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RESEARCH ARTICLE

Reactivation of the inactive X chromosome and post-transcriptional reprogramming of *Xist* in iPSCs

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ABSTRACT

Direct reprogramming of somatic cells to pluripotent stem cells entails the obliteration of somatic cell memory and the reestablishment of epigenetic events. Induced pluripotent stem cells (iPSCs) have been created by reprogramming somatic cells through the transduction of reprogramming factors. During cell reprogramming, female somatic cells must overcome at least one more barrier than male somatic cells in order to enter a pluripotent state, as they must reactivate an inactive X chromosome (Xi). In this study, we investigated whether the sex of somatic cells affects reprogramming efficiency, differentiation potential and the post-transcriptional processing of *Xist* RNA after reprogramming. There were no differences between male and female iPSCs with respect to reprogramming efficiency or their differentiation potential *in vivo*. However, reactivating Xi took longer than reactivating pluripotency-related genes. We also found that direct reprogramming leads to gender-appropriate post-transcriptional reprogramming – like male embryonic stem cells (ESCs), male iPSCs expressed only the long *Xist* isoform, whereas female iPSCs, like female ESCs, expressed both the long and short isoforms.

KEY WORDS: Reprogramming, iPSC, Pluripotency, *Oct4*, *Xist* isoform

INTRODUCTION

Induced pluripotent stem cells (iPSCs) can be derived directly from somatic cells by transduction of transcription factors (Takahashi and Yamanaka, 2006; Maherali et al., 2007; Okita et al., 2007; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007; Park et al., 2008; Shi et al., 2008). Among the various pluripotent cell types, iPSCs are the most similar to embryonic stem cells (ESCs), based on their molecular signature and differentiation potential, and they fulfill all the criteria for pluripotency – the formation of germline-competent chimeras (Okita et al., 2007), germline transmission (Kim et al., 2008; Shi et al., 2008) and full-term development after tetraploid complementation (Kang et al., 2009; Zhao et al., 2009). The generation of iPSCs also entails epigenetic modification, such as changes in genomic imprinting (Kim et al., 2013), histone modification (Maherali et al., 2007) and reactivation of the inactive X chromosome (Xi) (Maherali et al., 2007; Ohhata and Wutz, 2013). Generally, the efficiency of iPSC derivation using four transcription factors varies depending on the origin of the somatic cells, and ranges from 0.01 to 3.6% (Takahashi and Yamanaka, 2006; Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007; Huangfu et al., 2008; Kim et al., 2008). It has been shown that cells that already express some stem cell markers are most likely to be reprogrammed into iPSCs (Eminli et al., 2008; Kim et al., 2008), whereas fully differentiated cells are often unfavorable (Hanna et al., 2008). Therefore, the specific type of somatic cell selected for iPSC derivation is a factor that might affect reprogramming efficiency (Kim et al., 2008; Kim et al., 2009).

Cell reprogramming efficiency might also vary depending on the sex of somatic cells because female somatic cells must overcome at least one more barrier than male somatic cells during pluripotential reprogramming – Xi reactivation (Do and Schöler, 2009). In a previous study, we showed that reactivating the Xi takes longer than reactivating other pluripotency markers during fusion-induced reprogramming (Do et al., 2008). The presence of two X chromosomes also has been shown to affect early embryonic development in mammals, as female embryos develop more slowly than males (Mittwoch, 1993; Burgoyne et al., 1995). More ESCs or embryonic germ cells are derived from male embryos than from female embryos (Matsui et al., 1992), and the reprogramming efficiency of female primordial germ cells (PGCs) or somatic cells might be lower than that of male PGCs or somatic cells. Recent studies have also shown that the active X chromosome (Xa) in female ESCs interferes with differentiation from a pluripotent state (Schulz et al., 2014). Taken together, the presence of two X chromosomes seems unfavorable for early embryonic development, differentiation and reprogramming. However, to date, the reprogramming efficiency of iPSCs generated from male versus female somatic cells has not been compared.

Although iPSCs are morphologically and functionally indistinguishable from ESCs, iPSCs are distinguishable from ESCs by their gene expression signature (Chin et al., 2009). Based on this observation, it is of interest to further assess the identity of iPSCs at the post-transcriptional level as compared to ESCs. During post-transcriptional processes, RNA can be spliced to produce different splice variants that code for multiple proteins (Black, 2003). Previously, Hong et al. reported that two different *Xist* RNA isoforms exist in cells – a long isoform (L-isoform) and a short isoform (S-isoform) (Hong et al., 1999; Hong et al., 2000). As *Xist* RNA does not encode a protein, RNA splicing is the final post-transcriptional process that *Xist* RNA undergoes. Here, we compared the reprogramming efficiency of male somatic cells with that of female cells and investigated the developmental

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potential of these cells and the mechanisms underlying the reactivation of the Xi. We also assessed whether iPSCs perform the same post-transcriptional processes as ESCs.

RESULTS

Reprogramming efficiency of male and female MEFs

Male and female murine embryonic fibroblasts (MEFs) were prepared from *OG2*$^{+/-}$/*ROSA26*$^{+/-}$ transgenic mice carrying (1) GFP under the control of the *Oct4* regulatory regions and (2) a ubiquitously expressed *neo/lacZ* transgene. To compare reprogramming efficiencies, these male and female cells were used as somatic cells for direct reprogramming with four transcription factors (Oct4, Sox2, Klf4 and c-Myc) in the absence of any small molecules for enhancing reprogramming efficiency. Oct4-GFP^{+} ESC-like colonies were first detected on day 9 postinfection in both the male MEFs (mMEFs) and the female MEFs (fMEFs; Fig. 1A). Colonies of GFP^{+} iPSCs were morphologically indistinguishable from those of normal ESCs after sorting and subsequent culturing (Fig. 1A). To assess the efficiency of MEF reprogramming, we counted the number of GFP^{+} colonies on days 9, 14, and 21 postinfection. GFP^{+} iPSCs derived from mMEFs were obtained with an efficiency of ~0.002% at day 9 and 0.07% at day 21 postinfection (Fig. 1B). The reprogramming efficiency of fMEFs was not different from that of the mMEFs (Fig. 1B); this result indicates that there was no difference in the reprogramming efficiency of endogenous *Oct4* (based on the strength of the Oct4-GFP signal) between mMEFs and fMEFs. In addition, we assessed the *in vivo* developmental potential of the male and female iPSCs by evaluating chimera formation efficiency. Both male and female iPSCs efficiently formed chimeric embryos at 13.5 days post coitum (dpc), at a rate of ~66% and 69%, respectively; the cells were also shown to contribute to germline formation (Fig. 1C).

We next compared the gene expression patterns of male and female iPSCs with those of male and female ESCs, respectively, in order to exclude any variation between male and female cells (Fig. 2A). Microarray analysis showed that male and female ESCs exhibited 99.6% similarity (0.4% difference) in RNA expression patterns. Male ESCs and male iPSCs showed 99.5% similarity in gene expression patterns, whereas female ESCs and female iPSCs showed 99.9% similarity (Fig. 2A). Next, male and female iPSCs forming dome-like colonies (iPSC-M1 and iPSC-F1 cells, respectively) or flat colonies (iPSC-M2 and iPSC-F2

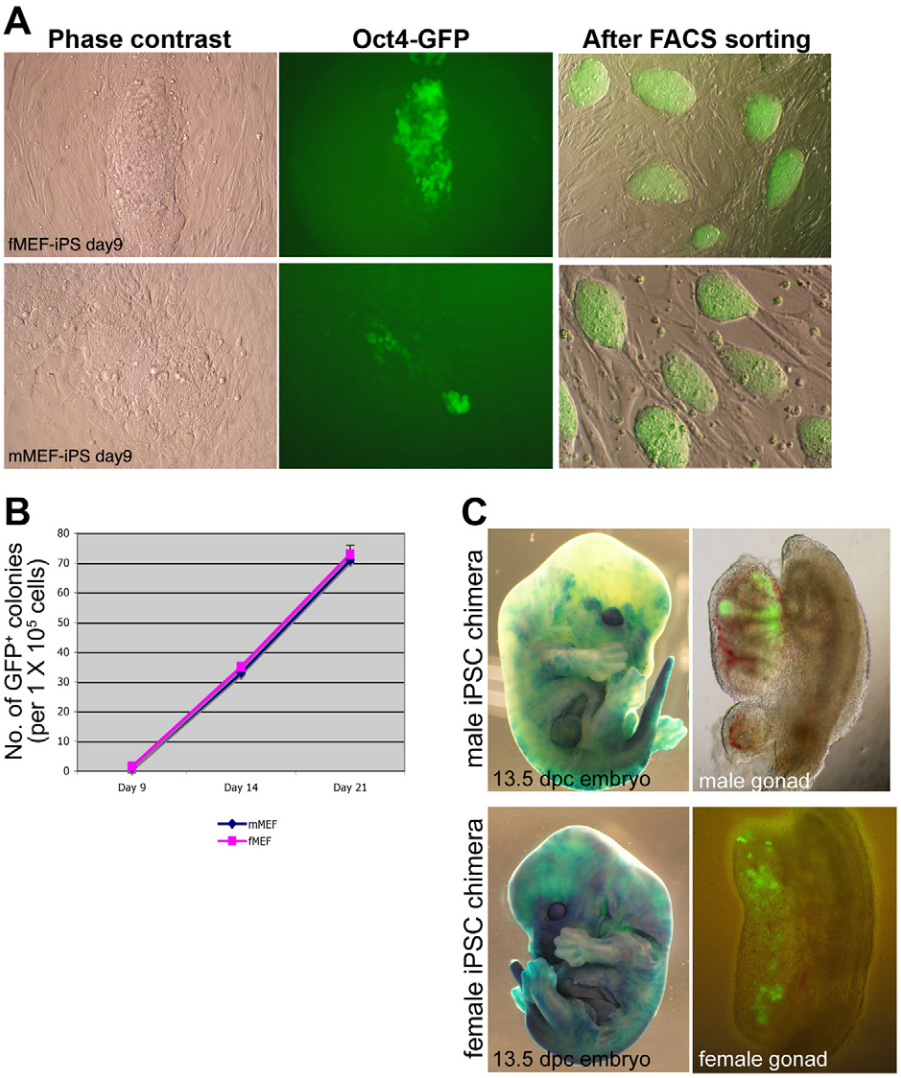

Fig. 1. Generation of male and female iPSCs and their characteristics. (A) Oct4-GFP^{+} iPSCs were first observed on day 9 during both male and female iPSC generation. The original magnification was ×100 for iPSCs at day 9 and ×200 after sorting. (B) Reprogramming efficiency of male and female mouse embryonic fibroblasts (mMEFs and fMEFs, respectively). GFP^{+} colonies were counted on days 9, 14 and 21 postinfection. The reprogramming efficiency of fMEFs was almost the same as that of mMEFs. (C) Chimera formation using iPSCs after aggregation with 8-cell embryos and transfer to a pseudopregnant mother. The chimera formation rate was ~66% (6/9) and 69% (18/26) using male and female iPSCs, respectively. These iPSCs also contributed to germline formation.

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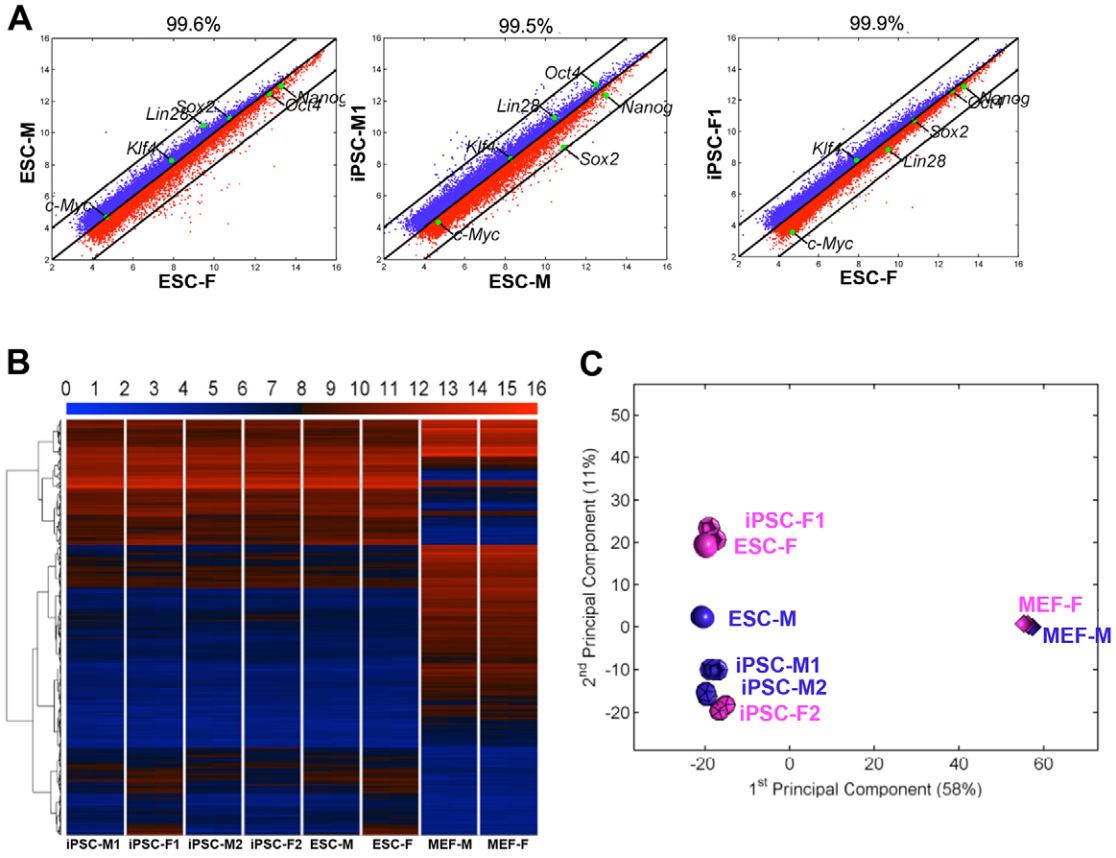

Fig. 2. Gene expression patterns of MEFs, ESCs and iPSCs. (A) Pairwise scatter plots comparing the global gene expression patterns between male and female ESCs (ESC-M and ESC-F, respectively), between male iPSCs (iPSC-M1) and male ESCs, and between female iPSCs (iPSC-F1) and female ESCs. Red data points show genes upregulated in *x*-axis samples; blue data points show *y*-axis samples. (B) Heat map of the global gene expression patterns of MEFs, ESCs and different iPSC lines. (C) Principal component analysis of male and female iPSC lines forming dome-like colonies (iPSC-M1 and iPSC-F1) and flat colonies (iPSC-M2 and iPSC-F2). The global gene expression pattern of the iPSC-F1 line was closer to that of female ESCs than that of male ESCs, and the global gene expression pattern of the iPSC-M1 line was closer to that of male ESCs than female ESCs. The gene expression patterns of the iPSC-M2 and iPSC-F2 lines, which form flat colonies, were distinct from those of the control male and female ESCs.

cells, respectively) were analyzed using heat map (Fig. 2B) and principal component analysis (Fig. 2C; supplementary material Fig. S1). The iPSC-F1 cells exhibited a global gene expression profile that was more similar to the profile of female ESCs than to that of male ESCs; similarly, the profile of iPSC-M1 cells was closer to the profile of male ESCs than to that of female ESCs (Fig. 2C). Interestingly, however, iPSC-M2 and iPSC-F2 were distinct from the control ESCs and clustered together (Fig. 2). Therefore, the formation of dome-like colonies seems to be characteristic of the global gene expression signature for standard pluripotent stem cells.

Xi reactivation is a slow process

Xi reactivation is one of the epigenetic milestones that occur during the process of pluripotential reprogramming (Do et al., 2008). We reported previously that the *Xist* gene took a longer time to be reprogrammed to a pluripotent state than other pluripotency markers in fusion-induced reprogramming (Do et al., 2008; Choi et al., 2012). Moreover, Xi reactivation is specific to naïve pluripotent cells and does not occur in primed pluripotent stem cells such as epiblast stem cells (EpiSCs) (Guo et al., 2009). Therefore, here, we investigated whether the Xi in female somatic cells could be efficiently reactivated to a pluripotent state, and whether the Xa in male somatic cells is changed to a pluripotent type of Xa expressing very low levels of *Xist*, given that it has been shown that the Xa of somatic cells does not express *Xist* (Do et al., 2008; Do et al., 2009).

The activity of X chromosomes can be estimated by quantifying the expression of *Xist* RNA, as *Xist* RNA is transcribed only from an Xi (Panning et al., 1997; Lee and Lu, 1999). Only female somatic cells contain an Xi; these become active when the cells acquire pluripotency *in vitro*, and female pluripotent cells carry two Xas until they differentiate (McBurney and Strutt, 1980; Stewart et al., 1994; Lyon, 1999). RNA expression levels were determined by real-time RT-PCR for fluorescence-activated cell sorting (FACS)-sorted iPSCs on day 20 and 30 postinfection, and the expression level of *Xist* was compared to that of pluripotency and tissue-specific marker genes. As shown by real-time RT-PCR and *Xist*/*Tsix* RNA fluorescent *in situ* hybridization (FISH), *Xist* RNA was undetectable in mMEFs but was highly expressed in fMEFs (Fig. 3A–D). *Xist* was not expressed in mMEFs, as they do not contain an Xi; however, as the mMEFs were reprogrammed, *Xist* RNA levels were upregulated and then reset to the male ESC level by day 30 (Fig. 3A). By contrast, the expression of *Xist* in female iPSCs was upregulated and maintained at a high level (approximately fivefold higher than in fMEFs) until day 20 postinfection, when the expression of the pluripotency markers

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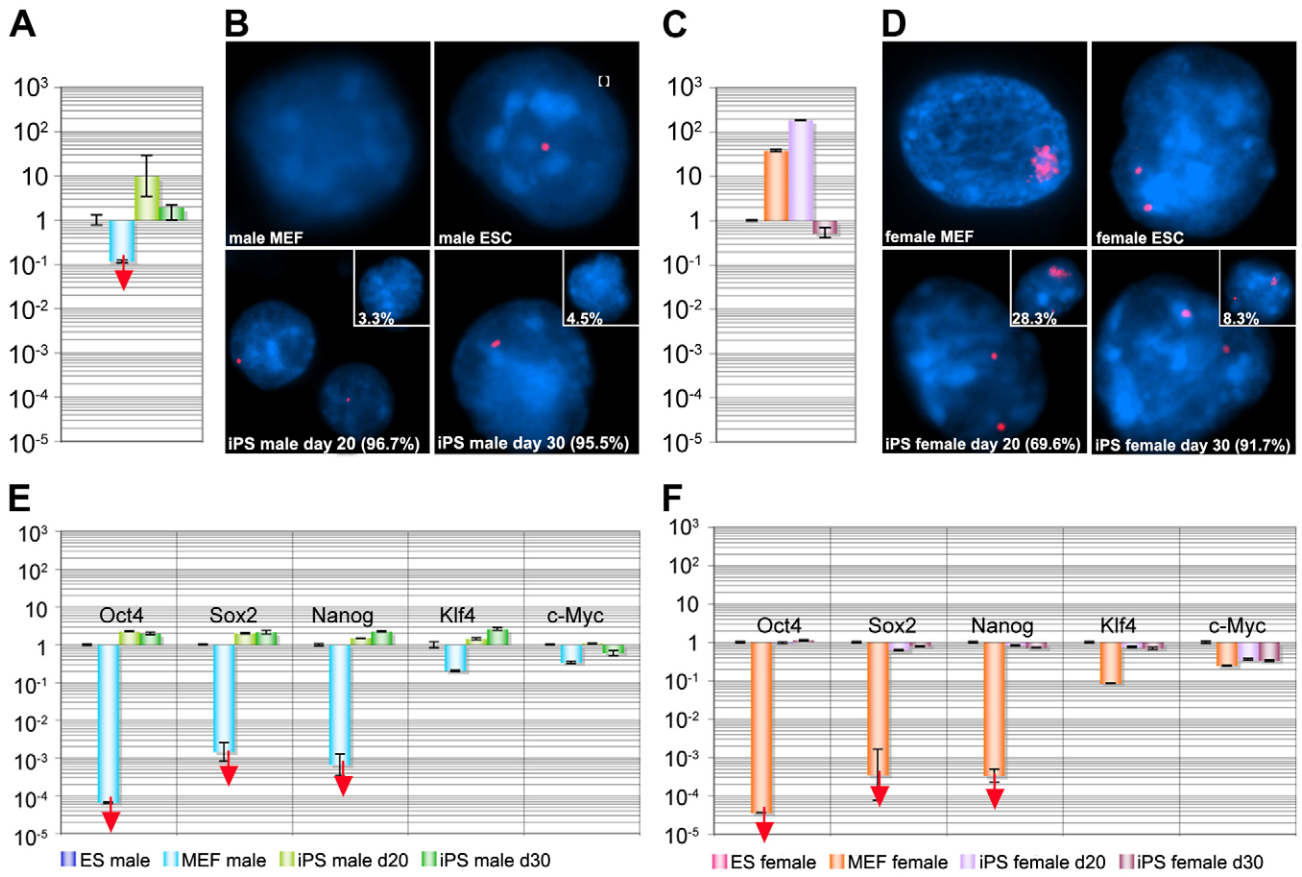

Fig. 3. Expression of *Xist* RNA and pluripotency-related genes and reactivation of inactive X chromosomes during reprogramming. (A) Real-time RT-PCR analysis of male MEFs, ESCs and iPSCs. *Xist* RNA, which was not expressed in male MEFs, was upregulated during reprogramming and then reset to the male ESC levels on day 30. (B) *Xist* RNA FISH of male MEFs, ESCs and iPSCs. At day 20 postinfection, *Xist* mRNA was not detected in male MEFs but was detected as a pinpoint signal in pluripotent male ESCs. Most of the male iPSCs showed a single pinpoint signal on day 20 postinfection, indicating that the active X chromosomes present in male MEFs changed into a pluripotent form of Xa. (C) Real-time RT-PCR analysis of female MEFs, ESCs and iPSCs. *Xist* expression in female MEFs was upregulated until day 20 postinfection but then downregulated to the level seen in female ESCs on day 30 postinfection. (D) *Xist* RNA FISH of female MEFs, ESCs and iPSCs. An *Xist* cloud was detected in female MEFs, but two pinpoint signals were detected in female ESCs. Approximately 28% of the female iPSCs still showed an *Xist* cloud (indicating Xi) on day 20 postinfection. On day 30 postinfection, ~92% of female iPSCs showed complete reactivation of the Xi. The original magnification of the data shown in B,D was × 400. (E) By day 20 postinfection, the pluripotency markers *Oct4*, *Sox2*, *Nanog*, *Klf4* and *c-Myc* were reset to the levels seen in ESCs in male and female cells (F). The red arrow denotes that no signal was detected after 45 cycles, set as the effective Ct value in the analysis software. The *y*-axis values are based on a logarithmic scale, and minor gridlines indicate a tenth of the value of each major gridline. Quantitative data show the mean±s.d. (three independent experiments).

Oct4, *Sox2*, *Nanog*, *Klf4* and *c-Myc* had been reset to the ESC level and the Oct4-GFP signal was present (Fig. 3E,F); this observation indicates that *Xist* was aberrantly reprogrammed in the early stages of iPSC formation. *Xist* levels were not downregulated to ESC levels until day 30 postinfection; this observation indicates that the reprogramming of the *Xist* gene by defined factors was delayed (Fig. 3).

Next, *Xist*/*Tsix* RNA FISH analysis was conducted to analyze Xi reactivation at the single cell level. *Xist*/*Tsix* RNA FISH analysis is better than strand-specific *Xist* RNA FISH in this experiment because *Xist*/*Tsix* RNA FISH can discriminate between the Xa from somatic cells and that of pluripotent cells. In pluripotent cells, *Xist*/*Tsix* RNA is detected as a large red signal in the Xi and as a red pinpoint signal in the Xa. However, in somatic cells, no signal is detected (Do et al., 2008). Therefore, by using *Xist*/*Tsix* RNA FISH, it is possible to distinguish a somatic cell Xa from a pluripotent cell Xa; in addition, this method can be used to analyze the X chromosome state in male as well as female cells. Thus, *Xist*/*Tsix* FISH in combination with strand-specific RT-PCR is a better technique for examining *Xist* expression and X chromosome inactivation state.

Xist was not detected in mMEFs but was detected as a pinpoint signal in pluripotent male ESCs (Fig. 3B). On day 20 postinfection, 96.7% of reprogrammed male iPSCs had a single pinpoint signal, indicating that the Xa in mMEFs had been changed into the pluripotent form of Xa. By contrast, an *Xist* cloud (large red signal) was detected in fMEFs, but two pinpoint signals were detected in pluripotent female ESCs (Fig. 3D). Approximately 28.3% of the reprogrammed iPSCs showed an *Xist* cloud (Xi) on day 20 postinfection, indicating that reactivation of the Xi in female iPSCs was not completed at this time-point. On day 30 postinfection, most iPSCs (91.7%) showed complete reactivation of Xi; thus, we checked the reactivation state of Xi at the time of Oct4-GFP activation. At an earlier stage of reprogramming, on day 11 postinfection, only 25.0% of the Oct4-GFP^{+} cells underwent reactivation of Xi (supplementary material Fig. S2). Strand-specific RT-PCR data also showed that *Xist* was downregulated in female cells and

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upregulated in male cells after reprogramming, indicating that the Xa somehow differs between somatic cells and pluripotent cells (supplementary material Fig. S3). These findings suggest that the reactivation of Xi takes a longer time than the reactivation of pluripotency genes during the direct reprogramming of somatic cells using defined factors.

Post-transcriptional reprogramming of *Xist*

It has been suggested that *Xist*, a noncoding RNA, is regulated by an unknown post-transcriptional event (Ma and Strauss, 2005) in which different splice variants are formed (Fig. 4A). There are two types of *Xist* RNA isoforms, the L-isoform and the S-isoform, which differ at their 3′ ends (Hong et al., 2000; Memili et al., 2001). We used primers that were specific for the *Xist* S-isoform (as they are positioned on either side of the region of *Xist* exon 7 that is deleted in the S-isoform) to discriminate between the S- and L-isoforms of *Xist* (Fig. 4A; supplementary material Fig. S4A). Ma and Strauss have suggested previously that although both *Xist* L- and S-isoforms are present in somatic cells, the *Xist* L-isoform predominates in early embryos (Ma and Strauss, 2005). Thus, we tested whether the presence of specific *Xist* isoforms could be used as a marker to distinguish pluripotent stem cells from somatic cells. First, we compared ESCs and MEFs. Surprisingly, contrary to previous reports (Ma and Strauss, 2005), we found that male ESCs expressed only the L-isoform, whereas female ESCs and fMEFs expressed both the L- and S-isoforms (Fig. 4B). To determine whether the RT-PCR product produced by our primer set represented a true *Xist* S-isoform, we sequenced it. The sequencing analysis confirmed that the RT-PCR product was the *Xist* S-isoform and, thus, that there was no pseudogene contamination present (supplementary material Fig. S4). These results indicate that the *Xist* isoform expression pattern does not distinguish pluripotent stem cells from somatic cells; however, it does distinguish between male and female cells. Therefore, *Xist* RNA expression might be regulated by different post-transcriptional mechanisms in male and female ESCs. We then determined whether this phenomenon was also true for iPSCs. Similar to male ESCs, male iPSCs expressed only the *Xist* L-isoform, whereas female iPSCs, similar to female ESCs, expressed both the L- and S-isoforms (Fig. 4B). These results indicate that direct reprogramming of somatic cells by transduction of transcription factors also leads to the appropriate reprogramming of post-transcriptional events.

DISCUSSION

During cell reprogramming, female somatic cells must overcome at least one more barrier to entering the pluripotent state than male somatic cells do – the reactivation of the Xi. Therefore, we investigated whether it is more difficult for female somatic cells to be reprogrammed to a pluripotent state than it is for male somatic cells. We also determined whether Xi reactivation during direct reprogramming by the transduction of transcription factors is a slow process.

During the reprogramming of female somatic cells by cell fusion with pluripotent cells, the reactivation of Xi has been shown to be a much slower process than the reactivation of other pluripotency-related genes; levels of expression of pluripotency- and tissue-specific marker genes were reprogrammed to ESC levels within 2 days of cell fusion, whereas complete reactivation of the Xi took at least 9 days (Do et al., 2008). In the present study, to determine whether the delayed reprogramming of *Xist* is a general feature of pluripotential reprogramming, we examined *Xist* expression and Xi reactivation status during iPSC induction, and we found that this phenomenon is also true for the direct reprogramming of somatic cells by the transduction of transcription factors.

Reprogramming driven by defined factors is a much slower reprogramming procedure than fusion-induced reprogramming. In fusion-induced reprogramming, all the factors needed for reprogramming are supplied from the ESC fusion counterpart. However, only a handful of defined reprogramming factors trigger pluripotential reprogramming during iPSC generation. In addition, it has been shown that the activation of the Oct4-GFP marker takes a week longer during iPSC generation than during fusion-induced reprogramming. Maherali et al. previously suggested that Xi reactivation in somatic cells is correctly induced during iPSC generation (Maherali et al., 2007). However, in this study, we showed that only a small population of iPSCs successfully reactivated the Xi at the initial stage of reprogramming and that it took ~30 days (3 weeks more than fusion-induced reprogramming) for >90% of iPSCs to complete Xi reactivation.

Although iPSCs are morphologically and functionally indistinguishable from ESCs, the two cell types are not identical and can be distinguished by their gene expression signatures (Chin et al., 2009). However, in the present study, we showed that the global gene expression profiles between iPSCs and ESCs were almost identical when male and female iPSCs were compared to male and female ESCs, respectively. Most previous studies have not considered the sex of iPSCs when comparing global gene expression profiles. Our results indicate that male iPSCs should be compared with male ESCs and likewise for female iPSCs and female ESCs.

In the present study, we focused on the reprogramming of differentiated somatic cells into the naïve pluripotent state. In female cells, the efficiency of reprogramming into a primed pluripotent state could be much higher than that for reprogramming into a naïve state, and it is possible that there is no difference in reprogramming efficiency between male and female cells because the reactivation of X chromosomes does not

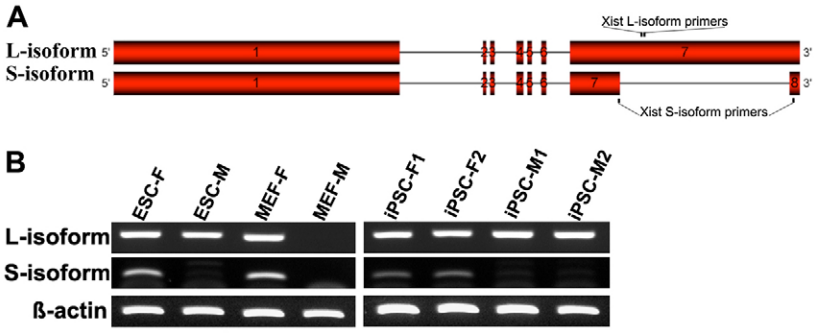

Fig. 4. Reprogramming of *Xist* RNA post-transcriptional processing. (A) The two *Xist* splice variants – the long isoform (L-isoform) and short isoform (S-isoform). The primers specific for the S-isoform span the deleted region in *Xist* exon 7. (B) Male ESCs (ESC-M) express only the *Xist* L-isoform, but female ESCs (ESC-F) and female MEFs (MEF-F) express both the L- and S-isoforms. Similar to male ESCs, male iPSCs (iPSC-M1 and iPSC-M2) express only the L-isoform, whereas, similar to female ESCs, female iPSCs (iPSC-F1 and iPSC-F2) express both the L- and S-isoforms. Male MEFs (MEF-M) express neither isoform.

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occur in primed pluripotent cells. However, we do not know whether differentiated somatic cells that are reprogrammed into naïve pluripotency pass through a primed state. Primed pluripotent EpiSCs can be obtained from fibroblasts by the transduction of *Oct4*, *Sox2*, *Klf4* and *c-Myc* when the cells are cultured in EpiSC medium (Han et al., 2010). However, early Oct4-GFP^{+} cells cultured in ESC medium (for reprogramming into naïve pluripotency) might be only partially reprogrammed or in the process of being reprogrammed into the completely naïve pluripotent state (which requires X chromosome reactivation), as Oct4-GFP is expressed in both primed and naïve pluripotent stem cells. Therefore, analyzing the efficiency of reprogramming cells into the primed pluripotent state is very difficult.

Ma and Strauss suggested previously that the *Xist* L-isoform is predominant during early embryonic development and is responsible for chromosomal silencing (Ma and Strauss, 2005). However, we found that expression of the isoforms is not dependent on the stage of development but rather on the sex of the cells; both the L- and S-isoforms were expressed in female cells (somatic cells, ESCs and iPSCs), whereas male cells (ESCs and iPSCs) expressed only the *Xist* L-isoform (Fig. 4B). However, because very little is known about the functional difference between the two different isoforms, further studies are needed to elucidate the function of the S-isoform and to determine why the S-isoform exists in cells if the *Xist* L-isoform is most important for X chromosome inactivation.

The genetic difference between male and female cells is based on the number of X chromosomes and the presence of a Y chromosome. Therefore, the presence of a Y chromosome might also affect the efficiency of reprogramming; this possibility, although unlikely, still needs to be addressed. Many researchers have shown that X chromosome dosage affects many cellular mechanisms, such as global DNA methylation pattern in ESCs (Zvetkova et al., 2005), early developmental differences after implantation (Burgoyne et al., 1995), ESC maintenance and differentiation ability (Schulz et al., 2014), and stem cell derivation efficiency from PGCs (Matsui et al., 1992). These mechanisms might be involved in the differences in pluripotential reprogramming seen in male and female cells (Do et al., 2008; this study). Thus, it is almost certain that Xi reactivation is one of the final barriers that must be overcome for complete cell reprogramming to occur.

MATERIALS AND METHODS

Generation and culture of iPSCs

In this study, pMX-based retroviral vectors encoding the mouse complementary DNA (cDNA) for *Oct4*, *Sox2*, *Klf4* and *c-Myc* (Takahashi and Yamanaka, 2006) were individually cotransfected with packaging-defective helper plasmids into 293T cells by using the Fugene 6 transfection reagent (Roche Diagnostics, Basel, Switzerland). At 48 h postinfection, virus supernatants were collected, filtered and concentrated as described previously (Zaehres and Daley, 2006). Then, fMEFs and mMEFs (*OG2*$^{+/-}$/*ROSA26*$^{+/-}$) were seeded at a density of 1×10^{5} cells per six-well plate and incubated for 24 h with viral supernatant that contained retroviral vectors for the four transcription factors (1:1:1:1) and was supplemented with 6 µg/ml protamine sulfate (Sigma-Aldrich, St Louis, MO). The cells were then replated onto mitomycin C (MMC)-treated MEF feeder layers in ESC medium. Oct4-GFP^{+} iPSCs were sorted using FACS and subcultured onto MMC-treated MEF feeders. Mouse iPSCs at passages 12–15 were used for further analysis.

Chimera formation

Male and female iPSCs were aggregated with denuded postcompacted eight-cell-stage embryos to obtain aggregate chimeras, as follows. Eight-cell embryos flushed from 2.5-dpc B6D2F1 female mice were cultured in microdrops of embryo culture medium under mineral oil. After cells were trypsinized for 10 s, aggregates of iPSCs (four to ten cells) were selected and transferred into microdrops containing zona-free eight-cell embryos. Morula-stage embryos aggregated with iPSCs were cultured overnight at 37℃, 55% CO_2. The aggregated blastocysts were transferred into 2.5-dpc pseudopregnant recipients in a single uterine horn for each recipient. Animals were maintained and used for experimentation under the guidelines established by the Institutional Animal Care and Use Committees of the Max-Planck Institute for Molecular Biomedicine and Konkuk University. This study was specifically approved by the Institutional Review Board (IACUC090003).

Fluorescence *in situ* hybridization

On day 30 of culture, pNSCs and iPSCs (GFP positive) were sorted by FACS and placed onto Roboz slides. The slides were fixed in 4% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature, rinsed in 70% ethanol and then stored in 70% ethanol at 4℃ until used for FISH. *Xist* RNA was detected with a Cy3-labeled *Xist* RNA FISH probe spanning the entire *Xist* cDNA. FISH was performed as described previously (Do et al., 2008). Images were obtained using an applied spectral imaging camera and analyzed with FishView software (Applied Spectral Imaging; ASI GmBH).

Microarray analysis

RNA samples for microarray analysis were prepared using Qiagen RNeasy columns with on-column DNA digestion. A quantity of 300 ng of total RNA per sample was used as the starting material for the linear amplification protocol (Ambion, Austin, TX), which involved synthesis of T7-linked double-stranded cDNA and 12 h of *in vitro* transcription incorporating biotin-labeled nucleotides. Purified and labeled cRNA was then hybridized for 18 h onto MouseRef-8 v2.0 Expression BeadChips (Illumina, Inc., San Diego, CA) according to the manufacturer's instructions. After the chips were washed, they were stained with streptavidin–Cy3 (GE Healthcare, Chalfont St Giles, UK) and scanned using an iScan reader and accompanying software. Samples were exclusively hybridized as biological replicates.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen GmbH) according to the manufacturer's instructions. Synthesis of cDNA was performed using the High Capacity cDNA Archive Kit (Applied Biosystems GmbH) according to the manufacturer's instructions, with a downscaled reaction volume of 20 µl. Transcript levels were determined using the ABI PRISM Sequence Detection System 7900 (Applied BioSystems) and the ready-to-use, 59-nuclease Assays-on-Demand. For each real-time amplification, 5 ng of total RNA template was used. Measurements were performed in triplicate, and a reverse-transcription-negative blank for each sample and a no-template blank served as negative controls. Amplification curves and gene expression were normalized to the housekeeping gene *Hprt1*, used as an internal standard. Oligonucleotides were designed using Taqman Assay-on-Demand for the detection of the following genes (Taqman primer ID): *Oct4* (Mm00658129_gH), *Sox2* (Mm00488369_s1), *c-Myc* (Mm00487803_m1), *Klf4* (Mm00516104_m1), β-actin (Mm00607939_s1) and *Hprt1* (Mm00446968_m1). Custom-designed oligonucleotides were used for the detection of *Nanog* and viral sequences. Quantification was normalized to the endogenous *Hprt1* gene within the log-linear phase of the amplification curve obtained for each probe or primer set using the ΔΔCt method (ABI PRISM 7700 Sequence Detection System, User Bulletin Number 2).

Reverse transcription-polymerase chain reaction

Synthesis of cDNA was performed using ~1 µg of total RNA, the SuperScript III reverse transcriptase enzyme (Invitrogen) and Oligo(dT)20 primers (Invitrogen) according to the manufacturer's instructions. The reverse transcription-polymerase chain reaction (RT-PCR) was carried out in a GeneAmp9700 PCR thermal cycler (Applied

Biosystems) as follows: preincubation at 94℃ for 5 min was followed by 27 cycles of 94℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s; postincubation was carried out at 72℃ for 7 min. The RT-PCR primers were as follows: *Xist* short isoform forward, 5′-AGAAGAGGA-ACTGTTTCTACAGG-3′; *Xist* short isoform reverse, 5′-GACTCC-AGTCTTTCAACAACAG-3′ (156 bp product); *Xist* long isoform forward, 5′-GACTAAGTACCTGAAGCTTTGTG-3′; *Xist* long isoform reverse, 5′-ACTGACTTGGAAGTTACAGTAGG-3′ (299 bp product); β-actin forward, 5′-CGCCATGGATGAGATATCG-3′; β-actin reverse, 5′-CGAAGCCGGCTTTGCACATG-3′ (68 bp).

Competing interests
The authors declare no competing interests.

Author contributions
J.S.K., H.W.C., H.R.S. and J.T.D. wrote the main manuscript text and designed the concept of the experiment. J.S.K., H.W.C., M.J.A.-B. and J.T.D. performed experiments and assembled data.

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