadherin knockout mice display embryonic lethality and embryos are unable to form adhesion complexes [20,21]. In this study, we also presented close correlations between lipid rafts enriched in MSGb5Cer and E-cadherin as well as the cortical actin network. In preimplantation embryos, MSGb5Cer and E-cadherin are similarly distributed at the interfaces between blastomeres in 8-cell embryos, while MbCD-treatment caused the release of MSGb5Cer into the perivitelline space, decompaction of compacted embryos and even distribution of E-cadherin on the cell surface. In 2-cell embryos lacking E-cadherin, however, the MSGb5Cer-enriched lipid rafts accumulated

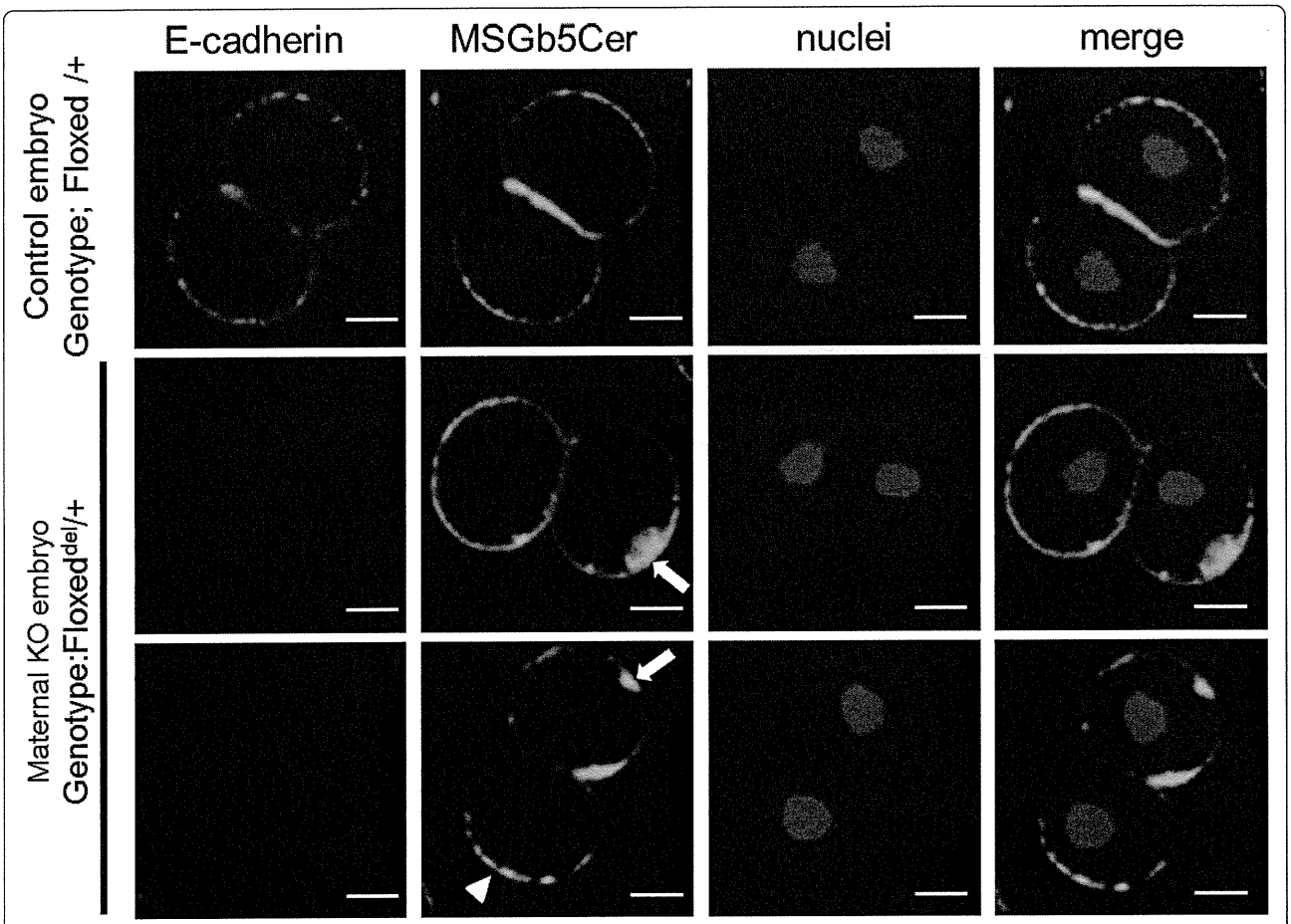

Figure 7 Absence of E-cadherin causes heterotopic localization of MSGb5Cer on the blastomere surface. Embryos expressing E-cadherin (Genotype; Floxed/+) and lacking E-cadherin (Genotype; Floxed^{del}/+) were dually immunostained with ECCD-2/Alexa Fluor® 546-conjugated Rabbit Anti-rat IgG and Alexa Fluor® 488-conjugated 6E2. 2D images of E-cadherin (red), MSGb5Cer (green) and nuclei (blue) are merged in the far right column. We used thirty maternal knockout embryos and seventeen control embryos in this experiment. MsGb5Cer represents heterotopic localization in all maternal knockout embryos.

heterotopically at sites other than the interface, indicating a close association with E-cadherin in maintaining the integrity of MSGb5Cer movement. On the other hand, disruption of polymerization of F-actin with CCD dose-dependently inhibited accumulation of MSGb5Cer at the interfaces, suggesting that the movement of MSGb5Cer-enriched lipid rafts into interfaces is driven by F-actin. During extensive aggregation of MSGb5Cer induced by cross-linking with specific antibody, all of the F-actin accumulated at the interfaces.

Although the details are still remaining to be clarified and further experiments are clearly needed, these results suggest that MSGb5Cer-enriched lipid rafts are driven into the furrow by the cortical actin network in close association with E-cadherin and play a role in cytokinesis in the early cleavage stage of mouse preimplantation embryos and our findings in this study should provide clues to the functional role of lipid rafts in early embryogenesis.

Methods

Embryo collection and culture

In all experiments, 6-8 week old female BDF1 mice purchased from CLEA Japan. Inc. (Tokyo, Japan) were induced to superovulated by intraperitoneal injections of pregnant mares' serum (ASKA Pharmaceutical Co., Ltd., Japan), and of human chorionic gonadotropin (hCG; ASKA Pharmaceutical Co., Ltd.) 48 hours later. Immediately after the hCG injection, each female mouse was mated with a male of the same strains. Zygotes and 2-cell and 8-cell embryos were collected by flushing out of the oviducts into M16 medium at 24 hours, 36 hours, and 60 hours, respectively, after the hCG injection and then cultured at 37°C in a conventional incubator. Animals were treated according to the institutional animal care and use guidelines of the National Research Institute for Child Health and Development. To obtain embryos lacking maternal E-cadherin, C57BL/6 mice were crossed as shown in Figure S2 in additional file 3

according to the procedure described by De Vries et al. [21]. The embryos did not express E-cadherin translated from paternal transcripts, at least until the 2-cell stage. Genotyping of all mice was performed by PCR using DNA extracted from ear snips of 28-day-old mice with an Automatic DNA Extraction System (NA-3000, KURABO, Tokyo, Japan). The following primer pair specific for cre recombinase was used to determine whether the Zp3-cre transgene was present: Cre1 (5'-ATG CCC AAG AAG AAG AGG AAG GT-3'), Cre2 (5'-GAA ATC AGT GCG TTC GAA CGC TAG A-3'). The primer pairs used to detect the different alleles of E-cadherin were as described previously by Boussadia et al. [22].

Antibodies and chemicals

Alexa Fluor® 488-conjugated 6E2, used for staining MSGb5Cer, was prepared as previously described [11]. Anti-mouse E-cadherin Mab ECCD-2 was purchased from TAKARA Bio. Co. (Tokyo Japan). Mab 15B2 was a generous gift of Dr. Taketo Yamada of Keio University School of Medicine [23]. Alexa Fluor® 633 phalloidin and Alexa Fluor® 546 CTX-B, used for staining actin and GM1, respectively, were purchased from Invitrogen. Alexa Fluor® 546 Rabbit Anti-rat IgG and Alexa Fluor® 546 streptavidin were also purchased from Invitrogen. Biotinylated-CTX-B of Sigma-Aldrich Inc. (St. Louis. MO) was also used. Filipin III, used for staining cholesterol, was purchased from Cayman Chem. Co. (Ann Arbor, MI). MbCD and CCD were obtained from Sigma and Calbiochem, respectively. GM1 and a monosialylganglioside mixture were purchased from Matreya Inc. (Pleasant Gap, PA).

Immunostaining of mouse preimplantation embryos

Immunostaining of "living" mouse preimplantation embryos was performed as previously described [11]. Briefly, cells were incubated in 30 μl drops of M16 medium containing 0.45 μg of Alexa Fluor® 488 or 546 -conjugated 6E2 (final concentration 15 μg/ml), and then they were washed three times in 30 μl drops of M16 medium. All staining steps were carried out at 37°C in a CO_2 incubator for fresh embryos. Cell nuclei were stained with DAPI (Invitrogen), which slowly permeates the living cell membrane and slowly leaks out after washing [24]. For actin and cholesterol staining, embryos were prefixed for 10 minutes at room temperature with 2% paraformaldehyde containing 0.1% glutaraldehyde in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline (HBS), then permeabilized with 0.01% Triton X-100 in HBS for 10 minutes at room temperature, and blocked with 3% bovine serum albumin. Staining of living embryos and fixed embryos was performed at 37°C and 4°C, respectively. The stained embryos were placed in a microdrop of M16 medium on a glass-bottom dish (IWAKI Glass, Tokyo, Japan), covered with liquid paraffin (NAKALAI TESQUE, Kyoto, Japan), and examined with a LSM510 Zeiss Confocal laser-scanning microscope (Carl Zeiss, Thornwood, NY) and a 40× objective lens so that only the embryo was included in the field of view. For three dimensional (3D) construction, two-dimensional (2D) images were captured as vertical sections (at approximately 2-μm intervals) by using a Z-axis motor, then processed with Zeiss Zen 2009 software (Carl Zeiss), and finally stacked into one picture.

Preparation of BODIPY®FL-MSGb5Cer

MSGb5Cer was purified from human renal cancer cell line ACHN cells by ion-exchange chromatography on DEAE-Sephadex A25 and preparative TLC/TLC blotting according to Taki et al. MSGb5Cer was conjugated to fluorescence reagents with Sphingolipid Ceramide N-deacylase (SCDase) according to the procedure described in the previous report [25]. To prepare lyso-MSGb5Cer, MSGb5Cer was incubated at 37°C for 16 hours with 8 μU of SCDase in 20 μl of 25 mM sodium acetate buffer, pH5.5, containing 0.2% Triton X-100 and 5 mM $CaCl_2$, and the reaction mixture was adsorbed onto an Oasis® MCX cartridge (Waters). The lyso-MSGb5Cer eluted from the cartridge with 5% ammonium hydroxide in methanol was subsequently incubated with BODIPY®FL-C12 (Invitrogen) in 20 μl of the condensation reaction mixture (8 mU SCDase, 25 mM Tris-HCl buffer, pH7.5, 5 mM $MgCl_2$, 0.1% Triton X-100) at 37°C for 16 hours. The reaction mixture was adsorbed to an Oasis MCX cartridge. The methanol eluate was dried, desalted with a DISCOVERY DC-18 cartridge (SUPELCO), and adsorbed to a DISCOVERY DSC-Si cartridge (SUPELCO). The condensed BODIPY®FL-MSGb5Cer product eluted from the cartridge with methanol was analyzed by high performance thin layer chromatography (HPTLC) by using chloroform/methanol/0.02% $CaCl_2$ (5:4:1, v/v) as the developing solvent, and it was viewed by using FLA-7000 (Fuji Film, Tokyo, Japan) (see Figure S3 in additional file 4). BODIPY®FL-GM1 was also prepared in the same manner as described above. MSGb5Cer and GM1 enzymatically conjugated with BODIPY®FL-C12 -fatty acid in ethanol were dissolved in 3.4% non-fatted BSA in Hank's balanced solution and diluted in M16 medium. Two-cell stage embryos were incubated in a drop of the medium for 20 minutes and examined.

Time-lapse imaging

MSGb5Cer was stained by incubating the zygote at 22-26 hours after the hCG injection for 20 minutes in a conventional incubator with Alexa Fluor® 488-conjugated 6E2 Mab and DAPI dissolved in M16 medium. Stained embryos were transferred to a microdrop of M16 medium on a glass-bottom dish covered with

liquid paraffin. During time-laps imaging, samples were placed in a 37°C stage-top incubator (INUBG2-PPZI, Tokai Hit, Japan) with a slow flow of air with 5% CO2 gas and high humidity. An objective heater (INUBG2-PPZI, Tokai Hit, Japan) was also used to maintain the objective temperature at 37°C to reduce temperature gradients within the sample. Time-lapse fluorescence images were taken at 15-minute intervals with a spinning disk confocal scanhead (Yokogawa) attached to an inverted fluorescence microscope (OLYMPUS). Images were captured as vertical sections (approximately 2-μm intervals) by using a Z-axis motor and processed by a deconvolution program using iQ software (Andor). Fluorescence intensity was measured with ImageJ software http://rsb.info.nih.gov/ij/.

Pretreatment of embryos with MbCD

A stock solution of MbCD was prepared in sterile water at 100 mM and diluted with M16 medium. Compacted embryos were preincubated with 0, 0.5, 2.5, 5, and 10 mM MbCD for 15 minutes, and then cultured for 48 hours. The embryos after preincubation with MbCD were immunostained with Alexa Fluor® 488-conjugated 6E2, Alexa Fluor® 546 CTX-B, and ECCD-2/Alexa Fluor® 546 conjugated-rabbit anti-rat IgG to examine the distribution of MSGb5Cer, GM1, and E-cadherin, respectively.

Embryo culture in the presence of anti-MSGb5Cer Mab

Two-cell stage embryos were cultured for 48 hours in M16 medium containing 100 μg/ml of anti-MSGb5Cer Mab 6E2, ECCD-2, or 15B2. ECCD-2 was used as a control Mab that binds to mouse embryos. 15B2 was used as a control Mab subclass-matched to 6E2 that do not binds to mouse embryos. The aliquots of embryos after culture for 2 hours in the presence of 6E2 or 15B2 were fixed and immunostained with Alexa Fluor® 633 phalloidin and ECCD-2/Alexa Fluor® 546 Rabbit Anti-rat IgG to examine the distribution of F-actin and E-cadherin, respectively.

Pretreatment of embryos with CCD

A stock solution of CCD was prepared in DMSO at 2 mg/ml and diluted with M16 medium. Two-cell stage embryos were preincubated for 30 minutes at 37°C with 0, 0.2, and 2 μg/ml of CCD in a CO_2 incubator. After washing with M16 medium, the embryos were immunostained for 1 hour with Alexa Fluor® 488-conjugated 6E2.

Additional material

Additional file 1: MSGb5Cer accumulates at the interface during cytokinesis. 6E2 stained cells were imaged by confocal microscopy during mitosis. Frames were taken every 15 minutes and are displayed at 2 frames per second.

Additional file 2: The data of blastocysts culture in the presence of 100 μg/ml of 6E2.

Additional file 3: Mating scheme used to generate embryos lacking E-cadherin.

Additional file 4: HPTLC showing the preparation of BODIPY®FL-GM1 and -MSGb5Cer.

List of abbreviations

GSL: Glycosphingolipid; MSGb5Cer: MonosialylGb5ceramide; MbCD: Methyl-beta-cyclodextrin; CCD: Cytochalasin D; Mab: Monoclonal antibody; SSEA-4: Stage-specific embryonic antigen-4; CTX-B: Cholera toxin B subunit; hCG: human chorionic gonadotropin; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBS: HEPES buffered saline; HPTLC: high performance thin layer chromatography; SCDase: Sphingolipid Ceramide N-deacylase; DIC: differential interference contrast.

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Author details

[1]Department of Pediatric Hematology and Oncology Research, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan. [2]Department of Reproductive Biology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan. [3]Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan. [4]Department of Anatomy and Developmental Biology, Inohana 1-8-1, Chuo-ku, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan.

Authors' contributions

BS designed and carried out most of experiments and drafted the manuscript. YUK, KT and NK designed the experiments and edited the manuscript. KM, HA, JF and AU helped conceive the experiments and commented on the manuscripts. NO and MI helped in the preparation of fluorescence labeled-glycosphingolipid. JF and NK obtained funding. All authors read and approved the final manuscripts.

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References

1. Albertson R, Riggs B, Sullivan W: **Membrane traffic: a driving force in cytokinesis.** *Trends Cell Biol* 2005, **15(2)**:92-101.
2. Burgess DR: **Cytokinesis and the establishment of early embryonic cell polarity.** *Biochem Soc Trans* 2008, **36(Pt 3)**:384-386.
3. Bauer T, Motosugi N, Miura K, Sabe H, Hiiragi T: **Dynamic rearrangement of surface proteins is essential for cytokinesis.** *Genesis* 2008, **46(3)**:152-162.
4. Vestweber D, Gossler A, Boller K, Kemler R: **Expression and distribution of cell adhesion molecule uvomorulin in mouse preimplantation embryos.** *Dev Biol* 1987, **124(2)**:451-456.
5. Fleming TP, Javed Q, Hay M: **Epithelial differentiation and intercellular junction formation in the mouse early embryo.** *Dev Suppl* 1992, 105-112.
6. Fleming TP, Sheth B, Fesenko I: **Cell adhesion in the preimplantation mammalian embryo and its role in trophectoderm differentiation and blastocyst morphogenesis.** *Front Biosci* 2001, **6**:D1000-7.
7. Simons K, Ikonen E: **Functional rafts in cell membranes.** *Nature* 1997, **387(6633)**:569-572.
8. Lingwood D, Simons K: **Lipid rafts as a membrane-organizing principle.** *Science* 2010, **327(5961)**:46-50.

9. Ng MM, Chang F, Burgess DR: **Movement of membrane domains and requirement of membrane signaling molecules for cytokinesis.** *Dev Cell* 2005, **9(6)**:781-790.
10. Comiskey M, Warner CM: **Spatio-temporal localization of membrane lipid rafts in mouse oocytes and cleaving preimplantation embryos.** *Dev Biol* 2007, **303(2)**:727-739.
11. Sato B, Katagiri YU, Miyado K, Akutsu H, Miyagawa Y, Horiuchi Y, Nakajima H, Okita H, Umezawa A, Hata J, Fujimoto J, Toshimori K, Kiyokawa N: **Preferential localization of SSEA-4 in interfaces between blastomeres of mouse preimplantaion embryos.** *Biochem Biophys Res Commun* 2007, **364(4)**:838-843.
12. Van Slambrouck S, Hilkens J, Steelant WF: **Ether lipid 1-O-octadecyl-2-O-methyl-3-glycero-phosphocholine inhibits cell-cell adhesion through translocation and clustering of E-cadherin and episialin in membrane microdomains.** *Oncol Rep* 2008, **19(1)**:123-128.
13. Ilangumaran S, Hoessli DC: **Effects of cholesterol depletion by cyclodextrin on the sphingolipid microdomains of the plasma membrane.** *Biochem J* 1998, **335(Pt 2)**:433.
14. Brown DA: **Isolation and use of rafts.** *Curr Protoc Immunol* 2002, **Chapter 11**:Unit 11.10.
15. Keller P, Simons K: **Cholesterol is required for surface transport of influenza virus hemagglutinin.** *J Cell Biol* 1998, **140(6)**:1357-1367.
16. Pike LJ: **Lipid rafts: heterogeneity on the high seas.** *Biochem J* 2004, **378(Pt 2)**:281-292.
17. Katsumata O, Kimura T, Nagatsuka Y, Hirabayashi Y, Sugiya H, Furuyama S, Yanagishita M, Hara-Yokoyama M: **Charge-based separation of detergent-resistant membranes of mouse splenic B cells.** *Biochem Biophys Res Commun* 2004, **319(3)**:826-831.
18. Lingwood D, Kaiser HJ, Levental I, Simons K: **Lipid rafts as functional heterogeneity in cell membranes.** *Biochem Soc Trans* 2009, **37(Pt 5)**:955-960.
19. Alford LM, Ng MM, Burgess DR: **Cell polarity emerges at first cleavage in sea urchin embryos.** *Dev Biol* 2009, **330(1)**:12-20.
20. Riethmacher D, Brinkmann V, Birchmeier C: **A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development.** *Proc Natl Acad Sci USA* 1995, **92(3)**:855-859.
21. De Vries WN, Evsikov AV, Haac BE, Fancher KS, Holbrook AE, Kemler R, Solter D, Knowles BB: **Maternal beta-catenin and E-cadherin in mouse development.** *Development* 2004, **131(18)**:4435-4445.
22. Boussadia O, Kutsch S, Hierholzer A, Delmas V, Kemler R: **E-cadherin is a survival factor for the lactating mouse mammary gland.** *Mech Dev* 2002, **115(1-2)**:53-62.
23. Nakano T, Umezawa A, Abe H, Suzuki N, Yamada T, Nozawa S, Hata J: **A monoclonal antibody that specifically reacts with human embryonal carcinomas, spermatogonia and oocytes is able to induce human EC cell death.** *Differentiation* 1995, **58(3)**:233-240.
24. Miyado K, Yoshida K, Yamagata K, Sakakibara K, Okabe M, Wang X, Miyamoto K, Akutsu H, Kondo T, Takahashi Y, Ban T, Ito C, Toshimori K, Nakamura A, Ito M, Miyado M, Mekada E, Umezawa A: **The fusing ability of sperm is bestowed by CD9-containing vesicles released from eggs in mice.** *Proc Natl Acad Sci USA* 2008, **105(35)**:12921-12926.
25. Furusato M, Sueyoshi N, Mitsutake S, Sakaguchi K, Kita K, Okino N, Ichinose S, Omori A, Ito M: **Molecular cloning and characterization of sphingolipid ceramide N-deacylase from a marine bacterium, Shewanella alga G8.** *J Biol Chem* 2002, **277(19)**:17300-17307.

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Differentiation of mesenchymal stem cells and embryonic stem cells into steroidogenic cells using steroidogenic factor-1 and liver receptor homolog-1☆

Takashi Yazawa[a,*], Shinya Kawabe[a], Yoshihiko Inaoka[a], Reiko Okada[a], Tetsuya Mizutani[a], Yoshitaka Imamichi[a], Yunfeng Ju[a], Yukiko Yamazaki[a], Yoko Usami[a], Mayu Kuribayashi[a], Akihiro Umezawa[b], Kaoru Miyamoto[a]

[a] Department of Biochemistry, Faculty of Medical Sciences, University of Fukui, Shimoaizuki 23, Matsuoka, Eiheiji-cho, Fukui 910-1193, Japan
[b] National Research Institute for Child Health and Development, Tokyo 157-8535, Japan

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ABSTRACT

Previously, we have demonstrated that mesenchymal stem cells could be differentiated into steroidogenic cells through steroidogenic factor-1 and 8bromo-cAMP treatment. Use of liver receptor homolog-1, another of the nuclear receptor 5A family nuclear receptors, with 8bromo-cAMP also resulted in the differentiation of human mesenchymal stem cells into steroid hormone-producing cells. The same approaches could not be applied to other undifferentiated cells such as embryonic stem cells or embryonal carcinoma cells, because the over-expression of the nuclear receptor 5A family is cytotoxic to these cells. We established embryonic stem cells carrying tetracycline-regulated steroidogenic factor-1 gene at the *ROSA26* locus. The embryonic stem cells were first differentiated into a mesenchymal cell lineage by culturing on collagen IV-coated dishes and treating with pulse exposures of retinoic acid before expression of steroidogenic factor-1. Although the untreated embryonic stem cells could not be converted into steroidogenic cells by expression of steroidogenic factor-1 in the absence of leukemia inhibitory factor due to inability of the cells to survive, the differentiated cells could be successfully converted into steroidogenic cells when expression of steroidogenic factor-1 was induced. They exhibited characteristics of adrenocortical-like cells and produced a large amount of corticosterone. These results indicated that pluripotent stem cells could be differentiated into steroidogenic cells by the nuclear receptor 5A family of protein *via* the mesenchymal cell lineage. This approach may provide a source of cells for future gene therapy for diseases caused by steroidogenesis deficiencies.

1. Introduction

Steroidogenic factor-1 (SF-1), also known as Ad4BP, and liver receptor homolog-1 (LRH-1) belong to the NR5A subfamily of nuclear receptors (Krylova et al., 2005). SF-1 is essential for adrenal and gonadal development, and SF-1 knockout mice exhibit adrenal and gonadal agenesis and impaired gonadotropin expression, resulting in postnatal death due to severe adrenal insufficiency (Luo et al., 1994; Sadovsky et al., 1995). SF-1 is expressed in the adrenal cortex, testicular Leydig and Sertoli cells, ovarian theca and granulosa cells, pituitary gonadotropes and hypothalamus (Parker and Schimmer, 1997; Morohashi, 1999; Schimmer and White, 2010). It regulates the cell-specific expression of a variety of different genes involved in steroidogenesis, including a number of steroid hydroxylases (Lala et al., 1992; Morohashi et al., 1992). With the aid of cAMP, it can induce the differentiation of bone marrow-derived mesenchymal stem cells (MSCs) into steroidogenic cells such as testicular Leydig cells and adrenocortical cells (Yazawa et al., 2006, 2008). However, the same approaches were inappropriate for other undifferentiated cells such as embryonic stem (ES) cells or embryonal carcinomas, because they barely survived after expression of SF-1. LRH-1 is mainly expressed in tissues of endodermal origin in adults (Fayard et al., 2004; Lee and Moore, 2008). Recently, elevated expression of LRH-1 has been demonstrated in gonads, suggesting the involvement of LRH-1 in steroidogenesis (Volle et al., 2007; Duggavathi et al., 2008).

In this study, we showed the differentiation of steroidogenic cells from MSCs and ES cells by SF-1 and LRH-1. Treatment with LRH-1 and 8br-cAMP resulted in the differentiation of human MSCs (hMSCs) into steroidogenic cells, with similar results exhibited using SF-1. This method could not be applied to ES cells. In this

Abbreviations: SF-1, steroidogenic factor-1; LRH-1, liver receptor homolog-1; MSC, mesenchymal stem cell; ES cells, embryonic stem cells; Tc, tetracycline; RA, retinoic acid; Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

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* Corresponding author. Tel.: +81 776 61 8316; fax: +81 776 61 8102.
E-mail address: yazawa@u-fukui.ac.jp (T. Yazawa).

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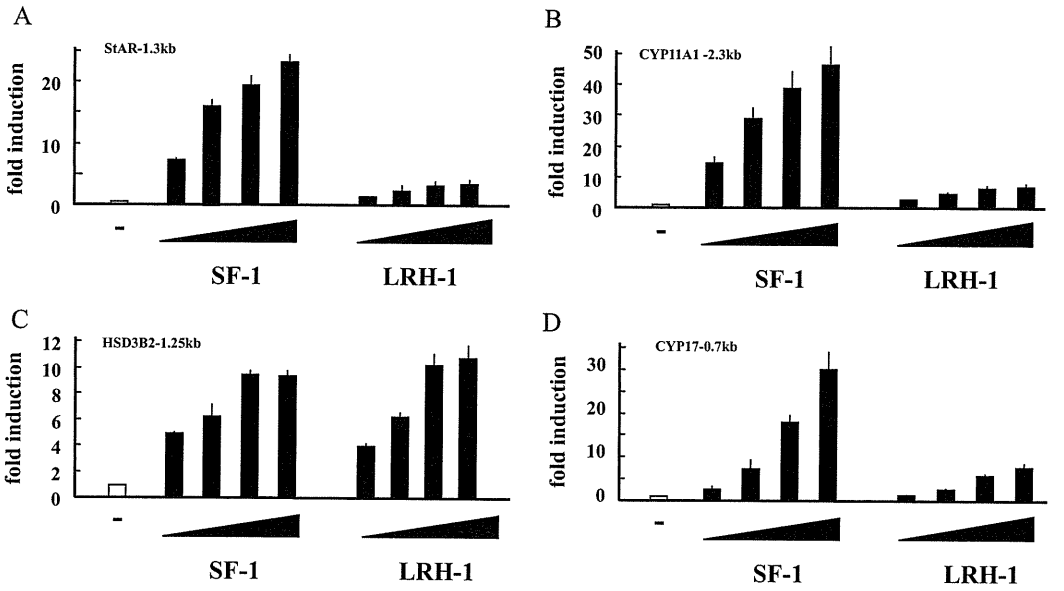


Fig. 1. Activation of the promoter activities of steroidogenic genes by SF-1 and LRH-1 (0, 1.25, 2.5, 5 ng). HEK293 cells were transiently transfected with each reporter and with 0, 1.25, 2.5, 5 ng of expression vector as indicated. Luciferase activities were measured after 48 h and relative activities are shown. Data are expressed as the mean ± SEM of at least four independent experiments.

study we developed a method for differentiation of ES cells into steroidogenic cells using a tetracycline (Tc)-regulated gene expression system for SF-1 (Masui et al., 2005).

2. Materials and methods

2.1. Cell culture, transfection and luciferase assays

The hMSCs (Okamoto et al., 2002; Mori et al., 2005), HEK293 and Phoenix cells were cultured in DMEM with 10% fetal calf serum (FCS). HEK293 cells were transfected using Lipofectamine plus (Invitrogen, Carlsbad, CA, USA). Luciferase assays were performed as described previously (Yazawa et al., 2003). Each data point represents the mean of at least four independent experiments. The murine ES cells, EBRTcH3, were cultured as described before (Masui et al., 2005). Briefly, they were cultured in the absence of feeder cells in Glasgow minimal essential medium (GMEM; Sigma–Aldrich; St. Louis, MO, USA) supplemented with 10% FCS, 1 mM sodium pyruvate (Invitrogen), 10^{-4} M 2-mercaptoethanol (Nacalai Tesque; Kyoto, Japan), 1× nonessential amino acids (Invitrogen) and 1000 U/ml of leukemia inhibitory factor (LIF) on gelatin-coated dishes. Culture methods for the induction of MSCs have been described elsewhere (Takashima et al., 2007). Culture media were collected for the measurement of steroid hormone production by enzyme-linked immunosorbent (ELISA) assays (Cayman Chemical Co., Ann Arbor, MI, USA) as described previously (Yazawa et al., 2008, 2009, 2010).

2.2. Plasmids

The pGL2-StAR1.3kb vector was kindly provided by Dr. Teruo Sugawara (Hokkaido University Graduate School of Medicine, Sapporo, Japan). To introduce the *SF-1* gene into *ROSA-TET* locus, the exchange vector was created by the insertion of the *XhoI-NotI* fragment of human SF-1 cDNA into pPthC (Masui et al., 2005), which had been cleaved by *XhoI–NotI*. Exchange vector is necessary for introduction of genes of interest into mouse genomic *ROSA-TET* locus by homologous recombination. The pCAGGS-Cre plasmid has been described elsewhere (Araki et al., 1997). Each vector containing the entire coding region for SF-1 and LRH-1 was generated by RT-PCR and subcloned into pQCXIP (Clontech, Palo Alto, CA, supplied by Takara Bio Inc., Shiga, Japan). The other vectors have been described before (Yazawa et al., 2009, 2010).

2.3. RT-PCR

Total RNA from the cultured cells was extracted using Trizol reagent (Invitrogen). RT-PCR was performed as described previously (Yazawa et al., 2006). The RT-PCR products were subjected to electrophoresis on 1.5% (w/v) agarose gels, and the resulting bands were visualized by staining with ethidium bromide. The primers used have been described previously (Yazawa et al., 2006, 2009; Takashima et al., 2007).

2.4. Retrovirus preparation and infection

The Phoenix packaging cell line was transiently transfected with the retroviral plasmids using the Lipofectamine Plus reagent (Invitrogen). The supernatant was concentrated by centrifugation and the virus solution stored at −80 °C until required. MSCs were infected with the retrovirus in the presence of 8 μg/ml polybrene (Sigma) for 48 h. The cells were then replated and selected using puromycin.

2.5. Exchange reaction of the Tc-regulated unit

EBRTcH3 cells were seeded onto gelatin-coated 6-well plates in medium containing 1 μg/ml Tc (Tc^+ medium; Sigma–Aldrich). The circular plasmid DNA of the exchange vector, pCAGGS-Cre and Lipofectamine 2000 (Invitrogen) was separately mixed with GMEM and combined to make the transfection mixture. This was added and incubated for 3–5 h, and then re-plated onto 10 cm dishes containing Tc^+ medium. After two days, the medium was changed to Tc^+ medium with 1.5 μg/ml puromycin (Sigma).

2.6. Western blot analysis

The extraction of protein from cultured cells and subsequent quantification was performed as described previously (Yazawa et al., 2003, 2008). Equal amounts of protein (50 μg) were analyzed by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Western blot analyses of SF-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were carried out with antiserum directed against Ad4BP (kindly provided by Dr. Ken-ichirou Morohashi, University of Kyushu, Fukuoka, Japan) and GAPDH (6C5; Santa Cruz Biotechnology, Santa Cruz, CA). ECL Western blot reagents (Amersham Pharmacia Biotech, Piscataway, NJ) were used to visualize protein bands.

3. Results

3.1. Differentiation of MSCs into steroidogenic cells using SF-1 and LRH-1

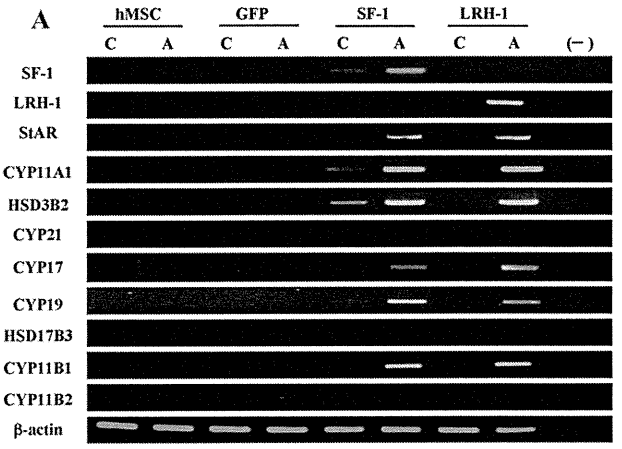
We investigated the effects of SF-1 and LRH-1 on activation of steroidogenesis-related gene promoters in HEK293 cells (Fig. 1). Consistent with many previous reports, SF-1 activated the promoters of steroidogenesis-related genes, such as StAR, CYP11A1, HSD3B2 and CYP17, in a dose-dependent manner. LRH-1 could also activate these promoters, although the extent was much lower than that of SF-1 except in the case of HSD3B2.

To examine abilities of SF-1 and LRH-1 to induce the differentiation of hMSCs into steroidogenic cells, hMSCs were transduced with SF-1 or LRH-1 by retrovirus-mediated transfection. Trans-

duction of SF-1 into hMSCs induced expression of the type II 3β-hydroxysteroid dehydrogenase (HSD3B2) gene (Fig. 2A), with these cells also producing progesterone (Fig. 2B). As we reported previously (Yazawa et al., 2006), 8br-cAMP treatment further induced various steroidogenic enzymes. Concomitantly, 8br-cAMP treatment markedly increased the production of progesterone. Similar results were obtained in LRH-1-transduced hMSCs. Expression of SF-1 was never induced in LRH-1-transduced cells, and *vice versa*. These results demonstrate that LRH-1 had similar potential as SF-1 for the induction of MSC differentiation into steroidogenic cells.

3.2. Differentiation of ES cells into steroidogenic cells using tetracycline

Our studies clearly indicate that the NR5A family can direct the differentiation of stem cells into steroidogenic cells. However, this approach was not appropriate for pluripotent stem cells such as ES cells (Yazawa et al., 2006), as they barely survive the expression of the NR5A family in the absence of LIF. To circumvent these problems, we used the ES cell line EBRTcH3, carrying a Tc-repressible transgene at the *ROSA26* locus (Masui et al., 2005). The SF-1 cDNA along with a gene encoding the yellow fluorescent protein, Venus, was integrated into the *ROSA-TET* locus by a knock-in method, and puromycin resistant clones were selected (Fig. 3A). Induction of the genes from the *ROSA-TET* locus was checked by RT-PCR and fluorescence of the reporter protein, Venus. Withdrawal of Tc from the culture medium resulted in the induction of Venus fluorescence in virtually all cells within 48 h (Fig. 3B), whereas no fluorescence was detected in cells cultured in the presence of Tc. Although SF-1 mRNA and proteins were also induced in ES cells in



B

Cells	8BrcAMP(-)	8BrcAMP(+) (fm/cell/hour)
GFP-hMSC	N.D.	N.D.
SF-1-hMSC	0.04±0.01	1.17±0.26
LRH-1-hMSC	0.07±0.02	1.31±0.17

Fig. 2. Differentiation of human BM-MSCs into steroidogenic cells by SF-1 and LRH-1. (A) RT-PCR analysis of each gene in each clone cultured with (lane A) or without (lane C) 8-br-cAMP for two days. (B) Production of progesterone by BM-MSCs stably expressing GFP, SF-1 or LRH-1 in the presence (+) or absence (−) of 8br-cAMP (1 mM). Means and SEM values of at least three independent experiments. N.D. indicates no detectable values.

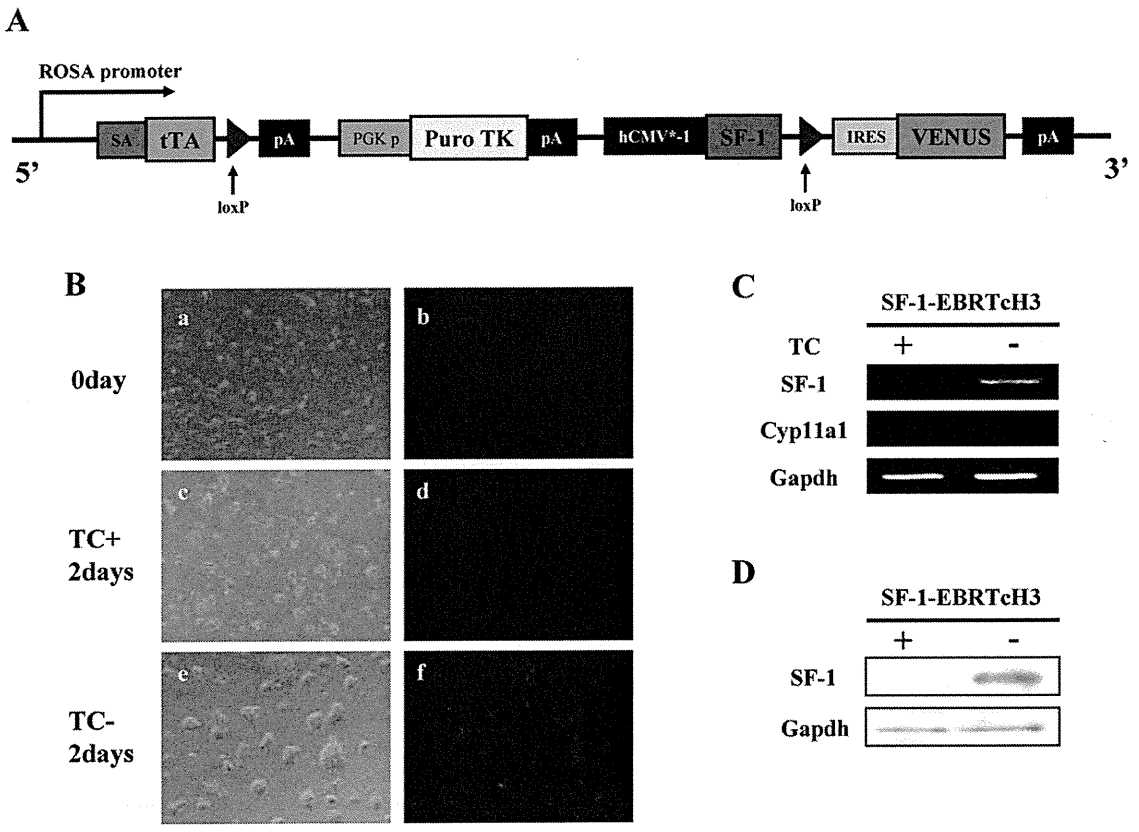


Fig. 3. Induction of SF-1 expression in the *ROSA-TET* system. (A) Schematic representation of the *ROSA-TET* locus exchanged with the SF-1-expression cassette in EBRTcH3 cells. (B) Induction of Venus fluorescence. Cells were observed before (panels a and b) or 48 h after induction (panels c–f) cultured with or without Tc. Bright field (panels a, c and e) and fluorescence images (panels b, d and f) are presented. (C and D) Induction of SF-1 expression in SF-1-EBRTcH3 cells. The mRNA (C) and protein (D) samples were prepared 48 h after the removal of Tc.

the absence of Tc, the cells were maintained in the undifferentiated state and never expressed any steroidogenic marker genes, including Cyp11a1 (Fig. 3Cand D). On the other hand, ES cells ceased to proliferate and died after several days when LIF was removed from culture medium. These results were in agreement with our previous observations in which steroidogenic cells could not be induced directly from ES cells (Yazawa et al., 2006).

We induced the expression of SF-1 after differentiation of the ES cells into MSCs. For differentiation of ES cells into MSCs, they were cultured on collagen IV-coated dishes and treated with pulse exposures to RA as described by Nishikawa and colleagues (Takashima et al., 2007) (Fig. 4A). Consistent with previous reports, molecular markers of the mesenchymal cell lineage such as PDGFRα, PDGFRβ and OB-CAD were robustly induced by RA treatment (Fig. 4B), indicating that the ES cells were successfully differentiated into mesenchymal cells. The cells were further cultured in the absence of RA and Tc for three days. In contrast to undifferentiated ES cells, the differentiated cells were able to survive following SF-1 expression in the absence of LIF.

As shown in Fig. 5A, expression of SF-1 in the differentiated cells resulted in the expression of various steroidogenesis-related genes, such as Cyp11a1, Hsd3b1, Cyp17, Cyp21 Cyp11b1 and Acthr. The gene expression pattern was quite similar to that in adrenocortical cells, especially fasciculata cells. Consistent with the gene expression profile, corticosterone was the most secreted steroid hormone from these cells (Fig. 5B), with Cyp17 expression barely detectable in the adult murine adrenal gland. Cortisol was also produced in these cells, although it was markedly lesser than corticosterone. These results indicate that ES cells could also be differentiated into steroidogenic cells by SF-1 *via* the mesenchymal cell lineage.

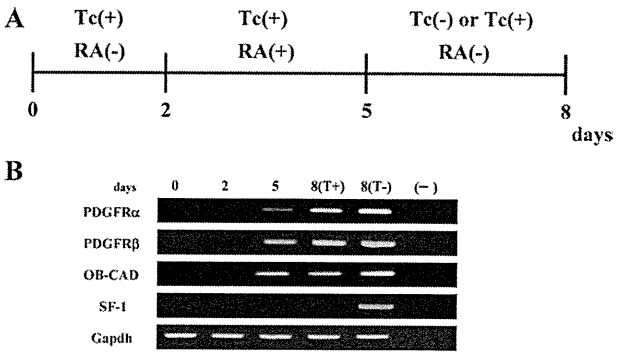
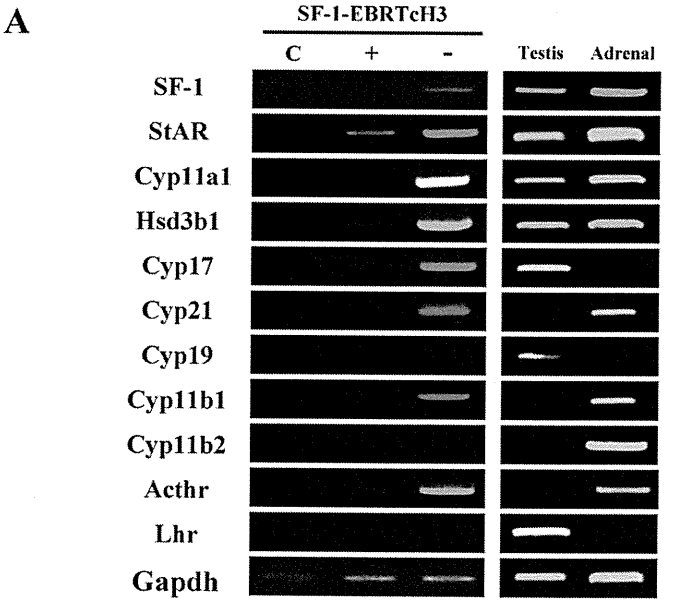


Fig. 4. Protocols for inducing steroidogenic cells from ES cells *via* the differentiation of ES cells into MSCs. Cells were cultured on collagen IV-coated dishes with differentiation medium containing Tc, with 10^{-7} M RA added to the medium from days 2 to 5. The medium was replaced with a RA- and Tc-free medium on day five. (B) RT-PCR analysis of marker genes for the MSC lineage and SF-1 genes at each time point.

4. Discussion

SF-1 has been clearly demonstrated to be a master regulator of steroidogenic organs. SF-1 knockout mice show agenesis of the primary steroidogenic organs, including the adrenal glands and gonads (Parker and Schimmer, 1997; Morohashi, 1999). SF-1 can induce the differentiation of MSCs into steroidogenic cells (Yazawa et al., 2006, 2008). In this study, we demonstrated that LRH-1 also has the capability to differentiate MSCs into steroidogenic cells. Consistent with our results, it has been reported that LRH-1 and SF-



B

Cells (Tet 1μg/ml)	progesterone	testosterone	estradiol	corticosterone	aldosterone (ng/ml)
SF-1-EBRTcH3(+)	N.D.	N.D.	N.D.	N.D.	N.D.
SF-1-EBRTcH3-MSCs(+)	N.D.	N.D.	N.D.	N.D.	N.D.
SF-1-EBRTcH3-MSCs(-)	638.2±65.98	65.1±5.33	0.05±0.04	896.7±90.25	12.4±2.19

1. N.D. means for no detectable values.
2. Data are means and SEM values of at least two independent experiments.

Fig. 5. Differentiation of ES cells into adrenocortical-like cells. (A) RT-PCR analysis of each gene in SF-1 induced (−) and uninduced (+) cells. Testis and adrenal represent a testis and an adrenal gland from an adult mouse. (B) Production of steroid hormones by each treatment. Means and SEM values of at least two independent experiments. N.D. means no detectable values.

1 could play similar roles in steroidogenesis in certain cells (Wang et al., 2001; Saxena et al., 2007; Yazawa et al., 2010). On the other hand, differentiated cells such as HEK293 cells stably transformed with SF-1 or LRH-1 did not express steroidogenic enzymes nor did they produce steroid hormones, suggesting that the expression of these genes in situ are controlled by additional factors.

LRH-1 is abundantly expressed in ES cells and is necessary for Oct-3/4 expression (an essential gene for maintenance of the inner cell mass and pluripotency of ES cells) at the epiblast stage (Gu et al., 2005). In addition, Oct-3/4 can be replaced by LRH-1 for the reprogramming of murine somatic cells into induced pluripotent stem (iPS) cells (Heng et al., 2010). As the differentiation of MSCs into steroidogenic cells, SF-1 and LRH-1 have similar potential for the regulation of Oct-3/4 expression (Barnea and Bergman, 2000; Gu et al., 2005), and therefore the same potential for induction of somatic cells into iPS cells (Heng et al., 2010). Niwa et al. (2000) demonstrated that quantitative expression of Oct-3/4 defines the fate of ES cells. A less than twofold increase in Oct-3/4 expression causes differentiation of ES cells into primitive endoderm and mesoderm, whereas repression of Oct-3/4 expression induces loss of pluripotency and causes de-differentiation of cells into the trophectoderm. It has been shown that DAX-1, a common transcriptional inhibitor of Oct-3/4, SF-1 and LRH-1 are also important for the pluripotency and survival of ES cells (Yu et al., 1998; Sun et al., 2009; Khalfallah et al., 2009). DAX-1 expression is detectable in ES cells and its expression is reduced upon differentiation of the cells into each germ layer. DAX-1 knockdown induces loss of pluripotency even under culture conditions for maintaining the undifferentiated state (Sun et al., 2009; Khalfallah et al., 2009), whereas complete deletion of DAX-1 by gene targeting results in cell death (Yu et al., 1998). Over-expression of the NR5A family was also cytotoxic to ES cells. These facts strongly suggest that regulated and coordinated expression of NR5A genes is essential for the pluripotency and survival of ES cells. These properties of the NR5A family are likely to cause difficulties in the induction of steroidogenic cells by NR5A members directly from ES cells.

In a previous study (Crawford et al., 1997), stable expression of SF-1 was shown to direct ES cells towards the steroidogenic lineage. However, the steroidogenic capacity of the cells was limited since a membrane-permeable substrate, 20α-hydroxycholesterol, was necessary to produce progesterone, the only steroid produced from the cells (Crawford et al., 1997). We demonstrated that regulated expression of SF-1 by the *ROSA-TET* system made it possible to derive steroidogenic cells from ES cells, with a capacity for autonomously secreting various steroid hormones.

It was reported that ES cells cultured on collagen-IV coated plates and treated with RA undergo differentiation into the mesenchymal cell lineage including MSCs, and that steroidogenic cells could be induced by SF-1 from MSCs (Takashima et al., 2007; Yazawa et al., 2006, 2008). Although we did not fully characterize the differentiated cells derived from the ES cells by RA treatment in this study, it is conceivable that the ES cell-derived steroidogenic cells must be produced *via* multipotent MSCs. Steroidogenic cells could be induced by SF-1 from MSCs, but not from differentiated cells such as fibroblasts, preadipocytes and HEK293 cells (Yazawa et al., 2006; Yanase et al., 2006). In support of this hypothesis, the ES cells could not be converted into steroidogenic cells *via* Tc-induced expression of SF-1 under culture conditions that induced differentiation of ES cells into preadipocytes, which also expressed PDGFRα as MSCs (data not shown). However, the exact origin of ES cell-derived steroidogenic cells should further investigated.

The ES cells-derived steroidogenic cells exhibited the very similar gene expression patterns to that of adrenocortical cells and produced a large amount of corticosterone, despite with Cyp17 expression was detectable. As in the case of other steroid hydroxylases, it is well-known that the expression of the CYP17/Cyp17 gene is regulated by SF-1 and LRH-1 (Zhang and Mellon, 1996; Lin et al., 2001; Yazawa et al., 2009). Hence, it is conceivable that the human CYP17 gene is expressed in both in gonadal and adrenal steroidogenic cells. In contrast, the murine Cyp17 gene is expressed only in gonadal cells in adults. However, it was shown that Cyp17 is expressed in the murine fetal adrenal gland (Heikkilä et al., 2002). Therefore, it is possible that steroidogenic cells derived from murine ES cells might reflect the fetal adrenal phenotype. Further studies are necessary for the determination of steroidogenic lineage and the regulation of Cyp17 expression.

In summary, we have shown that, as in the case of SF-1, LRH-1 could drive the differentiation of MSCs into steroidogenic cells. In addition, ES cells could also be differentiated into steroidogenic cells through the regulated expression of SF-1 using the *ROSA-TET* system. This approach might also provide the opportunity, through the use of MSCs, for the development of cell and gene therapy treatments in steroidogenesis deficiencies. Additionally, this approach could be a powerful tool for studies on the differentiation of steroidogenic cell lineages.

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References

Araki, K., Imaizumi, T., Okuyama, K., Oike, Y., Yamamura, K., 1997. Efficiency of recombination by Cre transient expression in embryonic stem cells: comparison of various promoters. J. Biochem. 122, 977–982.
Barnea, E., Bergman, Y., 2000. Synergy of SF1 and RAR in activation of Oct-3/4 promoter. J. Biol. Chem. 275, 6608–6619.
Crawford, P.A., Sadovsky, Y., Milbrandt, J., 1997. Nuclear receptor steroidogenic factor 1 directs embryonic stem cells toward the steroidogenic lineage. Mol. Cell Biol. 17, 3997–4006.
Duggavathi, R., Volle, D.H., Mataki, C., Antal, M.C., Messaddeq, N., Auwerx, J., Murphy, B.D., Schoonjans, K., 2008. Liver receptor homolog 1 is essential for ovulation. Genes Dev. 22, 1871–1876.
Fayard, E., Auwerx, J., Schoonjans, K., 2004. LRH-1: an orphan nuclear receptor involved in development, metabolism and steroidogenesis. Trends Cell Biol. 4, 23–34.
Gu, P., Goodwin, B., Chung, A.C., Xu, X., Wheeler, D.A., Price, R.R., Galardi, C., Peng, L., Latour, A.M., Koller, B.H., Gossen, J., Kliewer, S.A., Cooney, A.J., 2005. Orphan nuclear receptor LRH-1 is required to maintain Oct4 expression at the epiblast stage of embryonic development. Mol. Cell Biol. 25, 3492–3505.
Heikkilä, M., Peltoketo, H., Leppäluoto, J., Ilves, M., Vuolteenaho, O., 2002. S.S.V. Wnt-4 deficiency alters mouse adrenal cortex function, reducing aldosterone production. Endocrinology 143, 4358–4365.
Heng, J.C., Feng, B., Han, J., Jiang, J., Kraus, P., Ng, J.H., Orlov, Y.L., Huss, M., Yang, L., Lufkin, T., Lim, B., Ng, H., 2010. The nuclear receptor Nr5a2 can replace Oct4 in the reprogramming of murine somatic cells to pluripotent cells. Cell Stem Cell 5, 167–174.
Khalfallah, O., Rouleau, M., Barbry, P., Bardoni, B., Lalli, E., 2009. Dax-1 knockdown in mouse embryonic stem cells induces loss of pluripotency and multilineage differentiation. Stem Cell 27, 1529–1537.
Krylova, I.N., Sablin, E.P., Moore, J., Xu, R.X., Waitt, G.M., MacKay, J.A., Juzumiene, D., Bynum, J.M., Madauss, K., Montana, V., Lebedeva, L., Suzawa, M., Williams, J.D., Williams, S.P., Guy, R.K., Thornton, J.W., Fletterick, R.J., Willson, T.M., Ingraham, H.A., 2005. Structural analyses reveal phosphatidyl inositols as ligands for the NR5 orphan receptors SF-1 and LRH-1. Cell 120, 343–355.
Lala, D.S., Rice, D.A., Parker, K.L., 1992. Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor I. Mol. Endocrinol. 6, 1249–1258.
Lee, Y.K., Moore, D.D., 2008. Liver receptor homolog-1, an emerging metabolic modulator. Front. Biosci. 13, 5950–5958.
Lin, C.J., Martens, J.W., Miller, W.L., 2001. NF-1C, Sp1, and Sp3 are essential for transcription of the human gene for P450c17 (steroid 17alpha-hydroxylase/17,20 lyase) in human adrenal NCI-H295A cells. Mol. Endocrinol. 15, 1277–1293.
Luo, X., Ikeda, Y., Parker, K.L., 1994. A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell 77, 481–490.
Masui, S., Shimosato, D., Toyooka, Y., Yagi, R., Takahashi, K., Niwa, H., 2005. An efficient system to establish multiple embryonic stem cell lines carrying an inducible expression unit. Nucleic Acids Res. 33, e43.
Mori, T., Kiyono, T., Imabayashi, H., Takeda, Y., Tsuchiya, K., Miyoshi, S., Makino, H., Matsumoto, K., Saito, H., Ogawa, S., Sakamoto, M., Hata, J., Umezawa, A., 2005.

Combination of hTERT and bmi-1, E6, or E7 induces prolongation of the life span of bone marrow stromal cells from an elderly donor without affecting their neurogenic potential. Mol. Cell Biol. 25, 5183–5195.
Morohashi, K., 1999. Gonadal and extragonadal functions of Ad4BP/SF-1: developmental aspects. Trends Endocrinol. Metab. 10, 169–173.
Morohashi, K., Honda, S., Inomata, Y., Handa, H., Omura, T., 1992. A common transacting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. J. Biol. Chem. 267, 17913–17919.
Niwa, H., Miyazaki, J., Smith, A.G., 2000. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat. Genet. 24, 372–376.
Okamoto, T., Aoyama, T., Nakayama, T., Nakamata, T., Hosaka, T., Nishijo, K., Nakamura, T., Kiyono, T., Toguchida, J., 2002. Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. Biochem. Biophys. Res. Commun. 295, 354–361.
Parker, K.L., Schimmer, B.P., 1997. Steroidogenic factor 1: a key determinant of endocrine development and function. Endocr. Rev. 18, 361–377.
Sadovsky, Y., Crawford, P.A., Woodson, K.G., Polish, J.A., Clements, M.A., Tourtellotte, L.M., Simburger, K., Milbrandt, J., 1995. Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 sidechain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. Proc. Natl. Acad. Sci. U.S.A. 92, 10939–10943.
Saxena, D., Escamilla-Hernandez, R., Little-Ihrig, L., Zeleznik, A.J., 2007. Liver receptor homolog-1 and steroidogenic factor-1 have similar actions on rat granulosa cell steroidogenesis. Endocrinology 148, 726–734.
Schimmer, B.P., White, P.C., 2010. Minireview: steroidogenic factor 1: its roles in differentiation, development, and disease. Mol. Endocrinol. 24, 1322–1327.
Sun, C., Nakatake, Y., Akagi, T., Ura, H., Matsuda, T., Nishiyama, A., Koide, H., Ko, M.S., Niwa, H., Yokota, T., 2009. Dax1 binds to Oct3/4 and inhibits its transcriptional activity in embryonic stem cells. Mol. Cell Biol. 29, 4574–4583.
Takashima, Y., Era, T., Nakao, K., Kondo, S., Kasuga, M., Smith, A.G., Nishikawa, S., 2007. Neuroepithelial cells supply an initial transient wave of MSC differentiation. Cell 129, 1377–1388.
Volle, D.H., Duggavathi, R., Magnier, B.C., Houten, S.M., Cummins, C.L., Lobaccaro, J.M., Verhoeven, G., Schoonjans, K., Auwerx, J., 2007. The small heterodimer partner is a gonadal gatekeeper of sexual maturation in male mice. Genes Dev. 21, 303–315.
Wang, Z.N., Bassett, M., Rainey, W.E., 2001. Liver receptor homologue-1 is expressed in the adrenal and can regulate transcription of 11 beta-hydroxylase. J. Mol. Endocrinol. 27, 255–258.
Yanase, T., Gondo, S., Okabe, T., Tanaka, T., Shirohzu, H., Fan, W., Oba, K., Morinaga, H., Nomura, M., Ohe, K.H.N., 2006. Differentiation and regeneration of adrenal tissues: an initial step toward regeneration therapy for steroid insufficiency. Endocr. J. 53, 449–459.
Yazawa, T., Inanoka, Y., Mizutani, T., Kuribayashi, M., Umezawa, A., Miyamoto, K., 2009. Liver Receptor Homolog-1 regulates the transcription of steroidogenic enzymes and induces the differentiation of mesenchymal stem cells into steroidogenic cells. Endocrinology 150, 3885–3893.
Yazawa, T., Inanoka, Y., Okada, R., Mizutani, T., Yamazaki, Y., Usami, Y., Kuribayashi, M., Orisaka, M., Umezawa, A., Miyamoto, K., 2010. PPAR-gamma coactivator-1alpha regulates progesterone production in ovarian granulosa cells with SF-1 and LRH-1. Mol. Endocrinol. 24, 485–496.
Yazawa, T., Mizutani, T., Yamada, K., Kawata, H., Sekiguchi, T., Yoshino, M., Kajitani, T., Shou, Z., Miyamoto, K., 2003. Involvement of cyclic adenosine 5′-monophosphate response element-binding protein, steroidogenic factor 1, and Dax-1 in the regulation of gonadotropin-inducible ovarian transcription factor 1 gene expression by follicle-stimulating hormone in ovarian granulosa cells. Endocinology 144, 1920–1930.
Yazawa, T., Mizutani, T., Yamada, K., Kawata, H., Sekiguchi, T., Yoshino, M., Kajitani, T., Shou, Z., Umezawa, A., Miyamoto, K., 2006. Differentiation of adult stem cells derived from bone marrow stroma into Leydig or adrenocortical cells. Endocrinology 147, 4104–4111.
Yazawa, T., Uesaka, M., Inaoka, Y., Mizutani, T., Sekiguchi, T., Kajitani, T., Kitano, T., Umezawa, A., Miyamoto, K., 2008. Cyp11b1 is induced in the murine gonad by luteinizing hormone/human chorionic gonadotropin and involved in the production of 11-ketotestosterone, a major fish androgen; conservation and evolution of androgen metabolic pathway. Endocrinology 149, 1786–1792.
Yu, R.N., Ito, M., Saunders, T.L., Camper, S.A., Jameson, J.L., 1998. Role of Ahch in gonadal development and gametogenesis. Nat. Genet. 20, 353–357.
Zhang, P., Mellon, S.H., 1996. The orphan nuclear receptor steroidogenic factor-1 regulates the cyclic adenosine 3′,5′-monophosphate-mediated transcriptional activation of rat cytochrome P450c17 (17 alpha-hydroxylase/c17-20 lyase). Mol. Endocrinol. 10, 147–158.

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A Distinct Role for Pin1 in the Induction and Maintenance of Pluripotency*

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Mayuko Nishi,[a] Hidenori Akutsu,[b] Shinji Masui,[c] Asami Kondo,[a] Yoji Nagashima,[d] Hirokazu Kimura,[e] Kilian Perrem,[f] Yasushi Shigeri,[g] Masashi Toyoda,[b] Akiko Okayama,[h] Hisashi Hirano,[h] Akihiro Umezawa,[b] Naoki Yamamoto,[i] Sam W. Lee,[j] and Akihide Ryo[a1]

From the Departments of [a]Microbiology, [d]Pathology, and [h]Supramolecular Biology, Yokohama City University School of Medicine, Yokohama 236-0004, Japan, the [b]Department of Reproductive Biology, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan, the [c]Department of Regenerative Medicine, Research Institute, International Medical Center of Japan, Tokyo 162-8655, Japan, the [e]Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo 208-0011, Japan, the [f]Commonwealth Scientific and Industrial Research Organization, P.O. Box 225, Dickson, Australian Capital Territory 2602, Australia, the [g]National Institute of Advanced Industrial Science and Technology, Osaka 563-8577, Japan, the [i]Department of Microbiology, National University of Singapore, 117597 Singapore, and the [j]Cutaneous Biology Research Center, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129

The prominent characteristics of pluripotent stem cells are their unique capacity to self-renew and pluripotency. Although pluripotent stem cell proliferation is maintained by specific intracellular phosphorylation signaling events, it has not been well characterized how the resulting phosphorylated proteins are subsequently regulated. We here report that the peptidyl-prolyl isomerase Pin1 is indispensable for the self-renewal and maintenance of pluripotent stem cells via the regulation of phosphorylated Oct4 and other substrates. Pin1 expression was found to be up-regulated upon the induction of induced pluripotent stem (iPS) cells, and the forced expression of Pin1 with defined reprogramming factors was observed to further enhance the frequency of iPS cell generation. The inhibition of Pin1 activity significantly suppressed colony formation and induced the aberrant differentiation of human iPS cells as well as murine ES cells. We further found that Pin1 interacts with the phosphorylated Ser[12]-Pro motif of Oct4 and that this in turn facilitates the stability and transcriptional activity functions of Oct4. Our current findings thus uncover an atypical role for Pin1 as a putative regulator of the induction and maintenance of pluripotency via the control of phosphorylation signaling. These data suggest that the manipulation of Pin1 function could be a potential strategy for the stable induction and proliferation of human iPS cells.

Stem cells are characterized by their ability to self-renew through mitotic cell division and to differentiate into a diverse range of specialized cell types (1, 2). Human pluripotent stem cell proliferation is maintained through the action of several transcription factors including Oct4 (octamer 4), SOX2, Klf-4, Nanog, and c-Myc, which perform reprogramming functions under the stimulatory effects of stem cell-specific growth factors, including basic fibroblast growth factor (3–5). Basic fibroblast growth factor signaling has been shown to be essential for pluripotency as its depletion from cell culture media leads to aberrant cell differentiation and cell death (6, 7). Fibroblast growth factors produce mitogenic effects in targeted cells via signaling through cell surface receptor tyrosine kinases (8). These kinases can initiate intracellular signaling in cells, which is transmitted and diffused by tyrosine phosphorylation of the assembled proteins and of cellular substrates, including protein kinases with specificity for serine/threonine residues (8, 9). Although this intracellular phosphorylation signaling might indeed contribute to the self-renewal and pluripotency of stem cells (10, 11), it has not yet been fully determined how these phosphorylated proteins are further regulated.

Protein phosphorylation is a fundamental mode of intracellular signal transduction in a variety of key cellular processes such as cell proliferation, differentiation, and morphogenesis (12). A pivotal signaling mechanism that controls the function of phosphorylated proteins is the *cis-trans* isomerization of phosphorylated Ser/Thr-Pro motifs by the peptidylprolyl isomerase Pin1 (13, 14). This modification regulates multiple intracellular signaling pathways, including ErbB2/Ras, Wnt/β-catenin, and NF-κB, and thus plays an important role in the etiology of several human diseases (15–18). These include various cancers, Alzheimer disease, and immune disorders (14, 17, 18). However, the role of Pin1 in regulating the properties of pluripotent stem cells has not been adequately investigated to date.

In our current study, we investigated the role of Pin1 in the self-renewal and stemness of pluripotent stem cells. We reveal that Pin1 is induced upon cellular reprogramming and that its blockade significantly inhibits the self-renewal and maintenance of human iPS[2] cells in addition to murine ES cells. We find also that Pin1 can interact with phosphorylated Oct4 at the

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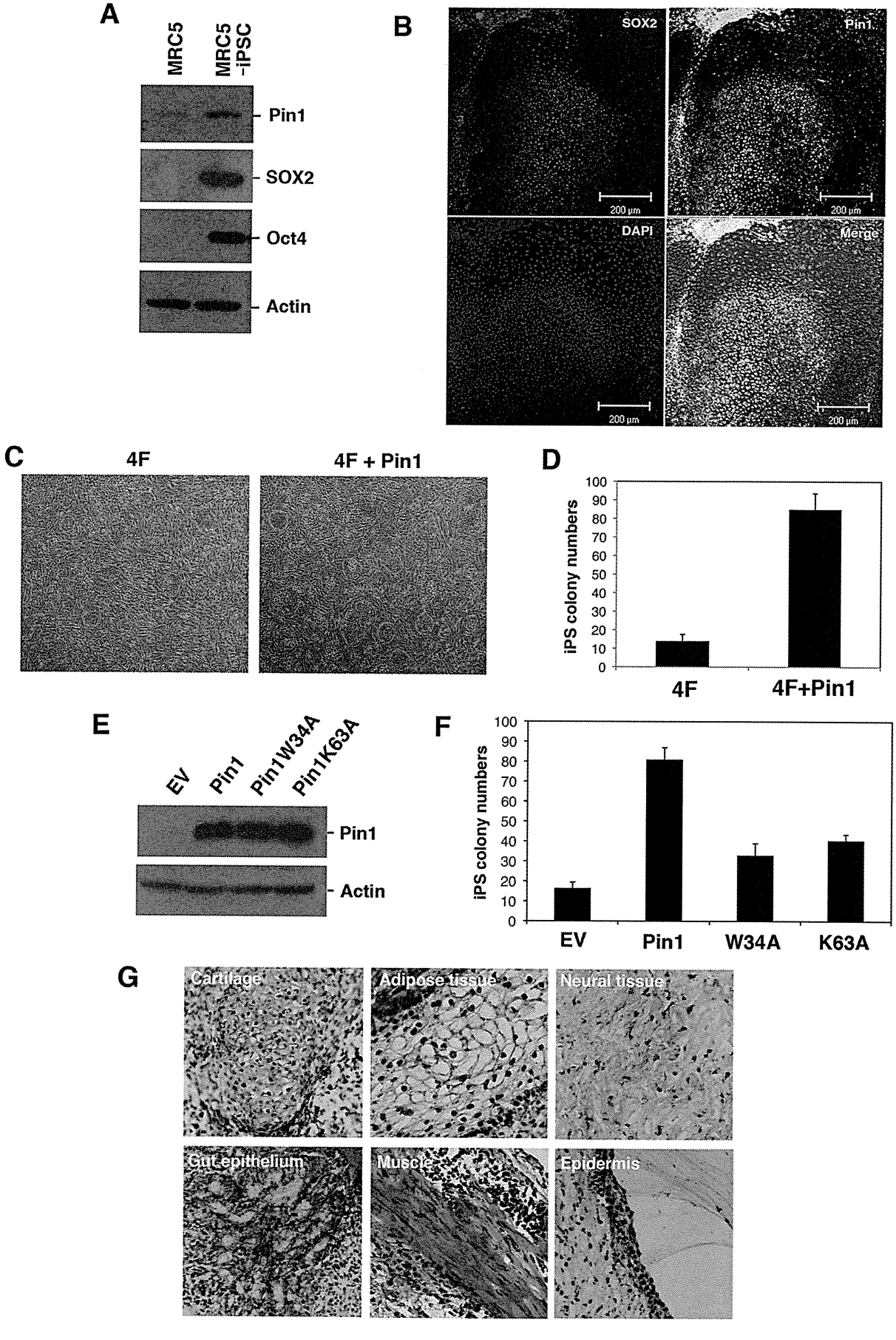
[1] To whom correspondence should be addressed: Dept. of Microbiology, Yokohama City University School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan. Tel.: 81-45-787-2602; Fax: 81-45-787-2851; E-mail: aryo@yokohama-cu.ac.jp.

[2] The abbreviations used are: iPS, induced pluripotent stem; AP, alkaline phosphatase; dnPin1, dominant-negative Pin1; 4F, four reprogramming factors; DMSO, dimethyl sulfoxide; SUMO, small ubiquitin-like modifier; Oct4, Octamer 4.



Ser^{12}-Pro motif in this protein. This enhances the stability and hence the transcriptional activity of Oct4. Our present data thus suggest that Pin1 is indeed a putative regulator of the self-renewal and proliferation of pluripotent stem cells.

EXPERIMENTAL PROCEDURES

Colony Formation Analysis—Human iPS cells were obtained from the RIKEN BioResource Center (clone no. 201B7) (19). Cells were cultured in human embryonic stem cell culture medium (KnockOut Dulbecco's modified Eagle's medium (Invitrogen)) supplemented with 20% KnockOut SR (Invitrogen), 1% GlutaMAX (Invitrogen), 100 μM nonessential amino acids (Invitrogen), 50 μM β-mercaptoethanol, and 10 ng/ml basic fibroblast growth factor). Murine ES cells were cultured in human embryonic stem cell culture medium (KnockOut Dulbecco's modified Eagle's medium supplemented with 15% KnockOut SR, 1% GlutaMAX (Invitrogen), 100 μM nonessential amino acids, 50 μM β-mercaptoethanol, and 1000 units/ml recombinant human leukemia inhibitory factor) (20). Colony formation was scored by counting the number of alkaline phosphatase (AP)-positive colonies as described previously (21). The number of cells per colony was determined by manually counting the number of DAPI-stained cells (21).

Cell Reprogramming—MRC5 fibroblasts were transduced with retroviral vectors encoding reprogramming factors as described previously (19). Briefly, the retroviral vector plasmids pMXs-hOct4, pMXs-hSOX2, pMXs-hKLF4, pMXs-hcMYC (Addgene), and pVSV-G were introduced into Plat-E cells using Effectene transfection reagent (Qiagen). After 48 h, virus-containing supernatants were passed through a 0.45-μm filter and supplemented with 10 μg/ml hexadimethrine bromide (polybrene). Cells were seeded at 6×10^5 cells per 60 mm dish at 24 h before incubation in the virus/polybrene-containing supernatants for 16 h. After 6 days, cells were plated on irradiated mouse embryonic fibroblasts, and culture medium was replaced with the hESC culture medium 24 h later. Cells were maintained at 37 °C and 5% CO_2 for 30 days.

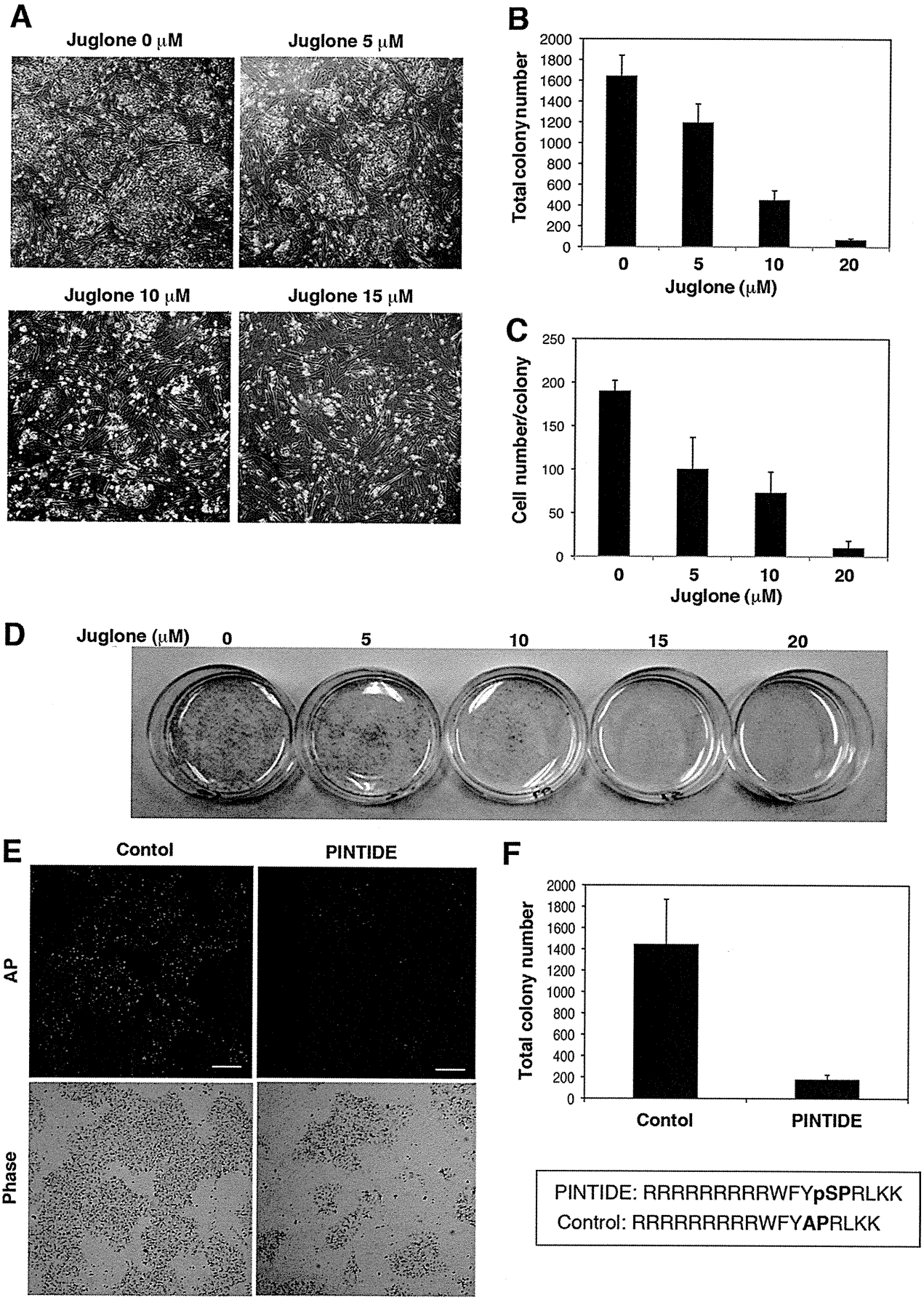
Construction of Expression Vectors—Oct4 cDNA was subcloned into pcDNA3-HA expression vector (Invitrogen). Expression constructs of Oct4 were as follows: pcDNA-HA-Oct4 wild-type, amino acids 1–360; pcDNA-HA-Oct4 ΔC, amino acids 1–297; pcDNA-HA-Oct4 ΔN1, amino acids 138–360; pcDNA-HA-Oct4 ΔN2, amino acids 113–360; and pcDNA-HA-Oct4 ΔN3, amino acids 34–360. pcDNA-HA-Oct4-S12A was generated by KOD-Plus Mutagenesis Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The primers were 5′-CGCCCCCTCCAGGTGGT-3′ (forward) and 5′-CGAAGGCAAAATCTGAAGCC-3′ (reverse).

Gene Reporter Assay—A pGL3-fgf4 reporter plasmid containing an Oct-SOX binding cassette and the firefly luciferase gene was transfected with pRL-CMV (22). The −2601/+1 (nucleotide positions indicated with respect to the +1 translation start site) genomic fragment of the Oct4 promoter upstream region was amplified by PCR from human lymphocyte genomic DNA and cloned into the KpnI/HindIII sites of the pGL4-basic reporter plasmid (Promega, Madison, WI) as described previously (23). The primer sets were as follows: 5′-CCTGGTACCAGGATGGCAAGCTGAGAAACACTG-3′ and 5′-TCGCAAGCTTGCGAAGGGACTACTCAAC-3′. Cells were transfected with reporter plasmid vectors using Effectene (Qiagen) or Xfect Stem (Clontech). One day after transfection, the cells were resuspended in passive lysis buffer (Promega) and incubated for 15 min at room temperature. Luciferase activities were measured with a Dual-Luciferase reporter assay system (Promega) in accordance with the manufacturer's instructions.

GST Pulldown Assay and Immunoprecipitation Analysis—Cells were lysed with GST pulldown buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.2 mM PMSF) and incubated with 30 μl of glutathione-agarose beads containing either GST-Pin1 or GST at 4 °C for 2 h. The precipitated proteins were then washed three times with lysis buffer and subjected to SDS-PAGE. For immunoprecipitation, cells were lysed with Nonidet P-40 lysis buffer (10 mM Tris HCl (pH 7.4), 100 mM NaCl, 0.5% Nonidet P-40, 1 mM Na_3VO_4, 100 mM NaF, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.2 mM PMSF). Cell lysates were incubated for 1 h with protein A/G-Sepharose·nonimmunized IgG complexes. Supernatant fractions were recovered and immunoprecipitated with 5 μg of anti-Myc antibody and 30 μl protein A/G-Sepharose. After washing three times with lysis buffer, the pellets were analyzed by SDS-PAGE.

Proteomics Analysis—Human iPS cell lysates were processed for immunoprecipitation with a monoclonal anti-Pin1 antibody (clone 257417, R&D Systems) at 4 °C for 3 h followed by SDS-PAGE. Gel lanes corresponding to the region from ~30 to 150 kDa were systematically excised, and the pieces were reduced, alkylated, and trypsinized. Peptides were analyzed by the linear ion trap Orbitrap hybrid mass spectrometer (Thermo Scientific). Protein identification was performed by peptide

FIGURE 1. **Pin1 is preferentially expressed in human iPS cells.** *A*, immunoblotting analysis of Oct4, SOX2, and Pin1 in MRC5 and MRC5-derived iPS cells. Actin was used as a loading control. *iPSC*, induced pluripotent stem cells; *EV*, empty vector. *B*, immunofluorescent analysis of Pin1 and SOX2 in human iPS cells. Representative images of phase-contrast microscopy and fluorescent immunocytochemistry for SOX2 (*red*) and Pin1 (*green*) are shown. Nuclei are indicated by DAPI staining (*blue*). Note that Pin1 is highly expressed in SOX2-positive pluripotent stem cells. *C* and *D*, Pin1 expression enhances 4F (Oct4, SOX2, Klf4, and c-Myc)-induced iPS cell induction. MRC5 fibroblasts were infected with retrovirus vectors encoding 4F and co-infected with those encoding either empty vector or Pin1. A representative picture of colony formations stained with AP is shown (*C*). The numbers of AP-positive colonies were scored in three independent experiments (*D*). Note that the co-introduction of Pin1 with 4F increases the frequency of iPS colony formation. *E* and *F*, MRC5 fibroblasts were infected with retrovirus vectors encoding 4F and co-infected with those encoding empty vector, HA-tagged wild-type Pin1, or its W34A or K63A mutants. The expression levels of HA-Pin1 or its mutants in infected MRC5 cells were analyzed by immunoblotting analysis with anti-HA antibody (*E*). The number of AP-positive colonies was scored in three independent experiments (*F*). *G*, teratoma tissue derived from human iPS cells induced by 4F and Pin1. iPS cells were transplanted subcutaneously into immunodeficient mice (2×10^6/mouse). Representative images of hematoxylin and eosin stained tumor with light microscope (200×) are shown.



mass fingerprinting with the Mascot and Aldente search algorithms.

Quantitative Real-time PCR—Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using a cDNA synthesis kit (Toyobo, Osaka, Japan) and subjected to RT-PCR analysis with the SYBR Premix Ex gent Kit TaqII (Takara Bio, Shiga, Japan) using an Applied Biosystems 7300 real-time PCR System. The primer sets used were as follows: mOct4, 5′-CGTGTGAGGTGGAGTCTGGAGACC-3′ and 5′-ACTCGAACCACATCCTTCTCTAGCC-3′; mGAPDH, 5′-CCATGGAGAAGGCTGGGG-3′ and 5′-CAAAGTTGTCATGGATGACC-3′.

Teratoma Formation—Cells were harvested using accutase, collected into tubes, and centrifuged. The pellets were then suspended in human ESC culture medium. Fox Chase severe combined immunodeficiency mice (CREA, Tokyo, Japan) were injected with 2×10^6 cells mixed with an equal volume of Matrigel (BD Biosciences). Frozen tumor tissues embedded in optimum cutting temperature compound were sliced by cryosectioning and stained with hematoxylin and eosin.

RESULTS

Pin1 Is Induced upon Cellular Reprogramming and Enhances Generation of iPS Cells—To examine the role of Pin1 in cellular reprogramming and pluripotency, we initially investigated the expression levels of this prolyl isomerase in human iPS cells. Pin1 was found to be significantly induced upon the generation of iPS cells derived from MRC5 human fibroblasts (Fig. 1*A*). Immunofluorescent analysis further revealed that Pin1 is selectively expressed in SOX2-positive pluripotent stem cells, whereas its expression was found to be significantly suppressed in the surrounding SOX2-negative differentiated cells (Fig. 1*B*). These results indicate that Pin1 is preferentially expressed in reprogramming stem cells.

We next evaluated whether Pin1 affects the reprogramming of somatic cells into iPS cells. The co-infection of a Pin1-encoding retrovirus vector with those encoding four defined reprogramming factors (4F; SOX2, Oct4, Klf-4, and c-Myc) (24) notably boosted the generation of AP-positive iPS cell colonies compared with an induction of human fibroblast MRC5 cells with only four iPS factors (Fig. 1, *C* and *D*). We next performed a parallel experiment using either a WW-domain (binding domain) mutant (W34A) or a peptidyl prolyl isomerase-domain (catalytic domain) mutant (K63A) of Pin1. We confirmed the equivalent expression of each of these mutants and wild-type Pin1 (Fig. 1*E*). Neither of these mutants could boost iPS cell colony formation to the level seen with wild-type Pin1 (Fig. 1*F*), indicating that both the WW and PPIase domains are required for this function.

To test pluripotency *in vivo*, we transplanted 4F plus Pin1-introduced iPS cells subcutaneously into the dorsal flanks of immunodeficient mice. Nine weeks after injection, we observed teratoma formation composed of various tissues including gut-like epithelial tissues (endoderm), striated muscle (mesoderm), cartilage (mesoderm), neural tissues (ectoderm), and epidermal tissues (ectoderm) (Fig. 1*G*). These results indicate that the expression of Pin1 with defined reprogramming factors accelerates the frequency of iPS cell generation.

Pin1 Is Required for Pluripotent Stem Cell Self-renewal and Colony Formation—We next addressed whether Pin1 indeed plays any roles in the self-renewal of human iPS cells. iPS cells were dissociated with accutase and then plated at a clonal density in the presence of several concentrations of the selective Pin1 inhibitor juglone (5-hydroxy-1,4-naphthoquinone) (25, 26). The blockade of Pin1 by juglone considerably reduced both the numbers and size of the colonies in a dose-dependent manner (Fig. 2, *A–C*). It was notable also that the concentration of juglone used did not illicit nonspecific toxic effects in the feeder mouse embryonic fibroblast cells (Fig. 2*A* and data not shown). The effect of Pin1 inhibition upon colony formation was also confirmed in feeder-free cultures of human iPS cells by AP staining (Fig. 2*D*). Moreover, treatment with the Pin1 inhibitory phosphopeptide PINTIDE (27), but not a nonphosphorylated control peptide, significantly reduced the colony formation of human iPS cells (Fig. 2, *E* and *F*).

We next investigated the effects of Pin1 inhibition upon colony formation in murine ES cells. The blockade of Pin1 by juglone significantly reduced the colony numbers in two different murine ES cell types, BDF2 and R1 (Fig. 3*A*). The adenovirus-mediated transduction of a GFP-fused dominant-negative Pin1 (GFP-dnPin1) (28), but not a GFP control, significantly suppressed colony formation in murine ES (R1) cells manifesting as a considerable reduction in both the numbers and colony size of the murine ES cells (Fig. 3, *B–D*). These results together demonstrate that Pin1 is indispensable for the self-renewal and proliferation of pluripotent stem cells.

Pin1 Functions in Maintenance of Pluripotency—We next asked whether Pin1 has any roles in the maintenance of pluripotency in stem cells. Human iPS cells were dissociated and then cultured for 5 days to form colonies. When human iPS cells are cultured in hES medium supplemented with basic fibroblast growth factor, the overwhelming majority of the cells in the colonies are undifferentiated (Fig. 4*A*). However, treatment with juglone resulted in aberrant cell differentiation resulting in a "mosaic pattern" of iPS cell colonies following AP staining (Fig. 4*A*). Similarly, the adenovirus-mediated transduction of GFP-dnPin1, but not a GFP control, prominently reduced the number of AP-positive undifferentiated cells in murine ES cell colonies (Fig. 4*B*). These results together indicate that Pin1 can sustain pluripotent stem cells in an undifferentiated state in addition to the enhancement of self-renewal.

FIGURE 2. **Defective self-renewal of human iPS cells caused by Pin1 inhibition.** *A–C*, human iPS cells were dissociated with accutase and then plated on a feeder cell layer at a clonal density in the presence of the indicated concentrations of juglone for 3 days. Colony formation was analyzed by phase-contrast microscopy (*A*). The number of colonies was counted at 3 days after treatment (*B*). The number of cells per colony was determined by manually counting the DAPI-stained cells (*C*). Data are the mean ± S.E. *D*, human iPS cells were plated at a clonal density on the feeder-free culture in the presence of the indicated concentrations of juglone followed by AP staining. *E* and *F*, human iPS cells were dissociated with accutase and then plated on feeder-free dishes at a clonal density in the presence of 50 μg/ml of the Pin1 inhibitory phosphopeptide PINTIDE (RRRRRRRRRWFYpSPRLKK) or a nonphosphorylated control peptide (RRRRRRRRRWFYAPRLKK) for 48 h (*E*). AP-positive colony numbers were scored (*F*). Data are the mean ± S.E. *Scale bar*, 50 μm.

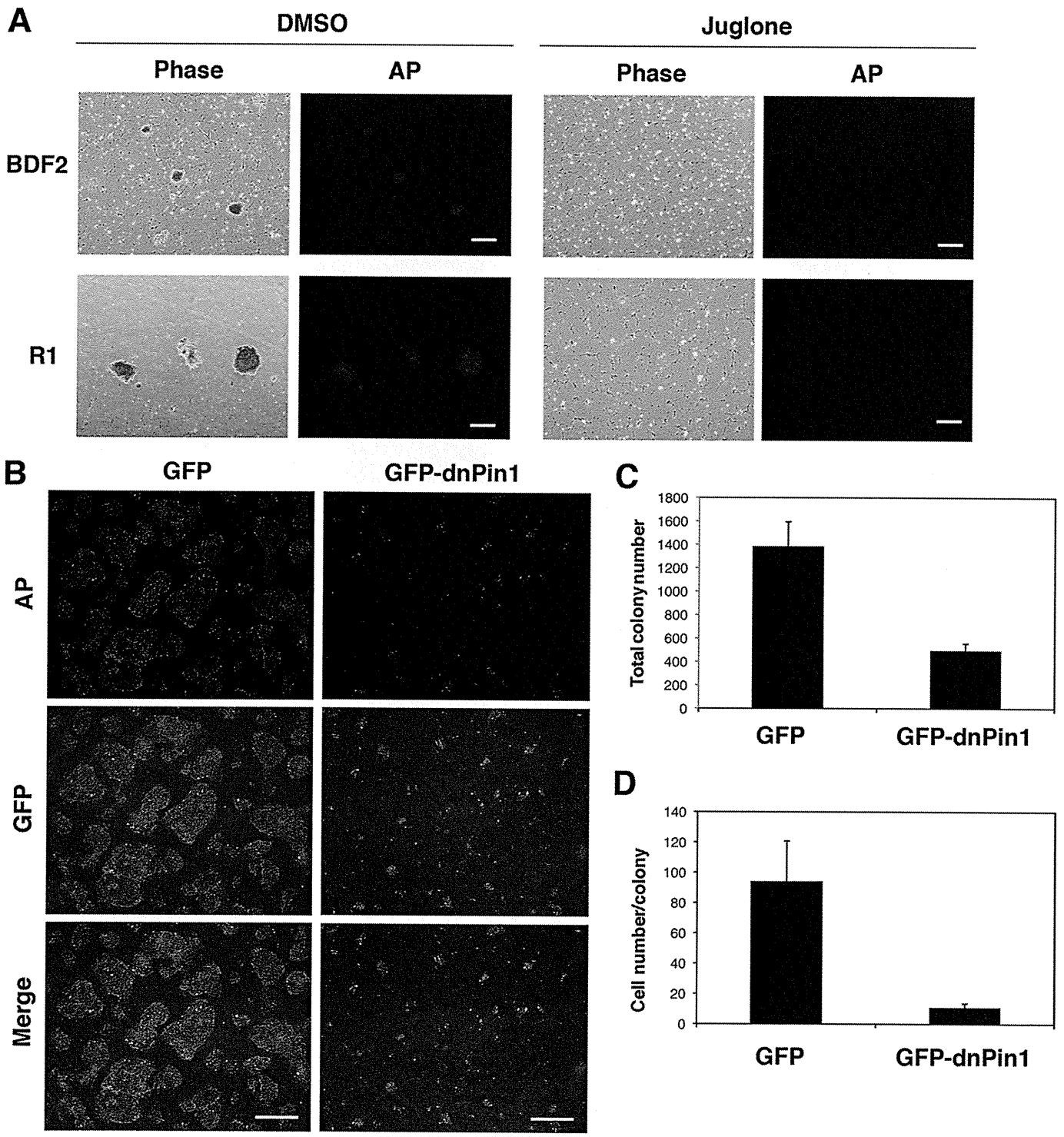


FIGURE 3. **Pin1 inhibition suppresses colony formation in murine ES cells.** *A*, two different murine ES cell types (BDF2 and R1) were plated on gelatin-coated dishes and treated with either DMSO or juglone (10 μM). Colonies were stained with AP (*red*). *Scale bar*, 200 μm. *B–D*, murine ES cells (R1) were infected with an adenovirus vector encoding either GFP or GFP-dnPin1 (3000 viral particles/cell). The cells were then stained with AP (*red*) and DAPI and analyzed by immunofluorescent microscopy (*B*). *Scale bar*, 200 μm. The total colony number (*C*) and the number of cells per colony (*D*) were then determined. Data are the mean ± S.E.

Identification of Pin1 Binding Proteins in Human iPS Cells—Our initial data indicated that Pin1 could enhance the function of reprogramming factors during the induction and maintenance of pluripotency. We next identified the substrates targeted by Pin1 in human iPS cells. Using a monoclonal Pin1 antibody, we co-immunoprecipitated proteins from human iPS cell lysates treated with a phosphatase inhibitor mixture. These isolated immune complexes were then boiled and resolved by one-dimensional SDS-PAGE, and the proteins were visualized using silver staining. Continuous regions of the gel corresponding to proteins of ~30 to 150 kDa in size were systematically excised (Fig. 5*A*), digested with trypsin, and analyzed in a linear