Connexin 43 is involved in the generation of human-induced pluripotent stem cells

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Although somatic cells can be successfully programmed to create pluripotent stem cells by ectopically expressing defined transcriptional factors, reprogramming efficiency is low and the reprogramming mechanism remains unclear. Previous reports have shown that almost all human connexin (CX) isoforms are expressed by human embryonic stem (hES) cells and that gap junctional intercellular communication (GJIC) is important for ES cell survival and differentiation. However, the CX expression profiles in human induced pluripotent stem (iPS) cells and the role of CXs in the process of reprogramming back to iPS cells remains unknown. Here, we determined the expression levels of most forms of CX in human embryonic fibroblasts (hEFs) and in the hEF-derived iPS cells. A scrape loading/dye transfer assay showed that human iPS cells contained functional gap junctions (GJs) that could be affected by pharmacological inhibitors of GJ function. We found that CX43 was the most dramatically upregulated CX following reprogramming. Most importantly, the ectopic expression of CX43 significantly enhanced the reprogramming efficiency, whereas shRNA-mediated knockdown of endogenous CX43 expression greatly reduced the efficiency. In addition, we found that CX43 overexpression or knockdown affected the expression of E-CADHERIN, a marker of the mesenchymal-to-epithelial transition (MET), during reprogramming. In conclusion, our data indicate that CX43 expression is important for reprogramming and may mediate the MET that is associated with the acquisition of pluripotency.

INTRODUCTION

Successful reprogramming of somatic cells, such as human embryonic or adult fibroblasts, to create pluripotent stem cells can be achieved by ectopically expressing defined transcriptional factors (1–6). The creation of induced pluripotent stem (iPS) cells that possess embryonic stem (ES) cell characteristics facilitates the generation of human patient-specific stem cell lines for modelling disease processes in vitro, providing a useful tool in studies of developmental biology and drug discovery (7–9). iPS cells can also be used in regenerative medicine while avoiding the ethical issues associated with the use of ES cells. Although great progress has been made in the iPS cell field, the human iPS cell technology is currently limited by its low reprogramming efficiency and time-intensive reprogramming process. Further exploration of the...
molecular reprogramming mechanisms may facilitate the development of more efficient and economical methods of iPS cell generation.

Connexin (CX), a component of vertebrate gap junctions (GJs), plays an important role in cell--cell communication. Six CX proteins form the so-called ‘connexon’, which is also termed as ‘hemichannel’. GJs facilitate the direct exchange of small molecules between adjacent cells, and each junctional channel is composed of two hemichannels (10). Low-molecular-weight molecules (<1–1.5 kD) may pass through GJs to enter neighbouring cells via passive diffusion; this process is termed as ‘gap junctional intercellular communication’ (GJIC) (11,12). Previous studies have identified CX43, CX45 and CX40 as components of functional GJs in human ES cells (13,14). The knockdown of CX expression in mouse ES cells reduces cell proliferation and downregulates the expression of pluripotency markers. Such data indicate that GJs play an essential role in maintaining mouse ES cells in the undifferentiated state (15).

More than 20 CXs have been identified in humans (16). Transcripts encoding almost all of the known CX isoforms are expressed by human ES cells, including CX25, CX26, CX30, CX30.2, CX30.3, CX31, CX31.1, CX31.9, CX32, CX36, CX37, CX40, CX43, CX45, CX46, CX47, CX59 and CX62 (14). CX43 and CX45 are highly enriched in undifferentiated human ES cells compared with either their differentiated counterparts or somatic tissues (17,18). In addition, CX43 has been identified as a downstream target of the key pluripotency transcription factors OCT4, SOX2 and NANOG (19). Wong et al. demonstrated that functional GJIC was characteristically present in undifferentiated human ES cells (20). Strong evidence suggests that CXs play an essential role in the maintenance of human ES cell pluripotency; however, the relevant molecular mechanisms by which CXs affect pluripotency are unclear.

iPS cells resemble ES cells in many respects. iPS cells express characteristic stem cell markers, and the two cell types are similar with respect to their chromatin methylation patterns and differentiation capacities both in vitro and in vivo. To date, however, no information on CX expression in iPS cells has been extensively reported. Huang et al. reported that adherens junctions, GJs, focal adhesions and tight junctions were involved in complicated intercellular crosstalk that occurs during reprogramming (21). Thus, the roles of CXs in the reprogramming process must be investigated further.

Here, we demonstrate that CX43 is specifically and highly enriched in undifferentiated human iPS cell lines during and after the reprogramming process. We also demonstrate that iPS cells display functional GJIC. CX43 expression is upregulated during the reprogramming process, and the ectopic expression of CX43 enhances the reprogramming efficiency, whereas the knockdown of CX43 reduces reprogramming efficiency, possibly by affecting the mesenchymal-to-epithelial transition (MET) process, as reported by changes in E-CADHERIN expression.

RESULTS
Generation and characterization of human iPS cells
To generate human iPS cells, we introduced retroviruses expressing human OCT4, SOX2, c-MYC and KLF4 (termed four factors) into human embryonic fibroblasts (hEFs). On day 5, samples containing 5 × 10⁴ cells were transferred into ES medium to 100 mm-diameter dishes carrying irradiated feeder layers. Approximately 3 weeks later, some flat, round, ES-cell-like colonies with distinct edges were observed. Twenty colonies were picked up, dissociated into small clumps without enzymatic digestion, and plated in ES medium onto irradiated feeder layers. Finally, six of the colonies derived from two hEF samples (hEF-1 and hEF-2) were successfully expanded to more than 30 passages. Because hEF-1 and hEF-2 showed similar results, all of the figures shown here come from hEF-1. Three fully characterized iPS cell lines derived from hEF-1 are shown in detail (Fig. 1 and Supplementary Material, Fig. S1).

Human iPS cells stained positive for alkaline phosphatase (AP), and immunofluorescence studies showed that they expressed the pluripotency markers OCT4, SSEA4, SOX2, TRA-1-60 and NANOG, as did H1 cells (Fig. 1A). RT–PCR experiments revealed that the human iPS cells expressed ES cell markers, including endogenous OCT4, SOX2, c-MYC, KLF4, and the other markers REX1, TERT, GDF, DNMT and ECAT1 (Supplementary Material, Fig. S1A). Real-time PCR experiments also revealed that the exogenous SOX2/KLF4/OCT4/c-MYC genes in the iPS cell lines were silenced (Supplementary Material, Fig. S1B). Like H1 cells, the human iPS cells formed embryoid bodies (EBs) in vitro, and RT–PCR analyses revealed that the EB cells expressed endodermal (CK19 and AFP), mesodermal (GATA4 and MEF2C) and ectodermal (NESTIN and MAP2) markers but not ES cell markers (OCT4 and NANOG) (Supplementary Material, Fig. S1C). To further analyse spontaneous differentiation, EBs were transferred to adherent culture on gelatin-coated plates. The cells displayed various morphologies and expressed the endodermal marker albumin (ALB), the mesodermal marker fibronectin (FN) and the ectodermal marker β-tubulin (TUJ1) (Fig. 1B). Approximately 2 months after the iPS cells were injected into nude mice, the animals were sacrificed, and the teratomas were analysed. The histological examination showed that the teratomas contained all the three germ layers, namely endoderm-derived glandular epithelium, mesoderm-derived cartilage and ectoderm-derived neural tubes (Fig. 1C).

An analysis of the methylation status of the OCT4 and NANOG promoters in these cells revealed that they were highly unmethylated in human iPS cells and H1 cells, whereas the CpG dinucleotides of these regions were highly methylated in the hEFs (Supplementary Material, Fig. S1D), indicating that the OCT4 and NANOG promoters are specifically active in both human iPS cells and H1 cells.

Both the in vivo and in vitro analyses revealed that these human iPS cells exhibited the essential characteristics of human ES cells, particularly the capacities of self-renewal and differentiation.

CX43 expression in human iPS cells
The human iPS cell lines synthesized mRNAs encoding almost all of the CXs, including CX25, CX26, CX30, CX30.2, CX30.3, CX31, CX31.1, CX31.9, CX37, CX40, CX43, CX45, CX46, CX47, CX59 and CX62 (Supplementary Material, Fig. S2). These results are similar to those of human ES (hES) cells (14). Real-time PCR experiments revealed the spectrum of relative CX mRNA expression
levels in H1, hEF and iPS cells. While all of the tested CX mRNA transcripts were expressed to some degree by each type of cell line, the CX43 mRNA showed the greatest upregulation in hEFs that were reprogrammed into iPS cells (Fig. 2A and Supplementary Material, Fig. S2). Western blotting experiments showed that the CX43 protein was expressed at similarly high levels in these human iPS cells and H1 cells, but it was expressed at low levels in hEFs and human foreskin fibroblasts (hFFs) (Fig. 2B and Supplementary Material, Fig. S3). The H1 cells showed a triplet of CX43 bands known to correspond to one non-phosphorylated (NP) and two phosphorylated (P1, P2) CX43 forms (13,22). The iPS cells showed the same triplet, suggesting that the post-translational processing of the CX43 protein was similar between the two cell types (Fig. 2B). In both the H1 and iPS cells, CX43 was primarily localized along the borders between neighbouring iPS cells in typical junctional punctae (Fig. 2C).

**Gap junctional intercellular communication (GJIC) in human iPS cells**

We next evaluated functional coupling among human iPS cells using the scrape loading/dye transfer assay. As shown in Figure 3, confluent cultures were scraped and incubated with the GJ-permeable fluorescent dye Lucifer yellow (LY; green) and the GJ-impermeable fluorescent dye rhodamine-dextran (RD; red). Extensive diffusion of LY was observed throughout the iPS colonies, and the average transfer distance of LY was ~0.9 mm. The pharmacological GJ inhibitors carbamolxolone (CBX) and 18-α-glycyrrhetinic acid (18-α-GA) and the CX43 mimetic peptide GAP27 were used to further confirm the existence of GJ communication among iPS cells. Both (CBX) and 18-α-GA significantly reduced the transfer of LY among cells; the LY transfer distances were ~0.2 and 0.3 mm, respectively. GAP27, which is homologous to a portion of the second extracellular loop of CX43 adjacent to the fourth transmembrane segment and likely acts by perturbing CX–CX interactions that maintain channel integrity (23,24), partially reduced the dye transfer (a decrease of ~0.4 mm) compared with the transfer level observed in the presence of the negative control GAP20. Taken together, these results suggest that iPS cells are coupled through functional GJs and that CX43 contributes to this function. Similar results were obtained when we performed this experiment in human iPS cells that were cultured on Matrigel (Supplementary Material, Fig. S4).
Increased CX43 expression during reprogramming

The average CX43 mRNA and protein levels in these three human iPS cell lines increased ≏5-fold and 4.5-fold with respect to the levels in hEFs, respectively (Fig. 2A and B). To explore the relationship between CX43 expression and the acquisition of pluripotency during the reprogramming process, we collected samples at days 0, 3, 9 and 15 after the viral transduction of reprogramming factors and assessed the mRNA and protein expression levels of both CX43 and the pluripotency marker NANOG. The immunoblotting and RT–PCR experiments revealed that CX43 and NANOG expression increased gradually during the period of iPS cell formation (Fig. 4A and B). When starting with hFFs, we also observed similar CX43 protein expression dynamics in the reprogramming process (Supplementary Material, Fig. S5). Real-time PCR experiments revealed that CX43 and NANOG expression increased gradually during the period of iPS cell formation (Fig. 4A and B). When starting with hFFs, we also observed similar CX43 protein expression dynamics in the reprogramming process (Supplementary Material, Fig. S5). Real-time PCR experiments revealed that CX43 mRNA level increased slightly on days 3 and 9 after transduction compared with the level observed on the day of the viral transduction. Strikingly, at day 15 after transduction, the CX43 mRNA level was 5.31-fold greater than that on day 0 (Fig. 4C), suggesting that CX43 may play an important role in the reprogramming process.

Ectopic expression of CX43 enhances human iPS cell generation

To further investigate the role of CX43 in the reprogramming process, we studied whether ectopic CX43 expression could promote iPS cell generation. pFinal/PGK-puro-EF1α-CX43-IRES-eGFP was constructed as an overexpression vector (abbreviated as hCX43, Supplementary Material, Fig. S6A), and the empty vector pFinal/PGK-puro-EF1α-eGFP (without CX43) was used as a negative control. Figure 5A shows that ectopic hCX43 expression significantly affected CX43 overexpression in hEFs; its expression was ≏3.5-fold greater than the control.

When the empty vector (GFP group) or CX43 expression vector (hCX43 group) plus four factors was added to the hEFs, >90% of the cells were infected as reflected by eGFP fluorescence at day 3 (Supplementary Material, Fig. S6B). On day 5, these cells were transferred in ES medium to six-well plates carrying irradiated feeder layers. Three weeks later, AP staining was performed. In accordance with previous reports, we counted the number of colonies that stained positive for AP as the total colony number and the number of colonies with positive AP staining and typical ES-cell-like morphology (ES-cell-like colonies with a flat, round shape and a distinct edge) as the highly AP-positive colonies (6,25). We obtained ≏83 ± 7 colonies from 10 000 cells for the total AP-positive colonies and 7 ± 2 colonies from 10 000 cells for the highly AP-positive colonies in the GFP group; in the presence of CX43, these numbers were 220 ± 14 and 18 ± 4, respectively. The TRA-1-60 antigen was then utilized to identify fully reprogrammed cells (26,27). On day 28, TRA-1-60 expression was assayed by immunohistochemical staining. By counting the TRA-1-60-positive colonies, we obtained ≏29 ± 4 TRA-1-60-positive colonies.

Figure 2. CX43 expression in human iPS cells. (A) Real-time PCR analysis of CX43 in H1 cells, hEF-1 and human iPS cells. The error bars represent SD, n = 3, *P < 0.05, **P < 0.01 versus H1 cells. (B) Western blot analysis of CX43 expression in H1 cells, hEF-1 and human iPS cells. Error bars represent SD, n = 3, *P < 0.05, ***P < 0.001 versus H1 cells. P1 and P2, phosphorylated; NP, nonphosphorylated. (C) Immunofluorescence analysis of CX43 in human iPS cells and H1 cells. The nuclei were visualized with DAPI staining (blue). Bar = 50 μm.
from 10,000 cells in the presence of CX43 compared with \( \approx 8 \pm 2 \) TRA-1-60-positive colonies from 10,000 cells when using only the four factors. We repeated the experiment and consistently obtained an \( \approx 3 \)-fold increase in the reprogramming efficiency when counting either the AP-positive or the TRA-1-60-positive colonies (Fig. 5B and C). These results indicate that the ectopic expression of CX43 can enhance iPS cell generation and that it may be essential for iPS cells generation. The picked colonies were maintained and further characterized for pluripotency and differentiation potential. Those colonies showed similar characteristics to the four-factor iPS cells (Supplementary Material, Fig. S7). We obtained similar results when we performed this experiment with hFFs (Supplementary Material, Fig. S8). By counting the total AP-positive, highly AP-positive and TRA-1-60-positive colonies, we obtained \( \approx 161 \pm 13, 20 \pm 4 \) and \( 23 \pm 4 \) colonies from 10,000 cells, respectively, in the presence of CX43 plus the four factors; we only obtained \( 77 \pm 8, 7 \pm 1 \) and \( 6 \pm 1 \) colonies, respectively, in the presence of the four factors alone.

**Ablation of endogenous CX43 reduces reprogramming efficiency**

To further investigate the role of CX43 in iPS cell generation, lentiviral vectors encoding CX43 short hairpin RNAs (shRNAs), termed SH1, SH2 and SH3, were constructed to knockdown CX43 expression. A vector containing non-specific shRNA, termed NTC (a negative control), was also constructed. The empty vector was used as an additional negative control (mock group). The pLL3.7 vector allowed shRNA to be stably expressed, and the transfection efficiency was reflected by eGFP fluorescence. More than 90% of the cells were transduced with either the control or shRNA lentiviruses (Supplementary Material, Fig. S9). SH1 and SH3 highly effectively knocked down CX43 (85% and 65%, respectively), while SH2 was significantly less effective. No knockdown was observed in the hFFs treated with either NTC or the empty vector (Fig. 6A).

We next investigated the effect of CX43 knockdown on iPS cell generation by counting the colonies after the viral transduction of reprogramming factors (Fig. 6B and C). When CX43 expression was disrupted by SH1 or SH3 after four-factor-induced reprogramming, we obtained \( 44 \pm 6 \) and \( 46 \pm 7 \) AP-positive colonies from 10,000 cells, respectively; 86 \( \pm 7 \) colonies were obtained from the mock group on day 21. Decreases of \( \approx 53\% \) and 47% were obtained in parallel experiments. By counting highly AP-positive colonies, we found that an \( \approx 70\% \) decrease occurred when CX43 expression was disrupted with SH1 (3 \( \pm 0.5 \) colonies) when compared with the negative control (10 \( \pm 1 \) colonies). By counting the TRA-1-60-positive colonies, we obtained similar results among these groups (mock versus SH1 was \( 10 \pm 2 \) and \( 4 \pm 1 \) colonies).
colonies, respectively). These data indicate that endogenous CX43 expression may be essential for iPS cell generation.

**Gap junction inhibition does not affect the efficiency of human iPS cells generation**

CX43 primarily functions as a component of GJs via GJIC formation; however, in some cases, it also plays a physiological role through a GJIC-independent pathway (26,28). To test whether the effect on cell reprogramming was due to CX43-mediated GJIC, we added GJ inhibitors into the reprogramming process. By counting the AP-positive and highly AP-positive colonies, we found that CBX, 18-α-GA and the CX43 mimetic peptide GAP27 did not affect the efficiency of iPS cell generation (Supplementary Material, Fig. S10). These results suggest that the effect of CX43 on the efficiency of iPS cell generation may be attributed to the CX43 protein itself but not to the function of GJIC.

Changes in CX43 influence the expression of E-CADHERIN during reprogramming

Mouse fibroblasts have been reported to undergo a mesenchymal—epithelial transition (MET) early in reprogramming, and the expression of the epithelial marker E-CADHERIN characteristically increases during the MET (27,29,30). To determine whether CX43 affects this process, we investigated E-CADHERIN synthesis at different time points during reprogramming and found that ectopic expression of CX43 can significantly enhance E-CADHERIN expression compared with the control group (Fig. 7A). From day 7, higher E-CADHERIN expression levels were detected in the hCX43-treated cells when compared with the negative controls. The higher E-CADHERIN expression levels were maintained in the hCX43-treated cells until the end of the experiment. The same results were observed by immunofluorescence (Fig. 7B).

In the RNA interference groups, E-CADHERIN began to express on day 9, and this expression did not occur in the CX43 shRNA-treated cells. By day 12, E-CADHERIN was expressed in both the NTC and CX43 shRNA-treated cells, but it was expressed at greatly reduced levels in the CX43 shRNA-treated cells. At day 17, E-CADHERIN expression was further increased, although treatment with CX43 shRNA continued to significantly reduce its expression (Fig. 8A and B). Thus, the knockdown of CX43 delayed E-CADHERIN expression during reprogramming.

**DISCUSSION**

Technical improvements to increase the efficiency of iPS cell generation have been greatly promoted. Previous studies have shown that many high-efficiency induction systems induce a number of extra factors with the typical ‘SY4’ transcriptional genes. These extra factors include small molecules (such as the histone deacetylase inhibitor valproic acid, the DNA demethylating agent 5-azacytidine, the antioxidantvitamin C and the histone methyltransferase inhibitor BIX01294) (31–34), microRNAs (such as mir21, mir29a and miR-302-367) (35,36), important transcription factors or genes (such as Tbx3, Glis1 and UTF1) and siRNA (such as siRNA against p53) (6,25,37). Although great progress has been made, the low efficiency of reprogramming and the unclear molecular mechanisms that control the reprogramming remain as large hurdles that limit the application of iPS cells. As a major member of the CX family, CX43 has been shown to play an important role in the proliferation and differentiation of Mouse embryonic stem cells (mESs) and hESs. However, it is unclear whether CX43 participates in the reprogramming process or plays a physiological function in iPS cells.
We first identified the expression levels of most members of the CX family and found that most tested CXs, including CX25, CX26, CX30, CX30.2, CX30.3, CX31, CX31.1, CX31.9, CX37, CX40, CX43, CX45, CX46, CX47, CX59 and CX62, are expressed in iPS cells. These results are similar to the CX expression profile of hES lines (13,38–40). We also observed functional GJIC formation in human iPS colonies through scrape loading/dye transfer assays and saw reduced dye transfer by the pharmacological GJ inhibitors.

Among these CXs, CX43 is the CX isoform that was the most highly enriched at both the mRNA and protein expression levels in the undifferentiated iPS cells compared with most highly enriched at both the mRNA and protein expression levels in the undifferentiated iPS cells compared with most highly enriched at both the mRNA and protein expression levels in the undifferentiated iPS cells. CX43 is the CX isoform that was the most highly expressed in mouse ES cells and was found to be a downstream target of the key pluripotent transcription factors OCT4, SOX2 and NANOG (19). Moreover, the downregulation of CX43 expression in mouse ES cells markedly affected cell morphology and aggregation while upregulating differentiation markers (15). Mouse embryonic fibroblasts (MEFs) have been identified as a cell source for investigating the reprogramming efficiency and mechanism and it seems more efficient than adult fibroblasts during the reprogramming (2,41). In this study, hEFs were derived from skin explants of aborted foetuses. The original intention that we used hEFs as the starting cell type is that hEFs are similar to MEFs on growth characteristics, and we considered hEFs as the counterpart of MEFs. Similar to MEFs, hEFs proliferated faster, aged more slowly and showed a little bit higher reprogramming efficiency than hFFs. But the following study showed that CX43 has a similar expression pattern and play an important role in reprogramming of both two cell lines. When hEFs and hFFs were reprogrammed by the four Yamanaka factors, we observed that the CX43 protein level increased gradually along with the expression of the pluripotency marker NANOG. Together, these results indicate that CX43 may not only be a pluripotency marker of iPS cells; it may also participate in the reprogramming process.

Most interestingly, we show here for the first time that the ectopic expression of CX43 can significantly increase the efficiency of iPS cell generation, whereas the knockdown of endogenous CX43 expression by RNAi reduced the reprogramming efficiency, suggesting an important role of CX43 in reprogramming. By performing a western blot analysis, we found that CX40 and CX45 were expressed in human iPS cells; this result has also been reported in human ES cells (14). Because the knockdown was designed specifically for CX43, and the knockdown or overexpression of CX43 did not affect the expression of CX40 and CX45 (Supplementary Material, Fig. S11), we inferred that CX43 may play a unique role in reprogramming that cannot be compensated by the presence of other CXs. Previous studies have reported that CX43 primarily functions as a component of GJs via GJIC formation. However, it was unclear whether the effects of CX43 on iPS cell generation were GJIC-dependent. By introducing GJ inhibitors into the reprogramming process, we found that CBX, 18-α-GA and the CX43 mimetic peptide GAP27 did not affect the efficiency of iPS cell generation, suggesting that GJIC may not be indispensable for reprogramming. CX is well known as a component of GJs is involved primarily in GJIC; however, some studies suggest that CX may also have a GJIC-independent function (26,28). Langlois et al. reported that in rat epidermal keratinocytes, CX43-mediated tumour suppression may be linked to its interaction with another reported tumour-suppressing molecule, caveolin 1, independent of GJIC function (42,43). Together, these data indicated that CX43-mediated GJIC may not be required for iPS cell generation.

To further investigate the possible mechanisms by which CX43 affect the efficiency of reprogramming, we examined the relationship between CX43 and the mesenchymal-to-epithelial transition (MET). After a spontaneous EMT in vitro, epicardium-derived cells were shown to acquire a spindle-shaped morphology, and CX43 expression was downregulated (44). In addition, the lack of both CX43 and E-CADHERIN expression and the presence of VIMENTIN are characteristic of the fibroblast-like cells that appear during reprogramming and that surround undifferentiated human ES cells. Among these CXs, CX43 is the CX isoform that was the most highly enriched at both the mRNA and protein expression levels in the undifferentiated iPS cells compared with most highly enriched at both the mRNA and protein expression levels in the undifferentiated iPS cells.
cell colonies, suggesting the occurrence of an EMT (45). De Boer et al. found that the MET was accompanied by increased levels of CX43-encoding mRNA and CX43 protein and enhanced metabolic and electrical coupling (46); we therefore hypothesized that CX43 may mediate reprogramming by facilitating MET. The appearance of the epithelial marker E-cadherin is a characteristic of the MET, and the deletion of E-CADHERIN prevents reprogramming (36,47,48). Xu et al. reported that the overexpression of CX43 significantly induced E-CADHERIN expression in human non-small-cell lung cancer cells (49), and the possibility that E-CADHERIN could enhance iPS cell generation has been reported (47,48). In our present work, we found that the ectopic expression of CX43 enhanced the expression of E-CADHERIN during reprogramming, while the knockdown of CX43 delayed E-CADHERIN expression. Therefore, the ectopic expression of CX43 may increase the efficiency of reprogramming partially by boosting the MET. Yu et al. recently reported that the reconstitution of CX43 in glioma stem cells reversed their malignant phenotypes by influencing E-CADHERIN and its coding protein, leading to changes in the expression of the WNT/β-CATENIN targeting genes (50). Overall, CX43 increases the efficiency of reprogramming, in part, by inducing a more efficient MET.

In conclusion, we first systematically investigated the expression of the CX43 protein in human iPS cells and the functional GJ formation within iPS colonies. We found that CX43 is specifically highly enriched when hEFs are reprogrammed into undifferentiated iPS cells. The CX43 protein level increased gradually during the reprogramming process, and its overexpression enhanced the reprogramming and the MET. CX43 may represent a pluripotency marker of iPS cells and may play an important role in this process. Together, our results assist in the future exploitation of the iPS cell
technology. Further work is needed to elucidate the exact mechanisms by which CX43 affects reprogramming and iPS cell biology.

MATERIALS AND METHODS

Cell culture

hEFs were prepared as previously described (51) from foetuses aborted after 2 to 3 months of gestation with informed parental consent. Skin explants (1 mm³) were placed into 100 mm-diameter tissue culture dishes containing hEFs medium (Dulbecco’s modified Eagle’s medium (DMEM) (Hyclone) containing 10% (v/v) FBS (Hyclone) and 1% (w/v) penicillin/streptomycin (Hyclone)) and incubated at 37°C in 5% CO₂ in an air atmosphere. The dishes were left undisturbed for three days, after which 2 ml aliquots of fresh medium were used to top up the existing media every 48 h. After one week, confluent primary monolayers were established, and the cells were maintained in hEF medium. hFFs from adult human foreskin were prepared and maintained as previously described with informed consent (52). All of these experimental procedures were approved by the Ethics Committee of Sun Yat-sen University. 293FT cells were cultured in DMEM containing 10% (v/v) FBS, 2 mM L-glutamine (Invitrogen), 1% (w/v) non-essential amino acids (Hyclone) and 1 mM sodium pyruvate (Sigma). Human iPS cells and the human ES cell line H1 were maintained on feeder layers of irradiated MEFs in ES medium using standard methods (53,54). The ES medium consisted of knockout DMEM (Invitrogen) supplemented with 15% (v/v) knockout serum replacement (Invitrogen), 5% (v/v) foetal bovine serum, 0.1 mM β-mercaptoethanol (Sigma), 1% (w/v) non-essential amino acids, 1% (w/v) penicillin/streptomycin, 2 mM L-glutamine and 10 ng/ml human basic fibroblast growth factor (Chemicon). When a feeder-free culture of H1 and human iPS cells was required, plates were coated with 0.3 mg/ml Matrigel (BD Biosciences) in MEF-conditioned ES medium. The MEF-conditioned ES medium was collected as previously described to allow feeder-free growth of undifferentiated human ES cells.
EBs were maintained in EB medium consisting of 80% (v/v) knockout DMEM supplemented with 20% (v/v) knock-out serum replacement, 0.1 mM β-mercaptoethanol, 1% (w/v) non-essential amino acids, 1% (w/v) penicillin/streptomycin and 2 mM L-glutamine. The colonies were harvested by treatment with dispase (Invitrogen).

Generation of human-induced pluripotent stem cells from hEFs

Retroviruses were produced by transfecting (using Lipofectamine™ 2000 (Invitrogen)) 293FT cells with pMX-based retroviral vectors containing sequences encoding human OCT4, SOX2, c-MYC, KLF4, pUMVC and pCMV-VSVG (AddGene). After 48 h, the supernatants were collected, filtered through filters with 0.45 μm diameter pores and added to hEF plates together with 8 μg/ml polybrene (Sigma). The titre of virus used in our study was \( \approx 5 \times 10^6 \) TU/ml (determined by the Retro-X™ qRT-PCR Titration Kit, Clontech). Each culture medium was replaced 12 h after infection. After a further 12 h, the hEFs were reinfected, and the culture medium replaced again; ‘day 0’ commenced at this time. On day 5, samples of \( 5 \times 10^4 \) cells were transferred in ES medium to 100 mm-diameter dishes bearing irradiated feeder layers. The medium was changed every other day. The colonies were selected morphologically, mechanically dissociated into small clumps by repeated pipetting and transferred to feeder layers growing in 24-well plates.

Alkaline phosphatase (AKP/AP) staining was performed using the BCIP/NBT Phosphatase Substrate System (Three-Component) (KPL) according to the manufacturer’s instructions. TRA-1-60 expression (1:100; Millipore) on colonies was analysed with immunohistochemistry using the Vectastain ABC kit (lgM) (Vector lab) to identify fully reprogrammed iPS cell colonies.

Embryoid body and teratoma formation from human iPS cells

Human iPS colonies were digested into small clumps in a solution of 1 mg/ml collagenase IV (Invitrogen) and incubated in

![Figure 8. Ablation of endogenous CX43 reduces the expression of E-CADHERIN during reprogramming. (A) Western blot analysis of E-CADHERIN and CX43 expression in hEF-1 transduced with the four factors and NTC or SH1 on the indicated days during the reprogramming process. The error bars represent SD, \( n = 4, ^* P < 0.05 \) versus NTC 9 days. (B) Immunofluorescence analysis of E-CADHERIN expression in hEF-1 transduced with the four factors and NTC or SH1 on the indicated days during the reprogramming process. Bar = 50 μm. NTC, containing nonspecific shRNA plus four factors; SH1, containing CX43 shRNA target sequences plus four factors.]
EB medium for 9 days to permit EB formation. The media was changed every other day. Next, EBs were either processed for RT–PCR (to confirm the expression of markers from each of the three germ layers) or transferred in EB medium onto gelatin-coated plates to facilitate adherent culture. After 8 days of incubation, these cultures were stained using immunofluorescent reagents to detect in vitro differentiation.

For teratoma production, ~1 × 10^7 human iPS cells were suspended in DMEM and injected subcutaneously into nude mice. Approximately 2 months later, teratomas were dissected and fixed overnight in 4% (v/v) paraformaldehyde. Paraffin-embedded tissues were sliced and subjected to H&E staining to detect in vivo differentiation. All of the animal experimental procedures were approved by the Animal Ethics Committee of Sun Yat-sen University.

Western blotting

Cell lysates were separated via SDS–PAGE using 8% (w/v) Tris-glycine mini-gels and transferred to polyvinylidene difluoride membranes as previously described (56). The primary antibodies used were as follows: mouse anti-CX43 (1:2000; CXN-6; Sigma), mouse anti-CX40 (1:500; Millipore), rabbit anti-CX45 (1:1000; Millipore), rabbit anti-NANOG (1:2000; D73G4; Cell Signaling), mouse anti-E-CADHERIN (1:1500; 36/E-CADHERIN; BD Biosciences) and mouse anti-β-ACTIN (1:10 000; AC-74; Sigma). Immunopositive bands were visualized on X-ray films (Kodak) after incubation with a reagent that enhanced chemiluminescence (Amersham). All of the western blot exposures were within the linear detection range, and the band intensities were quantified using the Quantity One software program and a GS-800 densitometer (Bio-Rad).

RT–PCR and real-time PCR

Total RNA was extracted using the RNeasy Plus Mini kit (QIAGEN) according to the manufacturer’s instructions. RNA was reverse-transcribed using an AMV First-Strand cDNA synthesis kit (Invitrogen). GoTaq™ Green Master Mix (Promega) was used to perform a semiquantitative PCR. A real-time quantitative PCR was performed using the SYBR qPCR mix (Life Technologies). An RT–PCR was performed under the following conditions: initial denaturation at 94°C for 3 min; 32 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 45 s and a final incubation at 72°C for 10 min. For a real-time quantitative PCR, the following conditions were used: 5 min at 94°C, 40 cycles of 15 s at 94°C, 15 s at 58–61°C, and extension at 72°C for 40 s. All of the gene expression values were normalized to those of internal controls (GAPDH).

RT–PCR (to confirm the expression of markers from each of the three germ layers) or transferred in EB medium onto gelatin-coated plates to facilitate adherent culture. After 8 days of incubation, these cultures were stained using immunofluorescent reagents to detect in vitro differentiation.

Scrape loading/dye transfer assay

GJIC activity was assessed using the scrape loading/dye transfer assay as previously described (57,58). Briefly, the cells were rinsed three times with prewarmed PBS. In some of the experiments, the cells were treated with CBX (100 μM; 4 h; Sigma), 18α-GA (25 μM; 4 h; Sigma) or the CX43 mimetic peptide GAP27 (amino acid sequence SRPTKEFIII, 300 μM; 4 h; CPG Biotech). The CX43 mimetic peptide GAP20 (amino acid sequence EIKKFKYG, 300 μM; 4 h; CPG Biotech) served as a negative control. The cell colonies were scraped with a scalpel and incubated for 5 min with Lucifer yellow (2 mg/ml, Sigma) and rhodamine-dextran (2 mg/ml, Invitrogen) diluted in PBS. Dye transfer was observed by fluorescence microscopy, and the distance between the dye transfer front and the scrape line was analysed.

Ectopic expression of CX43

pUp-EF1α and the entry clone containing the 1149 bp human GJ alpha-1 (GJA1, also called CX43) (Fulgenz) ORF (pDown-CX43) and pTail-ires-eGFP were cloned into pDest puromycin by the Gateway LR reaction as described previously (59) to generate the expression lentiviral vector, which is designated as pFinal/pgk-puro-EF1α-CX43-ires-eGFP (abbreviated as hCX43, Supplementary Material, Fig. S6A). The pFinal/PGK-puro-EF1α-eGFP lentiviral vector (abbreviated as GFP) acted as a control. The lentiviral vectors were transected into 293FT cells in the presence of packaging plasmids using Lipofectamine 2000 (Invitrogen). After 48–72 h, the supernatants were collected, filtered through filters with pores 0.45 μm in diameter and concentrated via ultracentrifugation (50 000 g; 120 min at 4°C). To address the role of CX43 in human iPS cell generation, the GFP or hCX43 vector plus four reprogramming factors were introduced into hEFs twice as indicated above. On day 5, the fibroblasts were trypsinized and seeded onto feeder layers in six-well plates at a density in the range of 10 000 or 30 000 cells per well in ES medium. AP and TRA-1-60-positive colonies were counted by three independent experiments for each group.
Lentiviral-mediated shRNA interference of CX43

A short-hairpin RNA (shRNA) designed to knock down CX43 synthesis was annealed and cloned into the pL3.7 lentiviral vector (a kind gift from Dr David L. Garbers, University of Texas Southwestern Medical Center, Dallas, TX, USA). After sequencing, the lentiviral vectors were transfected into 293FT cells in the presence of packaging plasmids using Lipofectamine 2000 (Invitrogen) to produce lentivirus as described above. hEFs were infected twice, and the knockdown efficacy of each shRNA-containing lentivirus was assessed after 2 days by western blotting. To explore the effects of CX43 knockdown on human iPS cell generation, hEFs were transduced with shRNA-bearing lentivirus 2 days after transduction with the four reprogramming factors. On day 5, the hEFs were collected using trypsin and seeded in ES medium onto feeder layers in six-well plates at a density in the range of 10,000 or 30,000 cells per well. AP-positive and TRA-1-60-positive colonies were counted by three independent experiments for each group.

Chemical and peptide inhibition

hEFs were collected using trypsin and seeded in ES medium onto feeder layers in six-well plates at a density in the range of 10,000 cells per well 3 days after they were transduced with the four reprogramming factors. On the next day, CBX, 18-α-GA, and the CX43 mimetic peptide were added at final concentrations of 100, 25 and 300 μM, respectively. The medium was changed every other day, and the inhibitors were continually used during the process. AP-positive colonies were counted by three independent experiments for each group.

Statistical analysis

All of the data were statistically examined using the unpaired Student’s t-test; significance levels of *P < 0.05, **P < 0.01 and ***P < 0.001 were sought. The data are presented as the means ± SD calculated using the Sigma Plot software program (Jandel Scientific).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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