Chemically defined conditions for human iPSC derivation and culture

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We re-examine the individual components for human embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) culture and formulate a cell culture system in which all protein reagents for liquid media, attachment surfaces and splitting are chemically defined. A major improvement is the lack of a serum albumin component, as variations in either animal- or human-sourced albumin batches have previously plagued human ESC and iPSC culture with inconsistencies. Using this new medium (E8) and vitronectin-coated surfaces, we demonstrate improved derivation efficiencies of vector-free human iPSCs with an episomal approach. This simplified E8 medium should facilitate both the research use and clinical applications of human ESCs and iPSCs and their derivatives, and should be applicable to other reprogramming methods.

Human embryonic stem cells (ESCs) and human induced pluripotent stem cells (iPSCs) can proliferate without limit and yet maintain the potential to generate derivatives of all three germ layers. These properties make them useful for understanding the basic biology of the human body, for drug discovery and testing, and for transplantation therapies[1–6].

The culture conditions used to support the derivation and expansion of human iPSCs have been based on conditions developed for human ESCs over the last decade, which have been extensively compared and summarized by The International Stem Cell Initiative Consortium[7]. Members of our laboratory previously described the development of a medium (TeSR) for human ESC culture, which has more recently been used for the derivation and culture of human iPSCs[8]. However, although we demonstrate that TeSR could be used to derive human ESCs in the complete absence of animal proteins, the inclusion of human serum albumin and human-sourced matrix proteins makes those conditions prohibitively expensive, impractical for routine use and not truly completely defined. Although cloned human serum albumin is available and defined surfaces have now been described, because of the relative costs involved, researchers in many laboratories, including our own, continue to culture human ESCs and iPSCs routinely in medium that includes bovine serum albumin (BSA) on Matrigel, a complex mixture of matrix proteins derived from Engelbreth-Holm-Swarm mouse tumors. However, the variation in sources of these medium components is substantial, making extensive quality control necessary for all new batches. Because of the batch variation in medium components, researchers in different labs making the same medium often report substantially different results[9,10]. The batch variability of albumin is particularly problematic, both because of the unusually high concentrations used in the culture medium compared to other proteins and because of its ability to bind lipids and other impurities[11].

Optimization of medium is a daunting challenge in combinatorics. TeSR has 18 components added to a DMEM/F12 base medium that itself has 52 components. In the initial development of TeSR, we had demonstrated that subtracting albumin or any of the growth factors from the medium led to a dramatic decline in human ESC culture performance. However, because of the combinatorial complexity involved, originally we had not examined pairwise interactions between each factor. Now we show that removal of albumin (BSA in this study) from the medium leads to toxicity by a second component, β-mercaptoethanol (BME), and we demonstrate that in the absence of BME, BSA is no longer necessary for human ESC or iPSC culture. We re-optimized the basic components of human ESC and iPSC culture in the absence of BSA and BME, and developed a practical, completely defined medium, E8 (eight components, including DMEM/F12), and surfaces that support established human ESCs and iPSCs, and which greatly improve the efficiency of human iPSC derivation from dermal biopsy samples. Using the E8-based medium, defined conditions can be used for all stages of iPSC derivation and culture.

RESULTS

Albumin-free E8 medium for human ESC and iPSC culture

In addition to the components of DMEM/F12 (**Supplementary Table 1**), TeSR has 18 components, the major protein component being BSA (~1% in weight). Tremendous variability exists in the ability of different batches of BSA to support the undifferentiated

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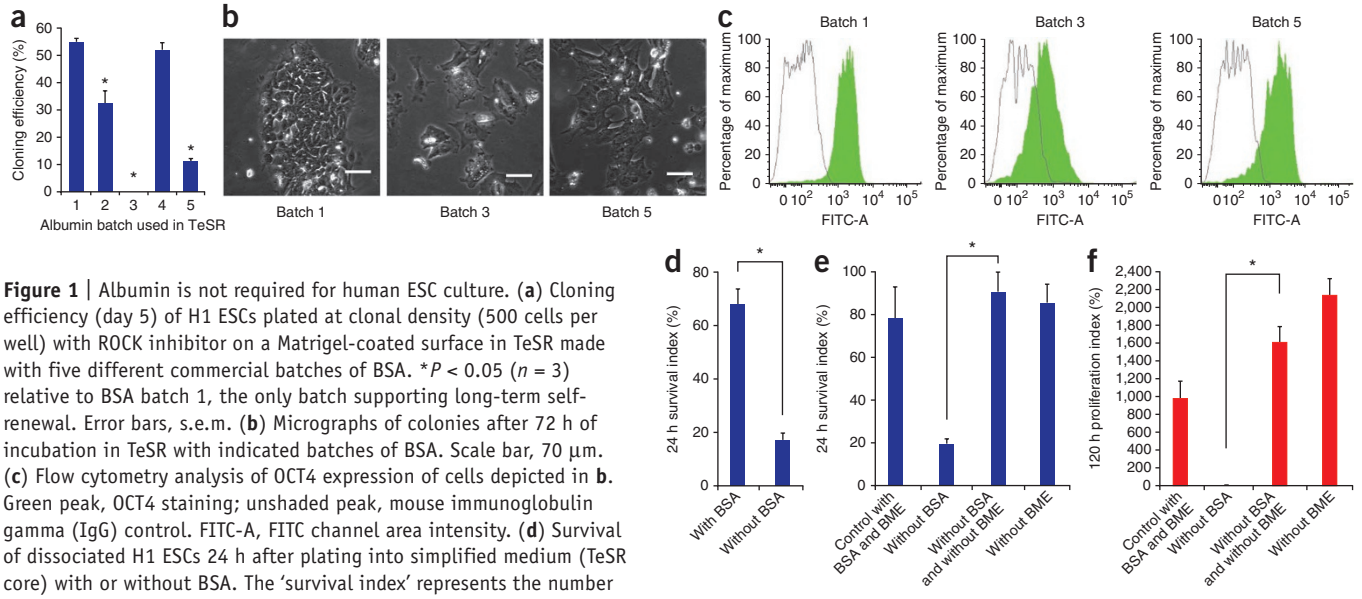

Figure 1 | Albumin is not required for human ESC culture. (**a**) Cloning efficiency (day 5) of H1 ESCs plated at clonal density (500 cells per well) with ROCK inhibitor on a Matrigel-coated surface in TeSR made with five different commercial batches of BSA. $*P < 0.05$ ($n = 3$) relative to BSA batch 1, the only batch supporting long-term self-renewal. Error bars, s.e.m. (**b**) Micrographs of colonies after 72 h of incubation in TeSR with indicated batches of BSA. Scale bar, 70 µm. (**c**) Flow cytometry analysis of OCT4 expression of cells depicted in **b**. Green peak, OCT4 staining; unshaded peak, mouse immunoglobulin gamma (IgG) control. FITC-A, FITC channel area intensity. (**d**) Survival of dissociated H1 ESCs 24 h after plating into simplified medium (TeSR core) with or without BSA. The 'survival index' represents the number of surviving cells divided by the number of input cells. $*P < 0.05$; $n = 3$. (**e**) Survival of dissociated cells 24 h after plating into TeSR-based medium with the indicated combinations of BSA and BME. The control medium is TeSR core. $*P < 0.05$; $n = 3$. (**f**) In the cell culture described in **e**, cell proliferation was measured 120 h after plating. $*P < 0.05$; $n = 3$. The 'proliferation index' represents the cell number at a specific time point divided by the number of input cells at time 0. Error bars, s.e.m.

proliferation of human ESCs (**Fig. 1a–c** and Online Methods). The absence of several growth factors (TGFβ, LiCl, GABA and pipecolic acid; see TeSR core in **Supplementary Table 1**) did not affect short-term cell survival and proliferation (**Supplementary Fig. 1a**). But the removal of BSA led to cell death of dissociated human ESCs (**Fig. 1d**). This suggests either that BSA contributes directly to ESC survival or that BSA counteracts the toxic effects of other medium components. Pairwise dropout experiments revealed that human ESCs survived without BSA when BME was also removed from the medium (**Fig. 1e**) and that cells subsequently proliferated well in such conditions (**Fig. 1f**).

We then re-examined the other medium components of TeSR in the absence of BSA and BME. Insulin and FGF2 were important for cell survival and proliferation (**Fig. 2a**,**b**). We found that L-ascorbic acid (vitamin C) promoted ESC proliferation (**Fig. 2c**) and that selenium was essential for sustained culture expansion (**Fig. 2d**). A comparative analysis of 12 different base media did not identify a base medium that performed better than DMEM/F12 (**Supplementary Fig. 1c**). We could expand human ESCs and iPSCs in a simple medium consisting only of insulin, FGF2, L-ascorbic acid and selenium in DMEM/F12 with pH adjusted with $NaHCO_3$, but cultures were often prone to sporadic differentiation after long-term passage (**Fig. 1e**). The addition of Nodal (100 ng ml^{-1}) or TGFβ (2 ng ml^{-1}) increased *NANOG* expression and led to consistent long-term culture stability of both human ESCs and iPSCs (**Fig. 2e**, **Supplementary Fig. 1b**,**d**,**e** and **Supplementary Table 2**). The inclusion of either a ROCK inhibitor (HA100 or Y27632)[12] or blebbistatin[13] improved initial survival and supported a high cloning efficiency (**Supplementary Fig. 1f**,**g**), which was increased by the addition of transferrin and by culture in hypoxic conditions (**Fig. 2f**,**g**).

After this optimization, the final E8 consisted just of insulin, selenium, transferrin, L-ascorbic acid, FGF2 and TGFβ (or Nodal) in DMEM/F12 with pH adjusted with $NaHCO_3$. This simplified medium supported undifferentiated proliferation of both human ESCs and iPSCs comparably to TeSR (**Fig. 2h**) and maintained pluripotency markers and normal karyotypes for over 25 passages and for more than 3 months in two ESC and five iPSC lines (**Supplementary Fig. 1h**,**i**). Both ESC and all five iPSC lines tested also formed teratomas in immunocompromised mice. Global gene expression analysis also demonstrated that cells maintained in E8 had an expression pattern similar to cells maintained in TeSR (**Supplementary Fig. 2**). Even without albumin, E8 was suitable for most common cell culture practices and had a 2 week shelf life if stored at 4 °C (**Supplementary Fig. 3**).

Vitronectin-coated surfaces support growth in E8

Multiple matrix proteins, such as laminin, vitronectin and fibronectin, support human ESC growth. Synthetic surfaces have also been developed for human ESCs. Most of these materials are too expensive for large-scale use. Because vitronectin is relatively easy to overexpress and purify[14], we tested several vitronectin variants and identified two, VTN-NC and VTN-N, that supported human ESC attachment and survival better than the wild type in E8 (**Fig. 3a**,**b**). We used VTN-NC for the rest of this study; VTN-NC supported initial attachment and survival of human ESCs well in E8 when we passaged cells in small clumps using EDTA (Online Methods), but less efficiently than Matrigel when cells were passaged as single cells (**Fig. 3c**). When we added a ROCK inhibitor or blebbistatin, VTN-NC supported both initial human ESC survival and cloning efficiency as effectively as Matrigel (**Fig. 3d**,**e**).

E8 improves the efficiency of iPSC derivation

Serum-containing media are often used during the derivation of dermal fibroblasts from biopsy samples from individuals, so we next examined whether E8 and vitronectin-coated surfaces could be used both for this purpose and for subsequent iPSC derivation.

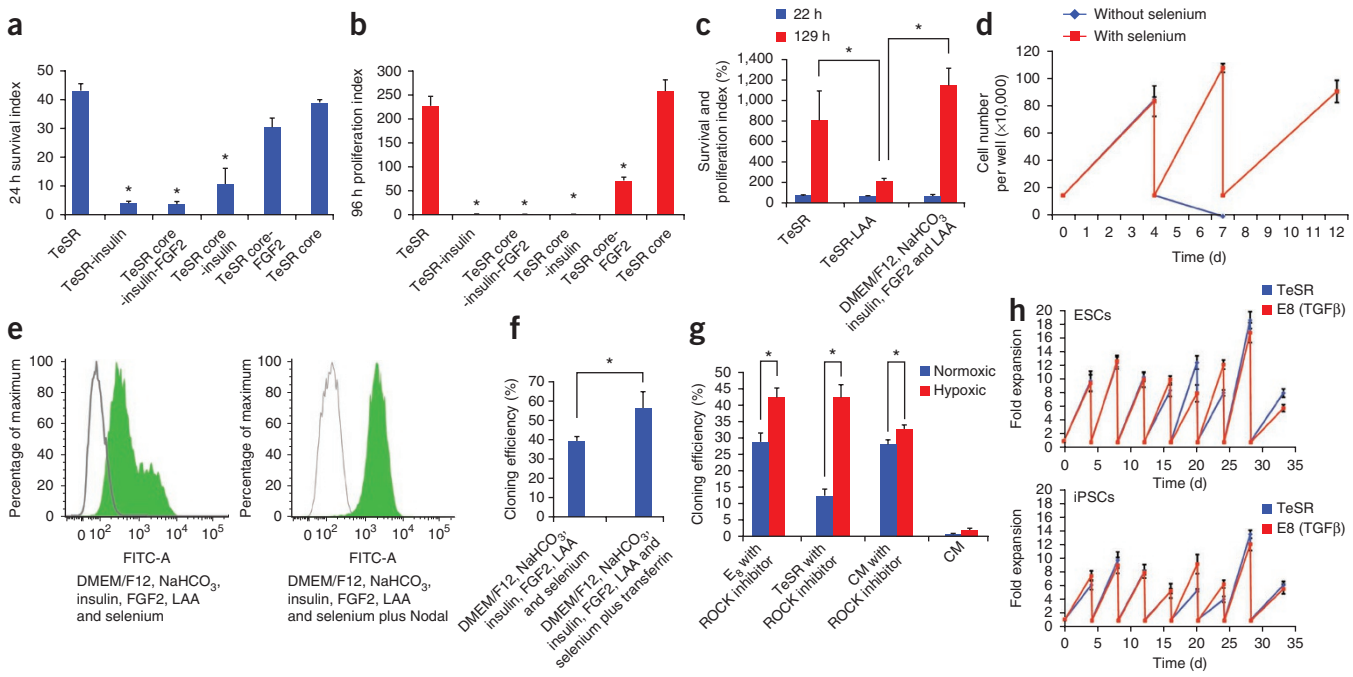

Figure 2 | Essential medium components for human ESC survival and proliferation. (**a**) Survival index of dissociated H1 ESCs 24 h after plating into the indicated medium on Matrigel-coated plates. *$P < 0.05$ ($n = 3$) relative to survival in TeSR. -insulin, medium without insulin; -FGF2, medium without FGF2. (**b**) Proliferation index of cells from **a** 96 h after plating and culture in the same medium with daily medium change. *$P < 0.05$ ($n = 3$) relative to proliferation in TeSR. (**c**) The plots show survival (22 h after plating) and proliferation (129 h after plating) of human ESCs dissociated and plated in the indicated media. *$P < 0.05$; $n = 3$. LAA, L-ascorbic acid. -LAA, medium without LAA. (**d**) Number of H1 cells maintained in defined media (DMEM/F12, $NaHCO_3$, insulin, FGF2 and LAA) for multiple passages with or without selenium (150,000 cells were seeded in each passage on days 0, 4 and 7). (**e**) Flow cytometry analysis of OCT4 expression in H1 cells grown in the indicated media for four passages. Green peak, OCT4 staining; unshaded peak, mouse IgG control. (**f**) Cloning efficiency of human foreskin iPSCs[29] grown in the indicated defined media, with ROCK inhibitor HA100, in hypoxic conditions (*$P < 0.05$, $n = 3$). MEF conditioned medium. (**g**) Cloning efficiency of ESCs in indicated media under hypoxic and normoxic conditions. CM, *$P < 0.05$; $n = 3$. Error bars, s.e.m. (**h**) Fold expansion of ESCs (H1) and iPSCs[15] cultured under hypoxic conditions in the indicated media (200,000 cells were plated at each passage).

FGF2 alone, which is already present in the E8 medium, supported the proliferation of dermal fibroblasts as effectively as any other FGF we tested but not as well as fetal bovine serum (**Supplementary Fig. 4a**). The addition of hydrocortisone and TGFβ further improved fibroblast growth (**Supplementary Fig. 4b**).

We then tested whether these defined fibroblast culture conditions would support subsequent iPSC derivation by an episomal approach[15] from established fibroblast cell lines. We found that electroporation of mRNA encoding EBNA1 (*EBNA*) along with *oriP* plasmids greatly improved subsequent expression from the *oriP* plasmids[16] (**Supplementary Fig. 4c**), and thus we subsequently used mRNA encoding EBNA during reprogramming with previously described *oriP* vectors[15]. We added hydrocortisone to E8 initially to facilitate robust fibroblast proliferation and later

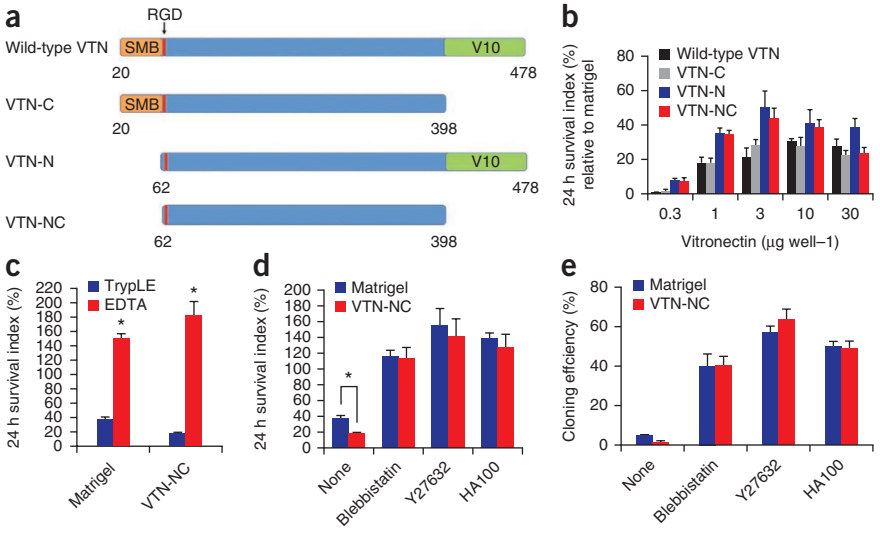

Figure 3 | Vitronectin-coated surfaces support human ESCs and iPSCs cultured in E8. (**a**) Schematics of wild-type vitronectin (VTN) and VTN-C, VTN-N and VTC-NC variants that were expressed and purified as coating materials for ESCs, with numbers indicating the amino acid number of the start and end of each construct. Somatomedin B domain[30] (SMB) and V10 are functional domains[31] of wild-type vitronectin; RGD, integrin-binding site. (**b**) Cell survival on the four vitronectin variants in E8 after 24 h. The survival index was normalized to cell survival on Matrigel. *$P < 0.05$; $n = 3$. (**c**) Cell survival after 24 h on VTN-NC and Matrigel in E8 (with TGFβ) medium after passaging with EDTA and a stable Trypsin-like enzyme, TrypLE (Invitrogen); *$P < 0.05$, $n = 3$. (**d**,**e**) Cell survival after 24 h (**d**) or cloning efficiency (**e**) on VTN-NC and Matrigel surfaces in the presence of the indicated chemical inhibitors. *$P < 0.05$; $n = 3$. Error bars, s.e.m.

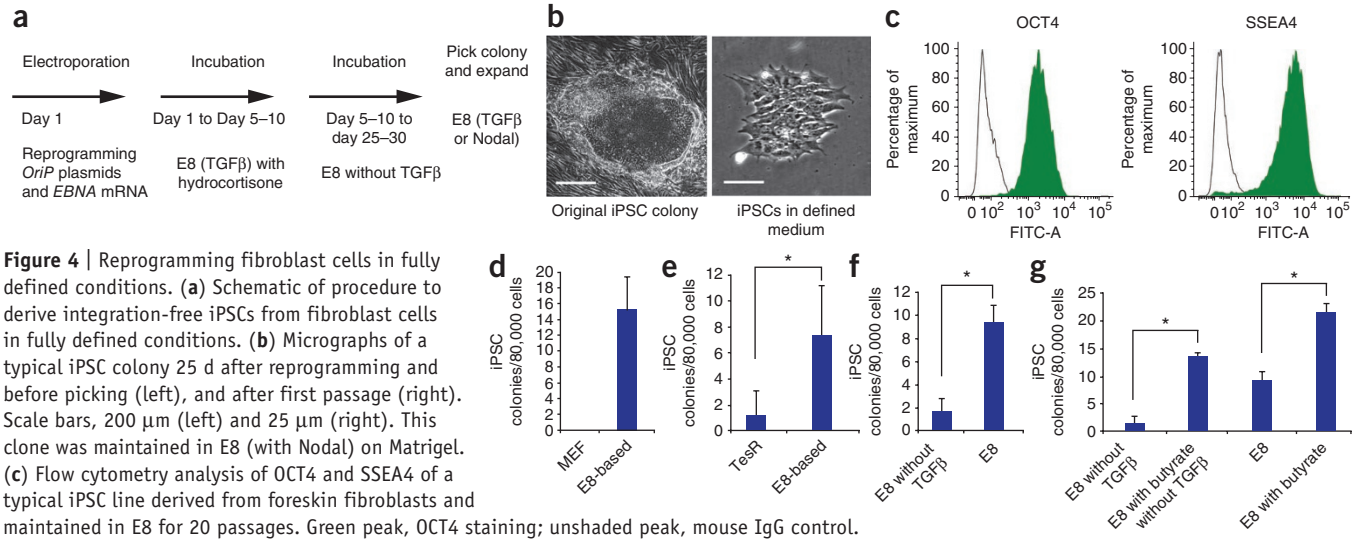

Figure 4 | Reprogramming fibroblast cells in fully defined conditions. (**a**) Schematic of procedure to derive integration-free iPSCs from fibroblast cells in fully defined conditions. (**b**) Micrographs of a typical iPSC colony 25 d after reprogramming and before picking (left), and after first passage (right). Scale bars, 200 μm (left) and 25 μm (right). This clone was maintained in E8 (with Nodal) on Matrigel. (**c**) Flow cytometry analysis of OCT4 and SSEA4 of a typical iPSC line derived from foreskin fibroblasts and maintained in E8 for 20 passages. Green peak, OCT4 staining; unshaded peak, mouse IgG control. (**d**–**g**) Reprogramming efficiency of human foreskin fibroblasts reprogrammed in the indicated media, scored after 30 d. In **d**, sodium butyrate (100 μM) was added to both conditions to improve efficiency. In **d**, **f** and **g**, $*P < 0.05$ ($n = 3$); experiments were repeated twice (**d**,**f**) and five times (**g**), with similar results. In **e**, owing to the inconsistency of reprogramming efficiency in TeSR, four independent experiments were each repeated three times; $*P < 0.05$ ($n = 12$). Error bars, s.e.m.

(5–10 d later) removed it, along with TGFβ, to inhibit fibroblast overgrowth of the iPSCs (**Fig. 4a**). iPSC colonies (**Fig. 4b**) intermingled with partially reprogrammed colonies (**Supplementary Fig. 4d**), appeared 20–30 d after transfection. We selected individual iPSC colonies and subsequently passaged and maintained them in complete E8 for long-term expansion. Because fibroblast overgrowth was reduced by hydrocortisone and TGFβ withdrawal, we could obtain iPSC colonies without secondary passage (**Supplementary Table 3**). We confirmed individual iPSC clones to be integration-free by PCR and, after ~20 passages, cells had normal karyotypes, expressed pluripotency markers (**Fig. 4c** and **Supplementary Fig. 4e**) and formed teratomas. E8-based medium supported improved reprogramming efficiencies compared to culture with mouse fibroblast feeder cells, FBS-containing medium or TeSR (**Fig. 4d**,**e** and **Supplementary Fig. 4f**).

TGFβ is a common growth factor in defined fibroblast growth media[17,18], and its presence in the first 6–8 d increased the number of iPSC colonies compared with reprogramming in the absence of TGFβ (**Fig. 4f**). It has been previously reported that TGFβ has an inhibitory effect when present at later stages of reprogramming[19], consistent with our observation that fibroblasts tended to overgrow and inhibit or obscure the emergence of iPSC colonies when TGFβ was present throughout the reprogramming procedure. To slow down fibroblast proliferation and to allow time for the emergence of iPSCs without fibroblast overgrowth, we removed both TGFβ and hydrocortisone at 5–10 d until iPSC colonies were selected and passaged into complete E8 with TGFβ or Nodal (**Fig. 4a**). Similar to the findings of others[20–22], we found that butyrate improved the efficiency of iPSC colony formation, which had an additive effect with TGFβ (**Fig. 4g**).

We used E8 to derive iPSC lines with both episomal and lentiviral methods from five banked fibroblast cell lines from commercial sources, obtained from individuals ranging in age from neonatal to 38 years of age (**Supplementary Table 3**). Using an episomal approach, all these fibroblast cell lines tested were reprogrammed at efficiencies of 4–200 iPSC colonies per 10^6 fibroblasts initially transfected. iPSC colonies were counted on the initial transfected fibroblast plates without additional passage, so the numbers represent independent clones. Global gene expression analysis also demonstrated that cells derived in E8 had an expression pattern similar to ESCs and iPSCs derived on feeder cells (**Supplementary Fig. 2b–d**).

Derivation of human iPSCs in defined conditions from biopsy

Next we developed fully defined conditions for processing dermal biopsies from individuals. We found that VTN-NC had a stimulatory effect on the growth of adult fibroblast cells (**Fig. 5a**). We also formulated a defined fibroblast medium based on E8 (**Fig. 5b**) to promote fibroblast proliferation from fresh biopsy samples. Combining all of these conditions, we derived integration-free iPSCs in totally defined conditions from the initial biopsy.

We treated the initial skin biopsy with recombinant enzymes and dissociated the dermis portion to derive skin fibroblasts in E8-based fibroblast medium (Online Methods). E8-based medium on vitronectin effectively supported initial fibroblast derivation from biopsy samples with robust proliferation (**Fig. 5c**). After expansion, we subjected the fibroblasts to the episomal reprogramming procedure described above (**Fig. 4a**). iPSC colonies appeared after ~25 d, and were passaged onto vitronectin-coated surfaces in E8 (**Fig. 5d**). We clonally expanded individual iPSCs under hypoxic condition on vitronectin-coated plates with a ROCK inhibitor at splitting and then screened them for a lack of vector integration. Pluripotency markers OCT4 and SSEA4 were maintained at high levels in iPSCs derived in these defined conditions (**Fig. 5e**,**f**).

Fibroblast cells freshly isolated from biopsy samples in E8-based fibroblast medium on vitronectin were consistently reprogrammed at a higher efficiency (60 to ~1,000 iPSC colonies per 10^6 transfected fibroblasts) than the established cell lines we had previously obtained from commercial sources (4–30 iPSC colonies per 10^6 transfected fibroblasts, excluding the neonatal foreskin fibroblasts; **Supplementary Table 3**). In the experiments

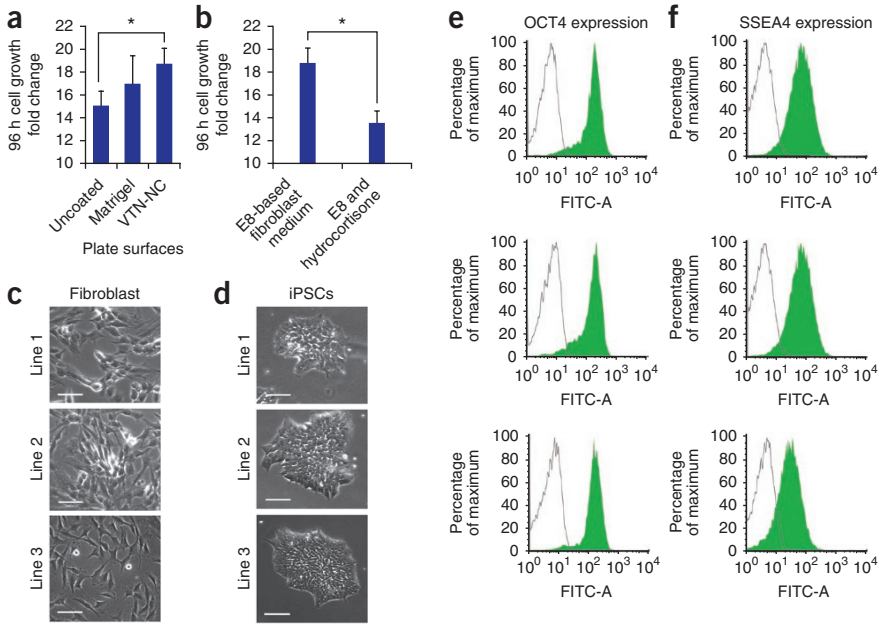

Figure 5 | Derivation of human iPSCs directly from biopsy samples in chemically defined conditions. (**a**,**b**) Analyses of growth of adult fibroblast cells plated on the indicated plate-coating materials in E8-based fibroblast medium and counted after 4 d (**a**) or cultured in the indicated media on vitronectin and counted 96 h after plating (**b**). $*P < 0.05$; $n = 3$. Error bars, s.e.m. (**c**) Micrographs of three fibroblast cell lines derived from skin biopsies in defined fibroblast medium on vitronectin-coated plates. Scale bar, 100 µm. (**d**) Micrographs of representative iPSC colonies obtained by reprogramming fibroblasts shown in **c** according to the procedure in **Figure 4a**, in the presence of butyrate. Colonies are shown after multiple passages in E8 (with TGFβ). Scale bars, 100 µm. (**e**,**f**) Flow cytometry analysis of pluripotency markers OCT4 and SSEA4 in iPSCs after ten passages.

illustrated in **Figure 5**, we expanded individual clones (10–24 clones for each patient sample) and analyzed them for genomic integration after iPSC derivation. We confirmed two integration-free clones of each sample to have normal karyotypes and then injected them into immunodeficient mice. Teratomas formed in all tested cell lines.

DISCUSSION

During these studies, we were most surprised by what human ESCs and iPSCs do not need and by how challenging it is to remove something from a medium once its inclusion becomes routine and accepted. Albumin, for example, has had many cell culture roles attributed to it, from lipid carrier and blocking reagent to physical protection against sheer force[11,23]. We found that the dominant role of BSA in TeSR is to prevent a toxic effect of BME and that in the absence of BME, BSA is not required. Because of a positive effect on cloning efficiency of mouse embryonal carcinoma cells described in 1978 (ref. 24), we had included BME in previous mouse ESC medium[25] and subsequently in early human ESC medium[1].

The finding of an interaction between BSA and BME underscores the challenge of optimizing media, as it is not sufficient to just examine the effects of the addition or subtraction of individual components. Our analysis of all other components of TeSR made without BSA or BME revealed that several (that is, pipocolic acid, GABA, LiCl, chemically defined lipids, trace elements B, trace elements C, glutathione and additional thiamine and L-glutamine) no longer had a positive effect. In spite of the much greater simplicity of E8, it supported the long-term undifferentiated proliferation of both human ESCs and iPSCs comparably to TeSR.

E8 supported higher reprogramming efficiencies for both viral and episomal approaches. In every attempt, from both established, banked fibroblasts (5) and from independent biopsy samples (4), we derived integration-free iPSC lines in E8-based medium on vitronectin (**Supplementary Table 3**). With the exception of the banked neonatal fibroblasts that reprogrammed at a high efficiency, the efficiencies obtained directly from biopsy samples grown in E8-based medium were consistently higher than those of established cell lines obtained from commercial sources, suggesting that fibroblast passage history is important. Although these efficiencies could be improved, the number of clones obtained per biopsy sample in these defined conditions using an episomal approach (60 to ~1,000 iPSC colonies per 10^6 fibroblasts) already greatly exceeded what is typically used for subsequent characterization.

It is important to distinguish mechanistically between culture components that improve reprogramming itself and those that merely improve the survival and proliferation of the resulting iPSC clones[19,26]. Because reprogramming occurs over time, it is often difficult to cleanly distinguish between these two effects, and indeed, they do not need to be mutually exclusive. We found, for example, that L-ascorbic acid promotes ESC proliferation and expansion. L-ascorbic acid has previously been reported to promote reprogramming[27], but our results are consistent with L-ascorbic acid only promoting iPSC growth and survival, with reprogramming itself being driven by other factors. It is also possible that ascorbic acid leads to epigenetic modifications essential to both reprogramming and cell survival in serum-free culture conditions[28]. Similarly, most derivation procedures have a splitting step before iPSC colonies are visible, and reprogramming efficiency is often calculated based on the final iPSC colony number. When splitting is required for the emergence of iPSCs in some situations (for example, fibroblast overgrowth), it can lead to confusion between factors that improve reprogramming itself and those that improve survival after splitting. If the iPSC colony number is only assessed after splitting, an independent clonal origin of distinct iPSC colonies cannot be confirmed, making calculations of true reprogramming efficiency problematic. Because our method removes TGFβ and hydrocortisone from the defined medium after 5–10 d, fibroblast overgrowth is inhibited, and truly independent iPSC clones can be isolated and counted without splitting.

Because E8 reduces medium cost and simplifies quality control, we now use it for all routine culture of both human ESCs and iPSCs. This simplified, defined medium also provides a much cleaner background for examining specific pathways in

self-renewal, cell death and differentiation[13], and it supports substantially improved reprogramming efficiencies. Although we only demonstrated improved efficiencies for viral and episomal reprogramming approaches, these conditions should be equally useful for other nonintegrative reprogramming approaches. Finally, because E8 is highly defined, it should also facilitate the transfer of basic research on human pluripotent stem cells to the clinic.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

G.C. and J.A.T. conceived the experiment; G.C., D.R.G., J.M.B., K.S.-O. and S.E.H. performed the reprogramming; Z.H., G.C. and N.E.P. produced vitronectin; G.C., D.R.G., J.M.B., N.R.D., G.O.L. and J.A.-B. performed the cell culture test; G.C., M.D.P. and R.W. derived fibroblasts; J.M.C.T. obtained the skin biopsy; V.R. and G.C. analyzed global expression; and G.C. and J.A.T. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://npg.nature.com/naturemethods/.

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1. Thomson, J.A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
2. Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A. & Bongso, A. Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. *Nat. Biotechnol.* **18**, 399–404 (2000).
3. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917–1920 (2007).
4. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872 (2007).
5. Park, I.H. *et al.* Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* **451**, 141–146 (2008).
6. Lowry, W.E. *et al.* Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc. Natl. Acad. Sci. USA* **105**, 2883–2888 (2008).
7. 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).
8. Sun, N. *et al.* Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. *Proc. Natl. Acad. Sci. USA* **106**, 15720–15725 (2009).
9. Skottman, H. & Hovatta, O. Culture conditions for human embryonic stem cells. *Reproduction* **132**, 691–698 (2006).
10. 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).
11. Garcia-Gonzalo, F.R. & Izpisua Belmonte, J.C. Albumin-associated lipids regulate human embryonic stem cell self-renewal. *PLoS ONE* **3**, e1384 (2008).
12. Watanabe, K. *et al.* A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat. Biotechnol.* **25**, 681–686 (2007).
13. Chen, G., Hou, Z., Gulbranson, D.R. & Thomson, J.A. Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells. *Cell Stem Cell* **7**, 240–248 (2010).
14. Braam, S.R. *et al.* Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alphavbeta5 integrin. *Stem Cells* **26**, 2257–2265 (2008).
15. Yu, J.Y. *et al.* Human induced pluripotent stem cells free of vector and transgene sequences. *Science* **324**, 797–801 (2009).
16. Howden, S.E. *et al.* Chromatin-binding regions of EBNA1 protein facilitate the enhanced transfection of Epstein-Barr virus–based vectors. *Hum. Gene Ther.* **17**, 833–844 (2006).
17. Assoian, R.K., Frolik, C.A., Roberts, A.B., Miller, D.M. & Sporn, M.B. Transforming growth factor-beta controls receptor levels for epidermal growth factor in NRK fibroblasts. *Cell* **36**, 35–41 (1984).
18. Roberts, A.B. *et al.* Type beta transforming growth factor: a bifunctional regulator of cellular growth. *Proc. Natl. Acad. Sci. USA* **82**, 119–123 (1985).
19. Lin, T. *et al.* A chemical platform for improved induction of human iPSCs. *Nat. Methods* **6**, 805–808 (2009).
20. Liang, G., Taranova, O., Xia, K. & Zhang, Y. Butyrate promotes induced pluripotent stem cell generation. *J. Biol. Chem.* **285**, 25516–25521 (2010).
21. Mali, P. *et al.* Butyrate greatly enhances derivation of human induced pluripotent stem cells by promoting epigenetic remodeling and the expression of pluripotency-associated genes. *Stem Cells* **28**, 713–720 (2010).
22. Ware, C.B. *et al.* Histone deacetylase inhibition elicits an evolutionarily conserved self-renewal program in embryonic stem cells. *Cell Stem Cell* **4**, 359–369 (2009).
23. Rothschild, M.A., Oratz, M. & Schreiber, S.S. Regulation of albumin metabolism. *Annu. Rev. Med.* **26**, 91–104 (1975).
24. Oshima, R. Stimulation of the clonal growth and differentiation of feeder layer dependent mouse embryonal carcinoma cells by beta-mercaptoethanol. *Differentiation* **11**, 149–155 (1978).
25. Martin, G.R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78**, 7634–7638 (1981).
26. 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).
27. Esteban, M.A. *et al.* Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. *Cell Stem Cell* **6**, 71–79 (2010).
28. Chung, T.L. *et al.* Vitamin C promotes widespread yet specific DNA demethylation of the epigenome in human embryonic stem cells. *Stem Cells* **28**, 1848–1855 (2010).
29. Yu, J.Y. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917–1920 (2007).
30. Seiffert, D. & Loskutoff, D.J. Evidence that type 1 plasminogen activator inhibitor binds to the somatomedin B domain of vitronectin. *J. Biol. Chem.* **266**, 2824–2830 (1991).
31. Hayman, E.G., Pierschbacher, M.D., Ohgren, Y. & Ruoslahti, E. Serum spreading factor (vitronectin) is present at the cell surface and in tissues. *Proc. Natl. Acad. Sci. USA* **80**, 4003–4007 (1983).

ONLINE METHODS

Human ESC culture. Human ESCs (H1 and H9) were usually maintained in specific media on Matrigel-coated tissue culture plates[32]. Cells were passaged routinely with EDTA as described previously[13]. Briefly, cells were washed twice with PBS-EDTA medium (0.5 mM EDTA in PBS, 340 mOsm), then incubated with PBS-EDTA for 5 min at 37 °C. PBS-EDTA was removed, and cells were washed off swiftly with a small volume of corresponding medium.

E8 medium contained DMEM/F12, 64 mg l^{-1} L-ascorbic acid-2-phosphate magnesium, 14 μg l^{-1} sodium selenium, 100 μg l^{-1} FGF2, 19.4 mg l^{-1} insulin, 543 mg l^{-1} $NaHCO_3$ and 10.7 mg l^{-1} transferrin, 2 μg l^{-1} TGFβ1 or 100 μg l^{-1} Nodal. Osmolarity of all media was adjusted to 340 mOsm at pH 7.4. All media were stored at 4 °C and were used within 2 weeks of production. L-ascorbic acid-2-phosphate magnesium is the stable form of L-ascorbic acid in cell culture.

Reagents. We used HA100 (Sigma), blebbistatin (Sigma), Y27632 (Tocris), sodium butyrate (Sigma), hydrocortisone (Sigma), sodium bicarbonate (Sigma), L-ascorbic acid 2 phosphate magnesium salt (Sigma), sodium selenite (Sigma), holo-transferrin (Sigma), DMEM/F12 (Invitrogen), insulin (Sigma), TGFβ1 (R&D), Nodal (R&D) and FGF2 (ref. 32). BSA batches we used were: batch 1, Sigma A2153-066K0738; batch 2, Sigma A2153 049K1585; batch 3, Sigma A7906-069K1653; batch 4, Sigma A2153-018K0665; and batch 5, Hyclone SH30574-090205068A. We used antibodies to OCT4 (Santa Cruz), to SSEA4 (Millipore) and Alexa Fluor 488–conjugated anti-mouse IgG (Invitrogen).

Survival assay. Survival assays followed the procedure previously described[13] unless specified. All experiments were done on 12-well plates, usually in triplicate for each treatment. Before the addition of cells, 500 μl of medium was loaded into each well. Cells were dissociated with TrypLE (Invitrogen) for 5 min or until fully detached from the plate, neutralized with equal volumes of media, counted, washed, diluted to 300,000–1,000,000 cells ml^{-1}, and 100 μl of cells were added into each well. Plates were normally placed into 5% O_2 and 10% CO_2 (hypoxic condition), 37 °C incubator, unless specified. Small chemical compounds or proteins were added or washed away according to the specified procedure. At each time point, cells were again dissociated with 0.4 ml TrypLE, neutralized with equal volumes of 10% FBS in DMEM, collected with pipettes and counted by flow cytometry. As an internal control, 5,000 Count-bright beads (Invitrogen) were added to each sample, and usually ~200 beads were counted for each sample. For proliferation experiments, media were changed daily until the day of analysis, and cells were counted as described above.

Cloning assay. The cloning assay has been described previously[13]. Briefly, triplicates were prepared in a 12-well plate format for each treatment. Before the addition of cells, 500 μl of medium were loaded in each well. Cells were dissociated with TrypLE for 5 min or until fully detached from the plate, neutralized with equal volumes of basic medium, counted, washed and then diluted to 5,000 cells ml^{-1}. Finally, a 100-μl suspension (500 cells) was added into each well. Plates were then placed into 5% O_2 and 10% CO_2 (hypoxic condition), 37 °C incubator unless otherwise specified. Small chemical compounds or proteins were added or washed away according to the specified procedure. Media were changed every 1–2 d if not specified. After 5–6 d, colonies were stained with an APS kit using a standard procedure (Vector Lab) and counted. Chemical concentrations used for cloning assays in this report were 10 μM blebbistatin, 10 μM Y27632 or 10 μM HA100.

Oxygen and carbon dioxide control. Cells were maintained in a water-jacketed CO_2 incubator (Forma Series II), in which O_2 and CO_2 were controlled through the injection of nitrogen and carbon dioxide. There are two conditions discussed in this paper: hypoxic condition (5% O_2 and 10% CO_2) and normoxic condition (5% CO_2). The hypoxic condition was used for most experiments in this report unless otherwise specified.

Statistics. In cloning or survival assays, triplicate data points were obtained for each condition. A *t*-test was performed to calculate *P* values for the difference between the means of the experimental conditions and control. Error bars in each figure represent s.e.m. of three individual experiments. Each finding was confirmed by independent biological replicates, unless specified.

iPSC derivation in defined condition. Episomal plasmids and methods have been described previously[15]. Plasmid combination 19 (pEP4-E-O2S-E-T2K, pEP4-E-O2S-E-N2K and pCEP4M2L) was used for most reprogramming unless mentioned otherwise. Plasmids and mRNA encoding EBNA were electroporated into fibroblast cells on Amaxa apparatus according to the manufacturer's instructions. One million cells were used in each electroporation, which were then plated into two six-well plates. E8 plus hydrocortisone medium was used for the first 5–10 d, according to cell survival and proliferation after electroporation. When confluency was reached (~20%), hydrocortisone was removed. ESC-like iPSC colonies usually appear after ~25 d. Cells were then picked into individual wells with E8 (TGFβ or Nodal). Cells were passaged for ~15 passages before subcloning with Y27632 on Matrigel or vitronectin.

Operation for skin biopsy. The area of biopsy on the left arm of the individual was prepped in a sterile fashion using 70% ethanol and 1% lidocaine with epinephrine (1:100,000 dilution) used for local anesthesia. The skin biopsy was obtained with a 4-mm punch. All specimens were obtained following standard Institutional Review Branch protocol approved by University of Wisconsin–Madison. All individuals consented to volunteer participation. For underage individuals, informed written consent was obtained from the individual's guardian.

Fibroblast derivation in defined conditions. After a skin punch biopsy was obtained from the individual, it was maintained in defined fibroblast medium (DMEM/F12 (Invitrogen), 64 mg l^{-1} L-ascorbic acid 2 phosphate magnesium salt (Sigma), 20 mg l^{-1} insulin, 14 μg l^{-1} sodium selenite, 10 mg l^{-1} transferrin (Sigma), 1 unit ml^{-1} thrombin (Sigma), 100 nM hydrocortisone (Sigma), 100 μg l^{-1} EGF, 100 μg l^{-1} hFGF2 and 2 μg l^{-1} TGFβ) and later immersed in TrypLE Select (Invitrogen) enzyme solution at 4 °C overnight. The epidermis was then peeled off the dermis, and the dermis was cut into small pieces before being incubated in enzyme mix (HEPES containing RPMI with 1 mM pyruvate, 10 mg ml^{-1}

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collagenase (Sigma), 0.5 AMP ml^{-1} hyaluronidase (Sigma) and 140 units ml^{-1} DNase I (Roche)) for 30 min at room temperature (23 °C). Cells were centrifuged and washed twice with fibroblast medium and then were plated onto vitronectin-coated tissue culture plates with fibroblast medium. After 3–7 d, fibroblast cells emerged and proliferated. TypLE was used for regular splitting to expand the cells before reprogramming.

Derivation of iPSCs from fibroblast cells in defined conditions. A schematic diagram of the procedure is shown in **Figure 4**. Episomal plasmids and methods have been described previously[15]. Plasmid combination 19 (pEP4-E-O2S-E-T2K, pEP4-E-O2S-E-N2K and pCEP4M2L) was used for most reprogramming unless mentioned otherwise. Plasmids and *EBNA* mRNA were electroporated into fibroblast cells on Amaxa apparatus according to company instructions. One million cells were used in each electroporation, which were then plated into two 6-well plates. E8 plus hydrocortisone media were used for the first 5–10 d, according to cell survival and proliferation after electroporation. When confluency was reached ~20%, hydrocortisone was removed. ESC-like iPSC colonies usually appear after ~25 d. Cells were then picked into individual wells with E8 (TGFβ or Nodal). Cells were passaged for ~15 passages before subcloning with Y27632 on Matrigel or vitronectin.

mRNA encoding EBNA for electroporation. mRNA encoding *EBNA* was synthesized and purified as previously reported[16]. A plasmid containing *oriP* sequence and *EGFP* gene was used to evaluate the improvement made by mRNA encoding EBNA during transfection. The electroporation was performed with Bio-Rad Gene Pulser II (**Supplementary Fig. 4c**). Unless specifically mentioned, all electroporation experiments mentioned in this work used Amaxa Nucleofector with Human Dermal Fibroblast Nucleofector Solution (Lonza). Program U-020 was used for foreskin fibroblast, and program U-023 was used for adult fibroblasts and fibroblasts freshly isolated from individuals.

Vitronectin expression and purification. Coding sequences for human vitronectin and various truncation mutants (**Fig. 3a**) were amplified from a cDNA clone purchased from Origene and cloned into NdeI and BamHI sites of pET3c (Novagen). All constructs were verified by sequencing. Protein expression was done in Rosetta2 (DE3) pLysS cells (Novagen) using Magic Media (Invitrogen) at 37 °C for 24 h. Vitronectin and its variants were purified using a protocol modified from a previous report[33]. Briefly, the *Escherichia coli* pellet was resuspended in PBS and lysed with FastBreak cell-lysis reagent (Promega). Insoluble material was pelleted by centrifugation at 10,000*g*. The pellet was washed once with PBS and 0.5 M NaCl and then solubilized in urea buffer (8 M urea, 20 mM Tris (pH 7.6), 150 mM NaCl and 3 mM DTT). Urea-solubilized vitronectin was loaded onto a Heparin sepharose column, and the column was then washed extensively with urea buffer. Protein was eluted with urea buffer with 500 mM NaCl and then dialyzed into PBS overnight.

Global gene expression analysis. Expression in ESC, iPSC and foreskin samples were measured by RNA sequencing (RNA-seq) via Illumina's Genome Analyzer GAIIx. The RNA-seq libraries were built following a previously described procedure[34]. The sequencing results were then normalized via the RSEM package[35]. Transcripts per million (TPM) values were obtained to measure the RNA expression. Spearman correlation coefficient (*R* values) was calculated to get a general overview of the expression profiles in each sample.

32. Ludwig, T.E. *et al.* Feeder-independent culture of human embryonic stem cells. *Nat. Methods* **3**, 637–646 (2006).
33. Wojciechowski, K., Chang, C.H. & Hocking, D.C. Expression, production, and characterization of full-length vitronectin in *Escherichia coli*. *Protein Expr. Purif.* **36**, 131–138 (2004).
34. Sengupta, S. *et al.* Highly consistent, fully representative mRNA-Seq libraries from ten nanograms of total RNA. *Biotechniques* **49**, 898–904 (2010).
35. Li, B., Ruotti, V., Stewart, R.M., Thomson, J.A. & Dewey, C.N. RNA-Seq gene expression estimation with read mapping uncertainty. *Bioinformatics* **26**, 493–500 (2010).