

for purification and increases the purity of recombinant proteins, compared with intact laminin-511. These advantages endow LM-E8s with a greater potential for scaled-up production, compared with intact laminins. Importantly, LM-E8s have a higher adhesion affinity for hESCs than do intact laminin-511 and laminin-332, although the reasons for this property are yet to be fully determined. The smaller size of LM-E8s may allow them to pack more closely to each other on the substrate and thereby increases the adhesiveness of the substrate for hESC attachment by either increasing the ligand-binding affinity of integrins and/or enhancing the signalling events triggered by integrin clustering.

Several groups have reported that vitronectin and one of its variants (VTN-NC) supports the maintenance of hESCs via $\alpha V\beta 5$ integrin^{4,13}. The binding affinity of vitronectin to $\alpha V\beta 5$ integrin was 10-fold lower than that of LM-E8 to $\alpha 6\beta 1$ integrin (Yuya Sato, K.S., unpublished data), suggesting that vitronectin is potentially inferior for hESC adhesion compared with LM-E8s. Indeed, although hESCs and hiPSCs have been cultured as colonies on fibronectin and vitronectin^{1,13,35,36}, hESCs and hiPSCs that had been completely dissociated showed less adhesion (Fig. 2a). It has been reported that AKT, ERK1/2 and kinases interacting with FAK are highly phosphorylated in human pluripotent stem cells³⁷. Furthermore, it is well known that AKT and FAK, stimulated via integrin activation, perform numerous cell survival functions³⁴. The phosphorylation of AKT, FAK and ERK1/2 confirms that LM-E8s have the potential to promote the survival of completely dissociated hESCs by robust adhesion via integrins, whereas vitronectin and fibronectin are unfavourable for culturing of completely dissociated hESCs and hiPSCs. The ROCK inhibitor Y-27632 has been shown to increase the survival of dissociated hESCs³³. Tsutsui *et al.*⁵ reported maintenance of hESCs on a fibronectin-coated surface by dissociated cell passaging, but their method requires a high dose of Y-27632 for cell attachment to fibronectin. It should be noted that a high concentration of Y-27632 increases toxicity and leads to a reduction of hESC numbers in some cases³⁸. Additionally, the maintenance of hESCs on fibronectin requires constant administration of Y-27632, even after cell attachment. In contrast, our method using LM-E8s does not require Y-27632 administration owing to sufficient stimulation of cell survival from the culture substrate. This method might be advantageous not only for supporting cell expansion but also for use in drug screening studies. This is because LM-E8s not only improve the culture condition to avoid transient adverse effects of untargeted chemicals, such as Y-27632, but also allow uniform seeding of routine numbers of hESCs and hiPSCs. Therefore, LM-E8s are an appropriate culture substrate for the culture of completely dissociated hESCs and hiPSCs. Although LM-E8s ensured hESC survival just after cell seeding, single hESCs exhibited poor survival over prolonged time periods, even on LM-E8s. It is known that E-cadherin signalling, which arises from cell–cell contact, has a key role in the self-renewal of hESCs as well as the generation of hiPSCs^{39–41}. LM-E8 signalling may be insufficient for prolonged survival of single hESCs, but LM-E8s enabled hESCs to actively migrate, and the cells aggregated promptly and restored cell–cell contacts to form small colonies within 24 h of culture initiation (Figs 2b, c and 5a). FAK is known to perform multiple functions and regulates cell migration⁴². The increased phosphorylation of FAK is indicative of active migration of dissociated hESCs on LM-E8s. It has been recently reported that cell–cell contact promotes hESC survival, and Y-27632 treatment accelerates the migration of hESCs to reconstruct cell–cell contacts⁴³. Here, we show that LM-E8s also activated dissociated hESC migration, but the mechanism is different from that of Y-27632 treatment, as shown by the MLC2 phosphorylation analysis. Collectively, LM-E8s support not only

strong adhesion to hESCs and hiPSCs but also promote cell–cell contact by inducing their migration ability, thereby ensuring cell survive and proliferation. Thus, our new protocol improves the adhesion of hESCs and hiPSCs and allows efficient propagation in a simple and rapid manner.

In conclusion, we demonstrate a defined xeno- and feeder-free culture system for hESCs and hiPSCs. LM-E8s derived from laminin isoforms support increased adhesion, survival, self-renewal and differentiation of hESCs. This simple culture system should facilitate bulk proliferation of hESCs and hiPSCs under defined xeno-free conditions. We believe that our protocol will be useful for clinical applications of hESCs and hiPSCs in compliance with good manufacturing practice and/or current good tissue practice.

Methods

Preparation of ECMs. Recombinant human laminins, laminin-111, -332 and -511 and LM-E8s (LM322-E8 and LM511-E8) were produced using a FreeStyle 293 Expression System (Invitrogen) as described previously^{21,29,30}. Laminin-111, -332 and -511 and LM-E8s were filtered through 0.22- μ m-pore-sized Ultrafree-CL Centrifugal Filter Units (Millipore) for sterilization. Using these methods, we purified an average of 0.74 mg intact LM511 ($n = 5$) and 4.1 mg LM511-E8 ($n = 4$) from 11 of culture supernatant. Thus, the yield was much higher for LM511-E8 (5.5-fold in terms of weight, or 27.7-fold per mol) than for intact laminin. Furthermore, purification of intact laminin-511 required 1–2 weeks, whereas purification of LM-E8s required only 2–3 days.

Human fibronectin and vitronectin were purified from plasma, as described elsewhere^{44,45}. Matrigel (Growth Factor Reduced) was purchased from BD Biosciences.

Coating of matrices. For the dose–response binding assay, 96-well flat-bottomed plates (non-treated, BD Falcon) were precoated with each of the matrices at a concentration of 0–25 μ g cm^{-2} for 3 h at room temperature just before cell seeding. For other experiments, we used 48-well plates (non-treated, BD Falcon) precoated with LM-E8s, fibronectin, or vitronectin at 1.5 μ g cm^{-2} or Matrigel at 25 μ g cm^{-2} for 3 h at room temperature for the culture of hESCs and hiPSCs. For conventional colony passaging as an experimental control, 12-well plates were coated with Matrigel at 25 μ g cm^{-2} . Coated dishes were not rinsed with buffer before use. To dilute the ECMs, Dulbecco's PBS was used for laminin-111, -332 and -511, LM-E8s, fibronectin and vitronectin. DMEM/F12 was used to dilute Matrigel.

Preparation of defined media. Commercially available defined media, mTeSR1 (mTeSR1; Stem Cell Technologies) and StemPro hESC SFM (StemPro, Invitrogen) and xeno-free medium, TeSR2 (TeSR2; Stem Cell Technologies), were used according to the manufacturer's instructions and freshly prepared every day. N2B27 medium was prepared daily, according to previous reports^{1,2}, and was composed of DMEM/F12 (Sigma, D6421), $1 \times$ N2 supplement (Invitrogen), $1 \times$ B27 supplement (Invitrogen), 1 mM non-essential amino acids (Sigma, M7145), 1 mM L-glutamine (Sigma, G7513), 0.1 mM 2-mercaptoethanol (Invitrogen) and 100 ng ml^{-1} FGF-2 (Wako, Japan, 060-04543).

Cell culture. hESC lines, KhES-1 at passage 22 and HES3 at passage 100, and hiPSC line 253G1 at passage 50 were maintained as previously described on mitomycin-C-treated mouse embryonic fibroblast (MEF) feeder cells⁴⁶. The hESC line H9 (WA09) at passage 36, hiPSC line iPS (IMR90) clone 1 (iPS(IMR90)-1) at passage 80 and iPS (foreskin) line at passage 64 were purchased from the National Stem Cell Bank. Cells were maintained in accordance with National Stem Cell Bank protocols, except for the use of mitomycin-C-treated MEFs from the ICR strain at a density of 3.2×10^4 cells per cm^2 .

Experimental control cells were obtained from conventional colony cultures that were maintained as follows. Near-confluent cells were incubated with 2 mg ml^{-1} dispase in DMEM/F12 at 37 °C for 2 min, and then rinsed twice with DMEM/F12. After addition of defined medium, weakly adherent colonies were detached using a cell scraper. Cells were collected and centrifuged at 200 g for 3 min at 4 °C. Small colonies were then passaged into Matrigel-coated vessels at a ratio of 1:4. Colonies showing morphological differentiation were manually removed under a microscope during each passage, except at the time of analysis. Completely dissociated cells were cultured after preculture in the appropriate defined medium for one passage on Matrigel-coated dishes to remove MEF feeders. This stage was defined as passage 0. Near-confluent cell clusters were treated with 4.8 mM EDTA/PBS for 2–5 min at room temperature and then TrypLE Select (Invitrogen) for 1 min at 37 °C. Cells in defined medium were pipetted to disperse the cells completely. After centrifugation at 200 g for 3 min at 4 °C, cells were seeded at the appropriate density (5×10^4 cells per cm^2 for passaging or as indicated) into new culture vessels

precoated with each matrix. The medium was changed daily for all experiments. hESCs and hiPSCs on LM-E8s were subcultured every 4–6 days, and those on Matrigel every 6–8 days.

Time-lapse recording. For time-lapse recording during initial attachment, completely dissociated H9 hESCs were seeded onto LM-E8-, fibronectin-, vitronectin- or Matrigel-coated 35-mm dishes at 2.5×10^4 cells per cm^2 in mTeSR1 medium. Recording began at 5 min after cell seeding and continued for 24 h. Movies were edited using AxioVisio software.

Cell adhesion assay. Dose–response binding assays were performed using a protocol described elsewhere¹³, with some modification. Briefly, hESCs or hiPSCs were cultured for one passage on Matrigel in mTeSR1 medium to remove feeder cells, and then the cells were detached using TrypLE Select, collected and counted. A total of 5×10^4 cells were seeded into each well of 96-well plates coated with the matrices described above. After 6 h of incubation, non-adherent cells were removed by rinsing the plates twice with DMEM/F12, and the remaining adherent cells were fixed with 10% formalin for 15 min, and postfixed in 100% ethanol for 5 min at room temperature. Cells were then stained with 0.4% crystal violet in methanol for 5 min at room temperature. After washing extensively with demineralized water, the remaining cells were solubilized by adding 100 μl of 1% SDS, and the optical density at 570 nm was measured by a multiwell plate reader. For the functional inhibition assay of integrins, H9 hESCs were seeded into each well of 96-well plates coated with the various matrices including LM511(EQ) in the presence or absence of $2 \mu\text{g ml}^{-1}$ integrin-blocking antibodies, anti- $\alpha 6$ integrin (R&D systems) and/or anti- $\beta 1$ integrin (R&D systems). After 30 min of incubation, adherent cells were screened as described above for the dose–response assay.

For the seeding density assay, hESCs or hiPSCs were precultured in mTeSR1 medium on Matrigel-coated vessels for one passage to remove feeder cells. Cells were detached using an EDTA solution and TrypLE Select as described above. Cells were seeded into 48-well plates coated with LM-E8s or Matrigel. After 6 h of incubation, non-adherent cells were rinsed twice with prewarmed DMEM/F12, and the remaining adherent cells were detached using 0.25% Trypsin/EDTA. Viable cells were counted by trypan blue exclusion.

For the proliferation assay, dissociated single cells were seeded at a density of 4×10^4 cells per cm^2 , and viable cells were counted every 24 h as described above. In parallel, colonies were seeded on 24-well plates coated with Matrigel at the same cell density.

To evaluate Y-27632 pretreatment, H9 hESCs cultured on Matrigel in mTeSR1 medium were incubated with $10 \mu\text{M}$ Y-27632 (Wako, 253-00513) for 1 h just before cell detachment. Together with the cultures without Y-27632 pretreatment, cells were detached by EDTA and TrypLE Select treatment and then seeded in multiwell plates coated with substrates in mTeSR1 medium containing $10 \mu\text{M}$ Y-27632.

To evaluate the effect of Y-27632 treatment on cell proliferation, H9 hESCs after complete dissociation or as colonies were seeded at a density of 5×10^4 cells per cm^2 with or without $10 \mu\text{M}$ Y-27632 in 48-well plates coated with each substrate. Viable cells were counted every 24 h. To evaluate a derivation of vitronectin, a Synthamax-R Surface 6-well plate (Corning) was used as a substrate.

Flow cytometric analysis. Cells were dissociated by treatment with 4.8 mM EDTA/PBS for 2–5 min, followed by further treatment with a 0.05% trypsin/EDTA solution for 1 min. After two washes with DMEM/10% FBS and one wash with staining buffer (0.1% BSA/PBS), 1×10^5 cells were incubated for 30 min at 4°C with primary antibodies diluted in staining buffer. Cells were then rinsed twice with staining buffer and incubated for 30 min at 4°C with secondary antibodies diluted in staining buffer. Then, cells were rinsed twice with staining buffer, and counterstained with propidium iodide just before analysis.

Fluorescence intensities were analysed on a FACSCalibur flow cytometer (Becton Dickinson), and the FL-1 positive ratios in the propidium iodide-negative region were monitored using Cell Quest software. The following primary antibodies were used: SSEA-3 ($2 \mu\text{g ml}^{-1}$; Developmental Studies Hybridoma Bank (DSHB)), SSEA-4 ($1 \mu\text{g ml}^{-1}$; DSHB), Tra-1-60 ($1 \mu\text{g ml}^{-1}$; Chemicon), Tra-1-81 ($1 \mu\text{g ml}^{-1}$; Chemicon), GCTM2 ($1 \mu\text{g ml}^{-1}$; Chemicon), Tra-2-54 ($1 \mu\text{g ml}^{-1}$, DSHB), and SSEA-1 ($2 \mu\text{g ml}^{-1}$; DSHB). Anti-mouse Ig/FITC conjugated ($10 \mu\text{g ml}^{-1}$; Becton Dickinson) and anti-rat IgM/Alexa 488 conjugated ($1 \mu\text{g ml}^{-1}$; Molecular Probes) were used as secondary antibodies.

Karyotyping. hESCs and hiPSCs were treated with 100 ng ml^{-1} colcemid (Invitrogen) for 2–3 h. After dissociation in 0.25% trypsin/EDTA solution, cells were treated with a hypotonic solution and then fixed in Carnoy's solution. Cells were spread onto glass slides and stained with Giemsa. Chromosome spreads were analysed by randomly counting 50 cells using the Ikaros Karyotyping System (META system). M-FISH analysis was performed using the 24XCyte Human Multicolor FISH (mFISH) Probe Kit (META system) following the manufacturer's instructions, and analysed by ISIS software (META system).

Statistical analysis. The statistical significance of differences was determined by the two-tailed Student's *t*-test for two group comparisons, or two-way analysis of

variance (two-way ANOVA) for multiple comparisons followed by Tukey's test. Differences with a value of $P < 0.05$ were considered significant.

Other methods. See Supplementary Methods and Supplementary Table S2 for remaining methods, including semi-quantitative RT–PCR, the differentiation assay, immunofluorescence staining and immunoblotting.

References

- Liu, Y. *et al.* A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human embryonic stem cells. *Biochem. Biophys. Res. Commun.* **346**, 131–139 (2006).
- Akopian, V. *et al.* Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells. *In Vitro Cell Dev. Biol. Anim.* **46**, 247–258 (2010).
- Ludwig, T. E. *et al.* Derivation of human embryonic stem cells in defined conditions. *Nature Biotech.* **24**, 185–187 (2006).
- Chen, G. *et al.* Chemically defined conditions for human iPSC derivation and culture. *Nat. Methods* **8**, 424–429 (2011).
- Tsutsui, H. *et al.* An optimized small molecule inhibitor cocktail supports long-term maintenance of human embryonic stem cells. *Nat. Commun.* **2**, 167 (2011).
- Baker, M. Stem cells in culture: defining the substrate. *Nat. Methods* **8**, 293–297 (2011).
- Thomson, J. A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
- Amit, M. *et al.* Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev. Biol.* **227**, 271–278 (2000).
- Pyle, A. D., Lock, L. F. & Donovan, P. J. Neurotrophins mediate human embryonic stem cell survival. *Nature Biotech.* **24**, 344–350 (2006).
- Draper, J. S. *et al.* Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nature Biotech.* **22**, 53–54 (2004).
- Mitalipova, M. M. *et al.* Preserving the genetic integrity of human embryonic stem cells. *Nature Biotechnol.* **23**, 19–20 (2005).
- Rodin, S. *et al.* Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. *Nature Biotechnol.* **28**, 611–615 (2010).
- Braam, S. R. *et al.* Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via $\alpha 5 \beta 1$ integrin. *Stem Cells* **26**, 2257–2265 (2008).
- Melkounian, Z. *et al.* Synthetic peptide-acrylate surfaces for long-term self-renewal and cardiomyocyte differentiation of human embryonic stem cells. *Nature Biotechnol.* **28**, 606–610 (2010).
- Villa-Diaz, L. G. *et al.* Synthetic polymer coatings for long-term growth of human embryonic stem cells. *Nature Biotech.* **28**, 581–583 (2010).
- Mei, Y. *et al.* Combinatorial development of biomaterials for clonal growth of human pluripotent stem cells. *Nat. Mater.* **9**, 768–778 (2010).
- Klim, J. R., Li, L., Wrighton, P. J., Piekarczyk, M. S. & Kiessling, L. L. A defined glycosaminoglycan-binding substratum for human pluripotent stem cells. *Nat. Methods* **7**, 989–994 (2010).
- Saha, K. *et al.* Surface-engineered substrates for improved human pluripotent stem cell culture under fully defined conditions. *Proc. Natl Acad. Sci. USA* **108**, 18714–18719 (2011).
- Miner, J. H. & Yurchenco, P. D. Laminin functions in tissue morphogenesis. *Annu. Rev. Cell Dev. Biol.* **20**, 255–284 (2004).
- Miyazaki, T. *et al.* Recombinant human laminin isoforms can support the undifferentiated growth of human embryonic stem cells. *Biochem. Biophys. Res. Commun.* **375**, 27–32 (2008).
- Ido, H. *et al.* Molecular dissection of the α -dystroglycan- and integrin-binding sites within the globular domain of human laminin-10. *J. Biol. Chem.* **279**, 10946–10954 (2004).
- Smirnov, S. P. *et al.* Contributions of the LG modules and furin processing to laminin-2 functions. *J. Biol. Chem.* **277**, 18928–18937 (2002).
- Doi, M. *et al.* Recombinant human laminin-10 ($\alpha 5 \beta 1 \gamma 1$). Production, purification, and migration-promoting activity on vascular endothelial cells. *J. Biol. Chem.* **277**, 12741–12748 (2002).
- Kortessmaa, J., Yurchenco, P. & Tryggvason, K. Recombinant laminin-8 ($\alpha 4 \beta 1 \gamma 1$). Production, purification, and interactions with integrins. *J. Biol. Chem.* **275**, 14853–14859 (2000).
- Kariya, Y. *et al.* Efficient expression system of human recombinant laminin-5. *J. Biochem.* **132**, 607–612 (2002).
- Vuoristo, S. *et al.* Laminin isoforms in human embryonic stem cells: synthesis, receptor usage and growth support. *J. Cell Mol. Med.* **13**, 2622–2633 (2009).
- Nishiuchi, R. *et al.* Ligand-binding specificities of laminin-binding integrins: a comprehensive survey of laminin-integrin interactions using recombinant $\alpha 3 \beta 1$, $\alpha 6 \beta 1$, $\alpha 7 \beta 1$ and $\alpha 6 \beta 4$ integrins. *Matrix Biol.* **25**, 189–197 (2006).
- Meng, Y. *et al.* Characterization of integrin engagement during defined human embryonic stem cell culture. *FASEB J.* **24**, 1056–1065 (2010).

29. Ido, H. *et al.* The requirement of the glutamic acid residue at the third position from the carboxyl termini of the laminin gamma chains in integrin binding by laminins. *J. Biol. Chem.* **282**, 11144–11154 (2007).
30. Taniguchi, Y. *et al.* The C-terminal region of laminin beta chains modulates the integrin binding affinities of laminins. *J. Biol. Chem.* **284**, 7820–7831 (2009).
31. Deutzmann, R. *et al.* Cell adhesion, spreading and neurite stimulation by laminin fragment E8 depends on maintenance of secondary and tertiary structure in its rod and globular domain. *Eur. J. Biochem.* **191**, 513–522 (1990).
32. Takagi, J. Structural basis for ligand recognition by integrins. *Curr. Opin. Cell Biol.* **19**, 557–564 (2007).
33. Watanabe, K. *et al.* A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nature Biotech.* **25**, 681–686 (2007).
34. Vachon, P. H. Integrin signaling, cell survival, and anoikis: distinctions, differences, and differentiation. *J. Signal Transduct.* **2011**, 738137 (2011).
35. Prowse, A. B. *et al.* Long term culture of human embryonic stem cells on recombinant vitronectin in ascorbate free media. *Biomaterials* **31**, 8281–8288 (2010).
36. Lu, J., Hou, R., Booth, C. J., Yang, S. H. & Snyder, M. Defined culture conditions of human embryonic stem cells. *Proc. Natl Acad. Sci. USA* **103**, 5688–5693 (2006).
37. Phanstiel, D. H. *et al.* Proteomic and phosphoproteomic comparison of human ES and iPS cells. *Nat. Methods* **8**, 821–827 (2011).
38. Andrews, P. D. *et al.* High-content screening of feeder-free human embryonic stem cells to identify pro-survival small molecules. *Biochem. J.* **432**, 21–33 (2010).
39. Xu, Y. *et al.* Revealing a core signaling regulatory mechanism for pluripotent stem cell survival and self-renewal by small molecules. *Proc. Natl Acad. Sci. USA* **107**, 8129–8134 (2010).
40. Redmer, T. *et al.* E-cadherin is crucial for embryonic stem cell pluripotency and can replace OCT4 during somatic cell reprogramming. *EMBO Rep.* **12**, 720–726 (2011).
41. Chen, T. *et al.* E-cadherin-mediated cell-cell contact is critical for induced pluripotent stem cell generation. *Stem Cells* **28**, 1315–1325 (2010).
42. Schaller, M. D. Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. *J. Cell Sci.* **123**, 1007–1013 (2010).
43. Li, L. *et al.* Individual cell movement, asymmetric colony expansion, rho-associated kinase, and E-cadherin impact the clonogenicity of human embryonic stem cells. *Biophys. J.* **98**, 2442–2451 (2010).
44. Sekiguchi, K. & Hakomori, S. Domain structure of human plasma fibronectin. Differences and similarities between human and hamster fibronectins. *J. Biol. Chem.* **258**, 3967–3973 (1983).
45. Yatohgo, T., Izumi, M., Kashiwagi, H. & Hayashi, M. Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct. Funct.* **13**, 281–292 (1988).
46. Suemori, H. *et al.* Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. *Biochem. Biophys. Res. Commun.* **345**, 926–932 (2006).

Acknowledgements

We thank Ms Mari Hamao and Ms Ritsuko Goto for their valuable technical assistance and the members of the laboratories of N.N. and K.S. for insightful discussions. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO) of Japan, and partially supported by the Coordination, Support and Training Programme for Translational Research of the Ministry of Education, Culture, Sports, Science and Technology of Japan (to K.S.).

Author contributions

T.M., K.S. and E.K. designed research, T.M., S.F., Y.T., M.Y., M.K., M.H. and H.K. performed research, T.M., H.S., N.N., K.S. and E.K. analysed data and T.M., K.S. and E.K. wrote the paper.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at <http://npg.nature.com/reprintsandpermissions/>

How to cite this article: Miyazaki, T. *et al.* Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells. *Nat. Commun.* **3**:1236 doi: 10.1038/ncomms2231 (2012).



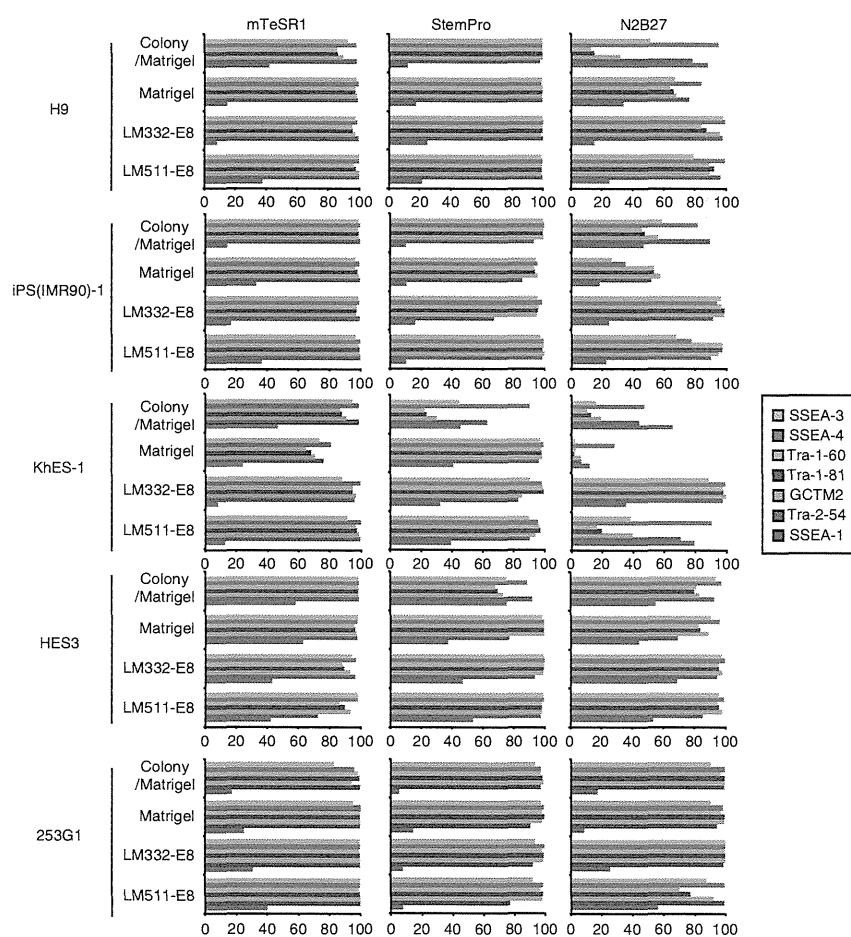
This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/3.0/>

Erratum: Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells

Takamichi Miyazaki, Sugiko Futaki, Hirofumi Suemori, Yukimasa Taniguchi, Masashi Yamada, Miwa Kawasaki, Maria Hayashi, Hideaki Kumagai, Norio Nakatsuji, Kiyotoshi Sekiguchi & Eihiro Kawase

Nature Communications 3:1236 doi: 10.1038/ncomms2231 (2012); Published 4 Dec 2012; Updated 29 Jul 2013

In the key to Fig. 3 of this Article, labels for the colours representing SSEA-1 and Tra-2-54 were inadvertently switched during the production process. The correct version of the figure appears below.





The SMAD2/3 corepressor SNON maintains pluripotency through selective repression of mesendodermal genes in human ES cells

Norihiro Tsuneyoshi, Ee Kim Tan, Akila Sadasivam, et al.

Genes Dev. 2012 26: 2471-2476

Access the most recent version at doi:10.1101/gad.201772.112

Supplemental Material <http://genesdev.cshlp.org/content/suppl/2012/11/12/26.22.2471.DC1.html>

References This article cites 33 articles, 8 of which can be accessed free at:
<http://genesdev.cshlp.org/content/26/22/2471.full.html#ref-list-1>

Email alerting service Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#)

TrueORF Gold
cDNA Clones

Validated for Protein Expression!

LEARN MORE >

ORIGENE
Your Gene Company

To subscribe to *Genes & Development* go to:
<http://genesdev.cshlp.org/subscriptions>

Copyright © 2012 by Cold Spring Harbor Laboratory Press

RESEARCH COMMUNICATION

The SMAD2/3 corepressor SNON maintains pluripotency through selective repression of mesendodermal genes in human ES cells

Norihiro Tsuneyoshi,¹ Ee Kim Tan,¹ Akila Sadasivam,¹ Yogavalli Poobalan,¹ Tomoyuki Sumi,^{2,3} Norio Nakatsuji,^{4,5} Hirofumi Suemori,² and N. Ray Dunn^{1,6}

¹Institute of Medical Biology, A*STAR (Agency for Science, Technology, and Research), Singapore 138648, Singapore;

²Department of Embryonic Stem Cell Research, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan; ³Department of Developmental Biology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan; ⁴Department of Development and Differentiation, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan; ⁵Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Kyoto 606-8501, Japan

Activin/Nodal signaling via SMAD2/3 maintains human embryonic stem cell (hESC) pluripotency by direct transcriptional regulation of *NANOG* or, alternatively, induces mesoderm and definitive endoderm (DE) formation. In search of an explanation for these contrasting effects, we focused on SNON (SKIL), a potent SMAD2/3 corepressor that is expressed in hESCs but rapidly down-regulated upon differentiation. We show that SNON predominantly associates with SMAD2 at the promoters of primitive streak (PS) and early DE marker genes. Knockdown of SNON results in premature activation of PS and DE genes and loss of hESC morphology. In contrast, enforced SNON expression inhibits DE formation and diverts hESCs toward an extraembryonic fate. Thus, our findings provide novel mechanistic insight into how a single signaling pathway both regulates pluripotency and directs lineage commitment.

Supplemental material is available for this article.

Received July 23, 2012; revised version accepted September 28, 2012.

Since the isolation of human embryonic stem cells (hESCs) more than a decade ago, numerous extrinsic signals have been implicated in the maintenance of their cardinal features of pluripotency and indefinite self-renewal (Pera and Tam 2010). These include growth factors belonging to the TGF β , FGF, WNT, and IGF families, which are often supplemented with culture

medium containing serum, feeder cell-conditioned medium, and/or ill-defined knockout serum replacement (Price et al. 1998). These various and complex platforms have obscured the identification of the minimal complement of extracellular cues that govern the intrinsic transcriptional regulators of pluripotency and self-renewal. Recently, however, it was shown that either recombinant TGF β 1 or the related ligand Activin A in combination with FGF2 can maintain hESCs in chemically defined and feeder-free conditions partly through direct regulation of the core pluripotency factor gene *NANOG* (Xu et al. 2008; Vallier et al. 2009).

Both TGF β and Activin signals are transduced intracellularly by phosphorylation of the cytoplasmic effector proteins SMAD2 and SMAD3 (Schmierer and Hill 2007). Once activated, they partner with SMAD4, translocate to the nucleus, and associate with tissue-specific transcription factors to regulate batteries of target genes. Chromatin immunoprecipitation (ChIP) experiments show that SMAD2/3 bind the *NANOG* promoter, and luciferase reporter assays confirm that this SMAD-binding element (SBE) is crucial for *NANOG* transcriptional activity (Xu et al. 2008; Vallier et al. 2009). Recently, SMAD2/3 ChIP combined with next-generation sequencing (ChIP-seq) revealed that, in addition to *NANOG*, SMAD2/3 occupy a broad number of genes both expressed in hESCs and associated with the maintenance of pluripotency (Brown et al. 2011; Kim et al. 2011). These include the additional core pluripotency factors *OCT4* and *SOX2* as well as other key regulators, such as *SUZ12*, *UTF1*, *TERT*, *DPPA4*, and *LIN28A*. Taken together, these findings emphasize the importance of continuous TGF β /Activin signaling for the maintenance of hESC pluripotency. However, it is a third divergent TGF β -related ligand, Nodal, that carries out this fundamental activity in vivo.

Nodal transcripts identify the pluripotent cells within the mouse blastocyst inner cell mass (ICM) and persist in these cells as they extensively reorganize into the cup-shaped epiblast epithelium after implantation (Mesnard et al. 2006; Arnold and Robertson 2009). In the absence of *Nodal*, null mutant embryos down-regulate *Oct4*, *Nanog*, and *Foxd3*, which are normally uniformly expressed throughout the epiblast; prematurely and ectopically up-regulate neural markers; and fail to gastrulate (Camus et al. 2006; Mesnard et al. 2006). With the onset of gastrulation, *Nodal* expression resolves to the posterior primitive streak (PS), where epiblast cells ingress and emerge as mesoderm and definitive endoderm (DE) (Arnold and Robertson 2009). Extensive genetic studies have shown that the highest levels of Nodal specify DE, while the assorted mesodermal lineages are patterned by intermediate to lower Nodal levels (Arnold and Robertson 2009). Thus, there is a remarkable conversion in a narrow developmental time span in how Nodal signals are interpreted intracellularly—from maintenance of pluripotency within the ICM/epiblast to inducer of differentiation within the PS. This raises the intriguing question of how Nodal/Smad2/3 signals in the ICM/epiblast engage only the pluripotency machinery and avert precocious activation of differentiation genes. This question also stringently applies to hESCs, which share many defining characteristics with the mouse epiblast and its derivative cell lines (termed epiblast stem cells [EpiSCs]) (Pera and Tam 2010),

[**Keywords:** Activin/Nodal; SNON; human embryonic stem cells; mesendoderm; pluripotency; repressor]

Corresponding author

E-mail ray.dunn@imb.a-star.edu.sg

Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.201772.112>.

Tsuneyoshi et al.

as the Nodal analog Activin A both regulates pluripotency and is nearly universally used to initiate mesendoderm differentiation in vitro to produce a variety of lineage-specific cell types (Sulzbacher et al. 2009).

We reasoned that one explanation for these contrasting effects is the existence of discriminating nuclear repressor proteins. Moreover, the expression of such a repressor would likely be under the direct control of the core pluripotency factors. Previous ChIP combined with DNA microarrays (ChIP-chip) identified numerous active and inactive genes that are triply bound by OCT4, SOX2, and NANOG in hESCs (Boyer et al. 2005). One of these is the SKI-related proto-oncogene *SNON* (*SKIL*) that encodes a potent negative regulator of TGF β -related signaling (Deheuninck and Luo 2009; Zhu and Luo 2012). *SNON* physically interacts with SMAD2, SMAD3, and SMAD4, disrupting their assembly into an activated heteromeric complex (Stroschein et al. 1999; Wu et al. 2002). In addition, *SNON* prevents SMAD2 and SMAD3 from associating with the transcriptional coactivator p300/CBP and recruits a repressor complex comprised of nuclear receptor corepressor (N-CoR) and histone deacetylase (HDAC) (Schmierer and Hill 2007). Thus, the presence of *SNON* at a particular SBE ensures transcriptional repression. Here, we found that *SNON* protein is highly abundant in hESCs and specifically enriched at differentiation gene promoters. Loss of *SNON* in hESCs prematurely activates PS and early DE genes and destabilizes pluripotency, while constitutive *SNON* expression restrains differentiation into DE. Taken together, our results identify *SNON* as a key component of the nuclear machinery that safeguards pluripotency through direct repression of differentiation genes downstream from Activin/Nodal.

Results and Discussion

SNON is abundant in hESCs and is targeted to the proteasome during early differentiation

We first sought to confirm the expression of *SNON* in pluripotent hESCs and examine its regulation during DE formation in vitro. Differentiation was carried out according to the schematic in Figure 1A (Teo et al. 2012) and, expectedly, resulted in the loss of pluripotency markers (*NANOG*, *OCT4*, and *SOX2*) and up-regulation of PS (*WNT3*, *FGF8*, *EOMES*, and *MIXL1*), mesendoderm (*GSC* and *LHX1*), and DE (*FOXA2* and *SOX17*) markers by day 5 (Supplemental Fig. 1A). These data were confirmed by Western analysis, immunohistochemistry, and FACS (Supplemental Fig. 1B–D). We observed that *SNON* transcript levels are robust in undifferentiated HES3 and on day 1 of differentiation but drop considerably by day 3 and decline further on day 5 (Fig. 1B).

This decrease in *SNON* levels in vitro closely mirrors the expression dynamics of *SnoN* in the early mouse embryo. Prior to gastrulation, *SnoN* transcripts label the pluripotent epiblast epithelium (Supplemental Fig. 1E). Mouse ESCs and EpiSCs also express *SnoN* (Supplemental Fig. 1F). With the onset of gastrulation, *SnoN* transcript levels are conspicuously down-regulated in the region of the incipient PS and continue to decline posteriorly as the PS lengthens (Supplemental Fig. 1E). Nascent mesoderm and DE also lack *SnoN* expression. By the late PS stage (embryonic day 7.5 [E7.5]), *SnoN* transcripts resolve to the anterior epiblast, but shortly

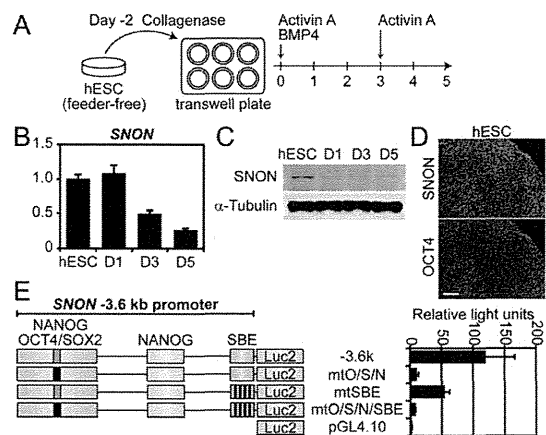


Figure 1. *SNON* expression in hESCs is controlled by SMAD2/3 and OCT4, SOX2, and NANOG. (A) hESCs were differentiated into DE for 5 d according to the schematic. Activin A and BMP4 (both at 50 ng/mL) were added on day 0. Only Activin A was replenished on day 3. (B) *SNON* expression levels during DE differentiation by qPCR. Data were normalized against *GUSB* and are shown relative to undifferentiated hESCs (= 1.0). (C) Western blot analysis for *SNON* protein levels during DE differentiation. (D) OCT4 and *SNON* immunofluorescence on undifferentiated HES3 (hESCs). Bar, 100 μ m. (E) Luciferase reporter assays of *SNON* promoter activity in HES3. Black shading indicates specific mutation of the OCT4/SOX2/NANOG-binding sites in the 5' distal enhancer or of the four SBEs proximal to the *SNON* transcriptional start site.

thereafter, this domain has largely disappeared. These expression data suggest that loss of the Smad2/3 co-repressor *SnoN* is an obligate step in the production of PS-derived lineages.

At the protein level, *SNON* is abundant in undifferentiated HES3 but, in contrast to our quantitative PCR (qPCR) data, drastically reduced by day 1 (Fig. 1C,D). *SKI*, which is closely related to *SNON*, is also present in hESCs, but its levels fluctuate negligibly between days 1 and 5 (Supplemental Fig. 1B,G). Several E3 ubiquitin ligases previously shown to target *SNON* to the proteasome are expressed in hESCs and during differentiation, including SMURF2, the RING domain-containing protein ARKADIA (RNF111), and the anaphase-promoting complex/cyclosome (APC/C) (Deheuninck and Luo 2009). *SMURF2* levels are up-regulated on day 1 and then plateau, while expression of *ARKADIA* and two APC/C subunits—*CDH1* (*FZR1*) and *ANAPC2*—increase gradually (Supplemental Fig. 1G). Similar results were observed by Western for SMURF2 and ARKADIA (Supplemental Fig. 1B). Other APC/C components, including *ANAPC11*, *CDC16*, and *CDC27*, were equally expressed at all time points (Supplemental Fig. 1G).

To confirm that the rapid loss of *SNON* protein is indeed due to proteasomal degradation, feeder-free HES3 cells were treated with two proteasome inhibitors: MG132 and lactacystin. When HES3 cells are differentiated in the presence of MG132 or lactacystin, *SNON* protein levels expectedly persist on day 1 (Supplemental Fig. 1H). Taken together, these data show that *SNON* expression is tightly linked to pluripotency and that *SNON* is targeted to the proteasome at the onset of DE formation.

SNON represses mesendoderm genes in hESCs

SNON expression in hESCs is controlled by SMAD2/3 and OCT4, SOX2, and NANOG

ChIP–chip, recent ChIP–seq, and phylogenetic alignments reveal a series of conserved SMAD2/3- and OCT4/SOX2/NANOG-binding regions within the ~5-kb *SNON* promoter/enhancer (Fig. 1E; Supplemental Fig. 1I; Boyer et al. 2005; Zhu et al. 2005; Brown et al. 2011; Kim et al. 2011). We investigated the contribution of each of these elements to *SNON* regulation in undifferentiated hESCs using luciferase reporter constructs. Serial deletion analysis showed that the distal 5' OCT4/SOX2/NANOG-binding region is essential for robust reporter activity (Supplemental Fig. 1I). The more proximal NANOG and SMAD2/3 elements contribute modestly to the regulation of *SNON* when compared with the –3.6-kb construct (Supplemental Fig. 1I). We also generated a series of –3.6-kb reporters in which OCT4/SOX2/NANOG- and SMAD2/3-binding sites were individually mutated (Fig. 1E; Supplemental Table 1). Consistent with our deletion studies, the mutated OCT4/SOX2/NANOG (mtO/S/N) construct in which distal OCT4/SOX2/NANOG binding is specifically disrupted shows significantly reduced (~13-fold) reporter activity (Fig. 1E). In contrast, mutating all four SBEs (mtSBEs) diminishes *SNON* reporter activity roughly twofold (Fig. 1E). Taken together, these data establish that *SNON* is TGF β -responsive and that OCT4/SOX2/NANOG serve as critical upstream regulators of *SNON* expression in hESCs.

SNON knockdown destabilizes pluripotency

To analyze *SNON* function, we first performed tetracycline (Tet)-inducible shRNA-mediated knockdown of *SNON* in S4TR5 cells, a derivative of the Shef4 hESC line that constitutively expresses the nuclear Tet repressor (TetRnls) (Zafarana et al. 2009). Two independent and previously validated human *SNON*-shRNA target sequences—shSNON(954) and shSNON(1307)—were transfected into S4TR5 cells (Sarker et al. 2005; Zhu et al. 2007). After selection, 11 of 12 clones for shSNON(954) and 10 of 12 clones for shSNON(1307) showed ~80% *SNON* knockdown by qPCR after 3 d of doxycycline (Dox) treatment (data not shown). Two clones, designated shSNON(954) and shSNON(1307), were chosen for further study. Both were indistinguishable by morphology (Supplemental Fig. 2A) and showed OCT4, SOX2, and NANOG levels comparable with the parental S4TR5 line by qPCR and Western (Supplemental Fig. 2B,C). Dox treatment resulted in a significant knockdown of *SNON* transcripts on day 1 (Fig. 2A,B). Interestingly, both shSNON clones lost their compact colony morphology after 5 d of continuous culture in Dox and appeared differentiated (Supplemental Fig. 2A). This was accompanied by a specific increase in PS (*BRACHYURY*, *MIXL1*, and *EOMES*), mesendoderm (*GSC*), and early DE (*FOXA2* and *SOX17*) markers beginning around day 3 (Fig. 2B; Supplemental Fig. 2B). Neuroectodermal gene expression was not above background (Supplemental Fig. 2B) and, importantly, levels of *OCT4*, *SOX2*, and *NANOG* remained unchanged (Supplemental Fig. 2B,C). In addition, loss of *SNON* did not impact SKI, SMURF2, SMAD2/pSMAD2, or SMAD3/pSMAD3 levels, suggesting that the genes encoding these proteins are not regulated by the *SNON* corepressor in hESCs (Supplemental Fig. 2C). Consistent with our qPCR data, Western analysis

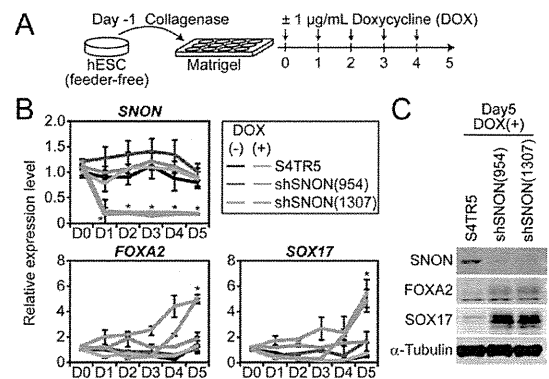


Figure 2. *SNON* knockdown induces differentiation in hESCs. (A) Schematic of the experimental design using two stable DOX-inducible S4TR5 hESC lines—shSNON(954) and shSNON(1307)—where DOX was replenished daily for 5 d. (B) *SNON*, *FOXA2*, and *SOX17* expression analysis by qPCR. RNA was collected on days 0–5 according to A. Data were normalized against *GUSB* and are depicted relative to S4TR5 (DOX–) at day 0 (= 1.0). *P*-values were calculated according to the Student's *t*-test. (*) *P* < 0.05. (C) Western blot analysis for *SNON*, *FOXA2*, and *SOX17*.

revealed that *SNON* is hardly detectable on day 1, and low levels of *FOXA2* were first detected on day 2, followed by *SOX17* on day 5 (Fig. 2C; Supplemental Fig. 2D). Immunofluorescence also confirmed loss of *SNON*, persistence of *OCT4*, and up-regulation of *SOX17* and *FOXA2* on day 5 (Supplemental Fig. 2E,F).

To provide additional evidence that loss of *SNON* results in differentiation, we performed a replating experiment whereby S4TR5 cells and both knockdown clones were passaged after 5 d of Dox treatment and cultured for four additional days in Dox. *OCT4*-DAB staining was used to identify hESC colonies and clearly revealed the dramatically reduced plating efficiency of the shSNON knockdown clones after passaging (Supplemental Fig. 2G). Taken together, our results show that diminished *SNON* levels in hESCs destabilizes pluripotency through precocious activation of mesendodermal genes.

SNON overexpression blocks differentiation into DE and mesoderm

We next performed the reciprocal experiment by overexpressing *SNON* in hESCs. After repeated attempts, we were unable to isolate HES3 subclones with levels greater than endogenous *SNON* (data not shown). We therefore chose to overexpress a stable truncated form of *SNON*—*SNON*(1–366)—that retains its ability to bind SMAD2/3 and is sufficient for transcriptional repression but lacks the C-terminal lysine residues critical for ubiquitination and proteasomal degradation (Stroschein et al. 1999, 2001). Two *SNON*(1–366) clones (#1 and #9) were isolated that produce roughly equivalent levels of *SNON*(1–366) (Fig. 3A) and remain undifferentiated, with *OCT4* immunoreactivity comparable with wild-type HES3 (Supplemental Fig. 3A).

Clones #1 and #9 were then subjected to DE differentiation according to the schematic in Figure 1A, along with two controls: wild-type HES3 and HES3 harboring pCAG-IRES-puro (vector control). *NANOG*, *OCT4*, *SOX2*, and *DPPA4* levels declined with similar kinetics in controls

Tsuneyoshi et al.

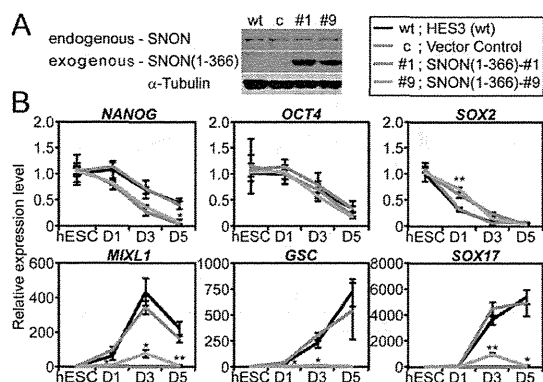


Figure 3. SNON overexpression inhibits DE formation. (A) Western blot analysis detects abundant C-terminally truncated, constitutively active SNON(1–366) in two stable, clonally selected lines (#1 and #9). (B) Expression analysis of the indicated genes by qPCR. hESCs were differentiated according to the schematic in Figure 1A. Data were normalized against *GUSB* and are shown relative to parental wild-type undifferentiated hESCs (= 1.0). The “vector control” HES3 line contains only the pCAG-IRES-Puro construct. *P*-values were calculated according to the Student’s *t*-test. (*) $P < 0.05$; (**) $P < 0.01$.

and clones #1 and #9 (Fig. 3B; Supplemental Fig. 3C). HES3 and the vector control cell line predictably showed up-regulation of the PS markers (*BRACHYURY*, *WNT3*, *FGF8*, *MIXL1*, and *EOMES*) as well as mesendodermal (*LHX1* and *GSC*) and DE (*FOXA2* and *SOX17*) markers beginning on day 1 (Fig. 3B; Supplemental Fig. 3C). Clones #1 and #9 failed to activate the expression of PS, mesendodermal, and DE marker genes, all of which are known Activin/Nodal targets and bound by SMAD2/3 (Fig. 3B; Supplemental Fig. 3C; Schmierer and Hill 2007; Arnold and Robertson 2009). This result is entirely consistent with the ability of SNON(1–366) to strongly suppress the synthetic ARE-lux Activin/Nodal pathway reporter, which contains three repeats of a *Xenopus* Activin response element (ARE) that binds SMAD2/4 and the forkhead transcription factor FOXH1. Human *GSC*-Luc2 and *MIXL1*-Luc2 reporters containing their respective endogenous AREs are similarly repressed by SNON(1–366) (Supplemental Fig. 3D). When confronted with culture conditions tailored to produce cardiomyocytes (Hudson et al. 2012), SNON(1–366)-overexpressing clones also fail to up-regulate early mesodermal progenitor markers (Supplemental Fig. 3E). SNON(1–366) therefore serves as a potent inhibitor of both mesoderm and DE formation downstream from Activin/Nodal signals. Consequently, SNON(1–366)-expressing clones initiate extraembryonic lineage differentiation in response to Activin A and BMP4 treatment, as evidenced by the up-regulation of the trophoblast marker genes *CDX2*, *CGA*, *CYP19A*, *GCM1*, *KRT7*, *LGALS16*, and *VGLL1* and the visceral/parietal endoderm marker *SOX7* (Supplemental Fig. 3C). In addition, no induction of early neuroectodermal markers (*NEUROD1* and *SOX1*) was observed (Supplemental Fig. 3C).

SNON occupies SBEs in mesendodermal genes

To formally test whether SNON binds and represses PS and mesendodermal genes in undifferentiated hESCs, we performed SMAD2, SMAD3, and SNON ChIP followed

by qPCR for enrichment at select SBEs. Given the high percentage of amino acid identity between SMAD2 and SMAD3 and the potential for antibody cross-reactivity, we used monoclonal anti-SMAD2 and anti-SMAD3 antibodies that specifically distinguish between these two proteins (Supplemental Figs. 1K, 4B). Among “stem cell factor” genes (*NANOG*, *OCT4*, *SOX2*, *DPPA4*, and *LIN28*) that are highly expressed in hESCs, SNON occupancy at their respective SBEs was low to undetectable (Fig. 4A; Supplemental Fig. 4A). Only *SUZ12* showed statistically significant enrichment for SNON ($P < 0.05$). However, *SUZ12*, like the other stem cell factor genes analyzed, was insensitive to SNON(1–366) overexpression (Supplemental Fig. 3C). In contrast, SNON highly occupied PS (*FGF8*, *WNT3*, *EOMES*, and *MIXL1*) and mesendodermal (*GSC* and *LHX1*) genes (Fig. 4A; Supplemental Fig. 4A). SMAD2 bound all SBEs analyzed by ChIP (Fig. 4A; Supplemental Fig. 4A). Consistent with the ability of SNON to recruit HDACs, acetylated H3 and H4 levels at PS/mesendodermal genes were low in undifferentiated HES3 cells but increased with differentiation (Supplemental Fig. 4C; Xi et al. 2011). The converse was observed at stem cell factor gene promoters, with declining acetylation mirroring the decrease in pluripotency gene expression during differentiation (Supplemental Figs. 1A, 3C, 4C). Taken together, these data are wholly consistent with SNON in combination with SMAD2/SMAD4 acting as a key transcriptional repressor of differentiation-specific genes in pluripotent hESCs (Fig. 4B).

Our ChIP data also reveal a bias of SMAD3 toward stem cell factor gene promoters (Fig. 4A; Supplemental Fig. 4A). In a very recent study, Mullen et al. (2011) reported that OCT4 and SMAD3 are predominantly associated with transcriptionally active genes in hESCs and, importantly, co-occupy the genome by binding adjacent DNA sites. Our results show that no repression of pluripotency genes was observed in hESC clones overexpressing SNON(1–366), which retains its ability to bind SMAD3 but cannot be targeted by E3 ligases (Fig. 3B; Supplemental Fig. 3C). These genes include *NANOG*, whose regulation by TGF β /Activin/SMAD2/3 in hESCs is extremely well characterized (Xu et al. 2008; Vallier et al. 2009). If SNON were a general repressor downstream from TGF β -related signaling, modulating the overall activity of the TGF β pathway in hESCs, ectopic expression of SNON(1–366) would be predicted to suppress *NANOG* and other crucial pluripotency genes and promote differentiation. This is not what we observed (Fig. 3B; Supplemental Fig. 3D). We therefore propose that OCT4 serves a central “protective” role in averting SNON repression of SMAD3-bound pluripotency genes (Fig. 4B). In contrast, selective recruitment of SNON represses PS/mesendoderm genes in hESCs until extrinsic cues trigger its degradation and allow differentiation to unfurl.

One outstanding question is thus the identity of the E3 ligases specifically responsible for the rapid clearance of SNON at the onset of differentiation. Genetic evidence in mice favors a role for Arkadia because *Arkadia*-null mutant embryos succumb to severe gastrulation defects due to impaired Nodal/Smad2/3 signaling (Episkopou et al. 2001; Mavrikakis et al. 2007). One interpretation of this phenotype is that in the absence of Arkadia, SnoN associates with crucial Nodal/Smad2/3 target genes,

SNON represses mesendoderm genes in hESCs

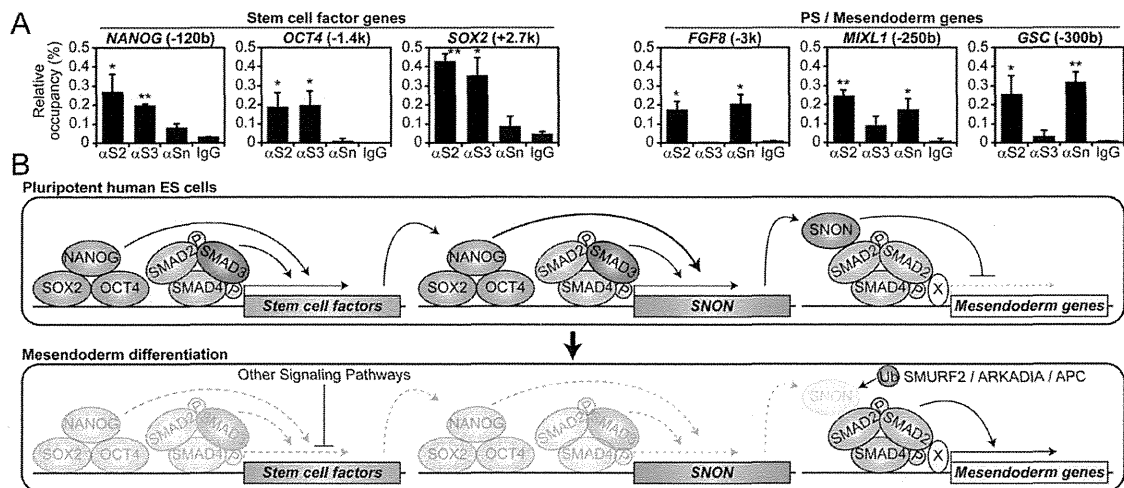


Figure 4. SNON highly and selectively occupies the promoters of PS and mesendodermal genes in hESCs. (A) ChIP-qPCR analysis of SNON-binding (α Sn), SMAD2-binding (α S2), and SMAD3-binding (α S3) sites at the indicated gene regions in HES3 cultured in mTeSR1 medium. Relative occupancy values are shown as the apparent immunoprecipitation efficiency (percentage) [ratio = immunoprecipitated DNA/input DNA]. (IgG) Normal rabbit IgG as negative control. *P*-values were calculated according to the Student's *t*-test. (* *P* < 0.05; ** *P* < 0.01). (B) Proposed model for SNON function in hESCs. In pluripotent hESCs, extrinsic signals (e.g., TGF β /Activin/Nodal) regulate stem cell factor gene expression through activated SMAD2/3/4 complexes. OCT4/SOX2/NANOG positively regulate their own promoters and activate *SNON* transcription. SMAD2/3 also regulate *SNON*. SNON is selectively recruited to SMAD2-bound mesendodermal genes and suppresses their transcription. During early differentiation, stem cell factor gene expression and SMAD2/3/4 occupancy at the SNON promoter/enhancer decline. Consequently, *SNON* expression decreases. Existing SNON protein is rapidly degraded in a ligand-dependent manner by E3 ubiquitin ligases (e.g., SMURF2, ARKADIA, or APC). This leads to the derepression of early PS and DE target genes (collectively referred to as mesoderm). (X) Tissue-specific transcriptional coactivators such as FOXH1.

preventing their activation. Consistent with this prediction, SnoN protein accumulates in *Arkadia*-deficient mouse ESC lines (Nagano et al. 2007). Thus, SNON degradation in hESCs may similarly require the stimulus-dependent activation of ARKADIA or the synergistic involvement of other ligand-dependent E3 ligases, such as SMURF2 (Bonni et al. 2001). It is important to note that there are >600 E3 ligases and substrate recognition subunits encoded by the human genome (Li et al. 2008), many of which are enriched in hESCs (Assou et al. 2009). This finding presages the heightened sensitivity that hESCs exhibit to proteasome inhibitors (Assou et al. 2009; Vilchez et al. 2012; data not shown) and further suggests that there are additional hitherto uncharacterized regulators that act alongside ARKADIA, APC, and SMURF2 to target SNON to the proteasome.

Materials and methods

Cell culture

HES3 cells were maintained as previously described (Suemori et al. 2006). For feeder-free culture, HES3 and S4TR5 cells (Zafarana et al. 2009) were maintained in mTeSR1 medium (Stem Cell Technologies) on 1:200 GFR-Matrigel-coated (Becton Dickinson) dishes. DE differentiation was carried out as previously described (Teo et al. 2012).

Tet-inducible shRNA knockdown

Previously validated human *SNON* shRNA target sequences were ligated into pSUPERIOR-Zeocin (Supplemental Material; Sarker et al. 2005; Zhu et al. 2007). S4TR5 cells were transfected with the shRNA constructs using FuGene HD (Roche) and selected for 2 wk using 2.5 μ g/mL Zeocin (Invitrogen).

SNON overexpression

Human *SNON*(1–366) cDNA was PCR-amplified and ligated into pCAG-IRES-Puro (Supplemental Material; Sumi et al. 2007). Feeder-free HES3 cells were transfected with pCAG-*SNON*(1–366)-IRES-Puro or empty vector using FuGene HD and selected for 2 wk using 1 μ g/mL puromycin (Sigma).

qPCR analysis

Total RNA was extracted using the RNeasy kit (Qiagen), and 0.5–2 μ g was reverse-transcribed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed using TaqMan Fast Advanced Master Mix or Power SYBR Green PCR Master Mix. Relative quantitation was performed using $2^{-\Delta\Delta CT}$ and normalized against *GUSB*. TaqMan gene expression assays and primers are listed in Supplemental Table 2.

Luciferase assay

An ~5-kb segment of the human *SNON* promoter was PCR-amplified (Supplemental Table 1) and subcloned into pGL4.10[*luc2*] (Promega). pTK-*hRluc* was used for data normalization (Supplemental Material). Molar equivalents of each *luc2* reporter vector and 0.05 μ g of pTK-*hRluc* were cotransfected into HES3 using FuGene HD. After 48 h, both firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega). Relative luciferase units were calculated by determining the ratio between firefly and *Renilla* luciferase activities and normalizing against the pGL4.10 vector control.

Immunofluorescence and immunocytochemical analysis

For immunofluorescence, hESCs were fixed in 3.7% formaldehyde/PBS for 15 min, permeabilized with 0.2% Triton X-100/PBS for 5 min, and incubated with primary antibodies (Supplemental Table 3). After incubation with Alexa Fluor 488- or 594-conjugated secondary antibodies

Tsuneyoshi et al.

(Invitrogen), nuclei were counterstained with DAPI (Sigma). For immunocytochemical staining of OCT4, hESCs were fixed, permeabilized, and incubated with anti-OCT4 antibody (Supplemental Table 3). After incubation with HRP-conjugated secondary antibody (Dako), antigens were visualized with DAB (Sigma).

Western blot analysis

Cell lysates were separated by SDS-PAGE, transferred to Immobilon-FL PVDf membrane (Millipore), and probed with primary antibodies (Supplemental Table 3). After incubation with IRDye 800CW- or 680LT-conjugated secondary antibodies (LI-COR), proteins were detected using the Odyssey infrared imaging system (LI-COR).

ChIP and quantitative real-time PCR

ChIP was performed as described previously (Boyer et al. 2005). Additional details are provided in the Supplemental Material.

Acknowledgments

We thank Stuart Avery and Peter Andrews for providing S4RT5 cells, Ludovic Vallier for mEpiSC RNA, and Alan Colman, Dmitry Bulavin, Leah Vardy, Matthew Lovatt, Oz Pomp, Elizabeth Robertson, Elizabeth Bikoff, and Oliver Dreesen for insightful discussions and comments on the manuscript.

References

- Arnold SJ, Robertson EJ. 2009. Making a commitment: Cell lineage allocation and axis patterning in the early mouse embryo. *Nat Rev Mol Cell Biol* 10: 91–103.
- Assou S, Cerecedo D, Tondeur S, Pantesco V, Hovatta O, Klein B, Hamamah S, De Vos J. 2009. A gene expression signature shared by human mature oocytes and embryonic stem cells. *BMC Genomics* 10: 10. doi: 10.1186/1471-2164-10-10.
- Bonni S, Wang HR, Causing CG, Kavsak P, Stroschein SL, Luo K, Wrana JL. 2001. TGF- β induces assembly of a Smad2-Smurf2 ubiquitin ligase complex that targets SnoN for degradation. *Nat Cell Biol* 3: 587–595.
- Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, et al. 2005. Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 122: 947–956.
- Brown S, Teo A, Pauklin S, Hannan N, Cho CH, Lim B, Vardy L, Dunn NR, Trotter M, Pedersen R, et al. 2011. Activin/Nodal signaling controls divergent transcriptional networks in human embryonic stem cells and in endoderm progenitors. *Stem Cells* 29: 1176–1185.
- Carrus A, Perea-Gomez A, Moreau A, Collignon J. 2006. Absence of Nodal signaling promotes precocious neural differentiation in the mouse embryo. *Dev Biol* 295: 743–755.
- Deheuninck J, Luo K. 2009. Ski and SnoN, potent negative regulators of TGF- β signaling. *Cell Res* 19: 47–57.
- Episkopou V, Arkell R, Timmons PM, Walsh JJ, Andrew RL, Swan D. 2001. Induction of the mammalian node requires Arkadia function in the extraembryonic lineages. *Nature* 410: 825–830.
- Hudson J, Titmarsh D, Hidalgo A, Wolvetang E, Cooper-White J. 2012. Primitive cardiac cells from human embryonic stem cells. *Stem Cells Dev* 21: 1513–1523.
- Kim SW, Yoon SJ, Chuong E, Oyolu C, Wills AE, Gupta R, Baker J. 2011. Chromatin and transcriptional signatures for Nodal signaling during endoderm formation in hESCs. *Dev Biol* 357: 492–504.
- Li VW, Bengtson MH, Ulbrich A, Matsuda A, Reddy VA, Orth A, Chanda SK, Batalov S, Joazeiro CA. 2008. Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. *PLoS ONE* 3: e1487. doi: 10.1371/journal.pone.0001487.
- Mavrakis KJ, Andrew RL, Lee KL, Petropoulos C, Dixon JE, Navaratnam N, Norris DP, Episkopou V. 2007. Arkadia enhances Nodal/TGF- β signaling by coupling phospho-Smad2/3 activity and turnover. *PLoS Biol* 5: e67. doi: 10.1371/journal.pbio.0050067.
- Mesnard D, Guzman-Ayala M, Constam DB. 2006. Nodal specifies embryonic visceral endoderm and sustains pluripotent cells in the epiblast before overt axial patterning. *Development* 133: 2497–2505.
- Mullen AC, Orlando DA, Newman JJ, Lovén J, Kumar RM, Bilodeau S, Reddy J, Guenther MG, DeKoter RP, Young RA. 2011. Master transcription factors determine cell-type-specific responses to TGF- β signaling. *Cell* 147: 565–576.
- Nagano Y, Mavrakis KJ, Lee KL, Fujii T, Koinuma D, Sase H, Yuki K, Isogaya K, Saitoh M, Imamura T, et al. 2007. Arkadia induces degradation of SnoN and c-Ski to enhance transforming growth factor- β signaling. *J Biol Chem* 282: 20492–20501.
- Pera MF, Tam PP. 2010. Extrinsic regulation of pluripotent stem cells. *Nature* 465: 713–720.
- Price PJ, Goldsborough MD, Tilkins ML. 1998. *Embryonic stem cell serum replacement*. International Patent Application WO 98/30679.
- Sarker KP, Wilson SM, Bonni S. 2005. SnoN is a cell type-specific mediator of transforming growth factor- β responses. *J Biol Chem* 280: 13037–13046.
- Schmierer B, Hill CS. 2007. TGF β -SMAD signal transduction: Molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol* 8: 970–982.
- Stroschein SL, Wang W, Zhou S, Zhou Q, Luo K. 1999. Negative feedback regulation of TGF- β signaling by the SnoN oncoprotein. *Science* 286: 771–774.
- Stroschein SL, Bonni S, Wrana JL, Luo K. 2001. Smad3 recruits the anaphase-promoting complex for ubiquitination and degradation of SnoN. *Genes Dev* 15: 2822–2836.
- Suemori H, Yasuchika K, Hasegawa K, Fujioka T, Tsuneyoshi N, Nakatsuji N. 2006. Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. *Biochem Biophys Res Commun* 345: 926–932.
- Sulzbacher S, Schroeder IS, Truong TT, Wobus AM. 2009. Activin A-induced differentiation of embryonic stem cells into endoderm and pancreatic progenitors—the influence of differentiation factors and culture conditions. *Stem Cell Rev* 5: 159–173.
- Sumi T, Tsuneyoshi N, Nakatsuji N, Suemori H. 2007. Apoptosis and differentiation of human embryonic stem cells induced by sustained activation of c-Myc. *Oncogene* 26: 5564–5576.
- Teo AKK, Ali Y, Wong KY, Chipperfield H, Sadasivam A, Poobalan Y, Tan EK, Wang ST, Abraham S, Tsuneyoshi N, et al. 2012. Activin and BMP4 synergistically promote formation of definitive endoderm in human embryonic stem cells. *Stem Cells* 30: 631–642.
- Vallier L, Mendjan S, Brown S, Chng Z, Teo A, Smithers LE, Trotter MW, Cho CH, Martinez A, Rugg-Gunn P, et al. 2009. Activin/Nodal signalling maintains pluripotency by controlling Nanog expression. *Development* 136: 1339–1349.
- Vilchez D, Boyer L, Morantte I, Lutz M, Merkwirth C, Joyce D, Spencer B, Page L, Masliah E, Berggren WT, et al. 2012. Increased proteasome activity in human embryonic stem cells is regulated by PSMD11. *Nature* 489: 304–308.
- Wu JW, Krawitz AR, Chai J, Li W, Zhang F, Luo K, Shi Y. 2002. Structural mechanism of Smad4 recognition by the nuclear oncoprotein Ski: Insights on Ski-mediated repression of TGF- β signaling. *Cell* 111: 357–367.
- Xi Q, Wang Z, Zaromytidou AI, Zhang XH, Chow-Tsang LF, Liu JX, Kim H, Barlas A, Manova-Todorova K, Kaartinen V, et al. 2011. A poised chromatin platform for TGF- β access to master regulators. *Cell* 147: 1511–1524.
- Xu RH, Sampsel-Barron TL, Gu F, Root S, Peck RM, Pan G, Yu J, Antosiewicz-Bourget J, Tian S, Stewart R, et al. 2008. NANOG is a direct target of TGF β /activin-mediated SMAD signaling in human ESCs. *Cell Stem Cell* 3: 196–206.
- Zafarana G, Avery SR, Avery K, Moore HD, Andrews PW. 2009. Specific knockdown of OCT4 in human embryonic stem cells by inducible short hairpin RNA interference. *Stem Cells* 27: 776–782.
- Zhu Q, Luo K. 2012. SnoN in regulation of embryonic development and tissue morphogenesis. *FEBS Lett* 586: 1971–1976.
- Zhu Q, Pearson-White S, Luo K. 2005. Requirement for the SnoN oncoprotein in transforming growth factor β -induced oncogenic transformation of fibroblast cells. *Mol Cell Biol* 25: 10731–10744.
- Zhu Q, Krakowski AR, Dunham EE, Wang L, Bandyopadhyay A, Berdeaux R, Martin GS, Sun L, Luo K. 2007. Dual role of SnoN in mammalian tumorigenesis. *Mol Cell Biol* 27: 324–339.

