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Unexpected X Chromosome Skewing during Culture and Reprogramming of Human Somatic Cells Can Be Alleviated by Exogenous Telomerase

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SUMMARY

Somatic tissues in female eutherian mammals are mosaic due to random X inactivation. In contrast to mice, X chromosome reactivation does not occur during the reprogramming of human female somatic cells to induced pluripotent stem cells (iPSCs), although this view is contested. Using balanced populations of female Rett patient and control fibroblasts, we confirm that all cells in iPSC colonies contain an inactive X, and additionally find that all colonies made from the same donor fibroblasts contain the same inactive X chromosome. Notably, this extreme "skewing" toward a particular dominant, active X is also a general feature of primary female fibroblasts during proliferation, and the skewing seen in reprogramming and fibroblast culture can be alleviated by overexpression of telomerase. These results have important implications for in vitro modeling of X-linked diseases and the interpretation of long-term culture studies in cancer and senescence using primary female fibroblast cell lines.

INTRODUCTION

During eutherian mammalian development, females randomly inactivate one of the two X chromosomes in a process called X chromosome inactivation (XCI) (Lyon, 1961). Most healthy human females consist of mosaic cell populations with respect to XCI pattern that follow a bell-shaped curve with a median value of 50% (Ozbalkan et al., 2005). In several heterozygous X-linked diseases, this mosaicism ensures the survival of females, whereas in males, the mutant gene on the single X chromosome proves lethal during embryonic development.

We are interested in modeling X-linked diseases by reprogramming fibroblasts from female donors. That way, one can generate both the perfect pair of control (expressing the normal allele) and experimental (expressing the mutant allele) cell types for investigation of the disease phenotype. Such modeling requires the production of cell populations exhibiting balanced XCI and the exact strategy for achieving this depends on the XCI status of the iPSC lines and their differentiated progeny. Murine female embryonic stem cells (ESCs) and iPSCs harbor two active Xs, but random XCI occurs during subsequent in vitro differentiation (Zvetkova et al., 2005; Maherali et al., 2007). Recently, Tchieu et al. (2010) reported that reprogramming of human fibroblasts is not accompanied by X chromosome reactivation (XCR), with the result that all cells in an iPSC colony display the same inactive X that was inactive in the original fibroblast. This claim was contested by Marchetto et al. (2010) who found that iPSC colonies derived from Rett Syndrome (RTT) patients had undergone XCR.

Here we investigate the status of the X chromosome in iPSC colonies derived from fibroblasts of RTT patients and healthy female controls. We confirm the results of Tchieu et al. (2010) that XCR does not occur during reprogramming. Furthermore, when starting from mosaic fibroblast populations with balanced XCI, all the iPSC clones derived from individual patients exclusively express the same X chromosome. We demonstrate that expression of a particular (dominant) X chromosome confers a distinct advantage to these cells during cellular reprogramming and that this same population becomes predominant during prolonged in vitro culture. Finally, we demonstrate, using various fibroblast populations, that exogenous telomerase can prevent skewing both in culture and during reprogramming. These results have important implications for in vitro modeling of X-linked diseases and the interpretation of long-term culture studies in cancer and senescence using primary female fibroblast cell lines.

RESULTS

Nonrandom XCI Pattern in Affected and Normal Human iPSCs

RTT is a neurological disease in females that is mainly caused by heterozygous mutations in the X-linked gene *MeCP2*. The various known *MeCP2* mutations provide an established genetic background to investigate X chromosome dynamics during somatic cell reprogramming. We obtained fibroblasts from two female RTT patients: GM11272 cells (patient-72; 3 years old) that are heterozygous for a 32 bp deletion within the 3′ coding region in the *MeCP2* gene (1155 del32), and GM17880 cells (patient-80; 5 years old) that harbor a single point mutation in the *MeCP2* methyl-binding domain (473: ACG → ATG) (Figure 1A). These mutations permit a clear distinction between both X chromosomes by RT-PCR fragment size or sequencing,

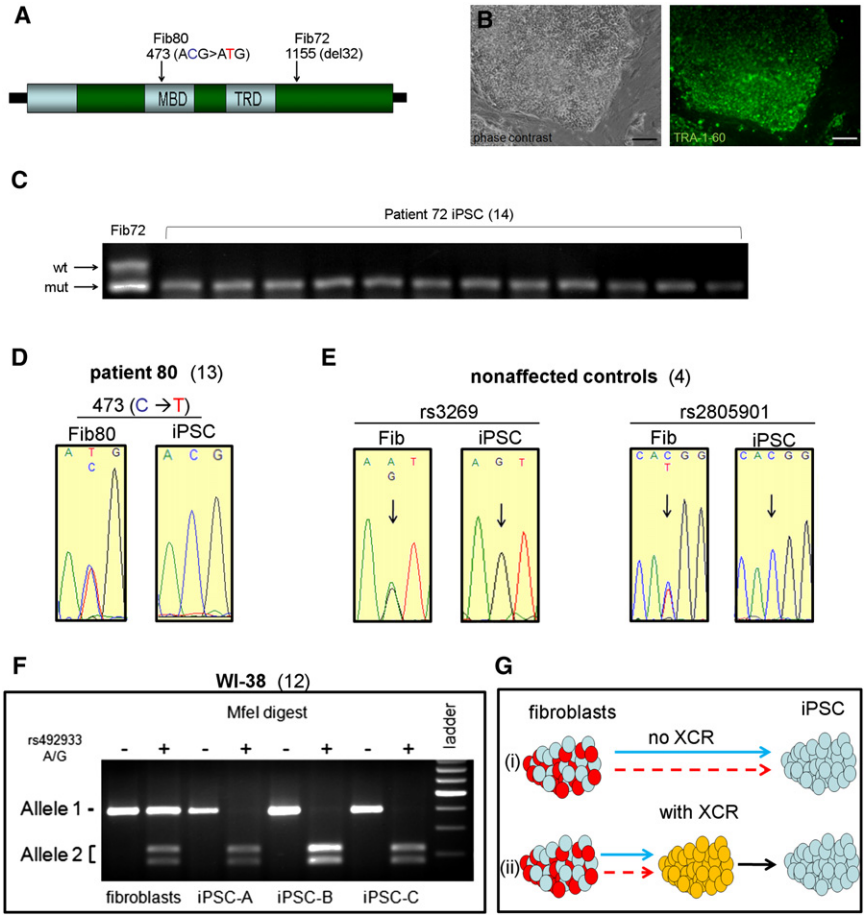

Figure 1. Nonrandom X Chromosome Inactivation Pattern in Human iPSCs

(A) Schematic representation of the *MeCP2* gene. Mutations in each patient are indicated.

(B) Representative iPSC colony 4–5 weeks after infection. Phase contrast micrograph (left panel); TRA-1-60 staining (right panel). Bars, 100 μm. See also Figures S1 and S2.

(C) Biallelic expression of *MeCP2* in original Fib72 population (lane 1) and 12 representative iPSC clones generated from five independent experiments. All iPSC clones express the mutant *MeCP2* allele. Identity of WT and mutant bands was confirmed by sequencing analysis.

(D) Sequencing analysis for Fib80 cells reveal biallelic *MeCP2* expression. A representative Fib80-derived iPSC clone expresses the WT *MeCP2* allele. All 13 iPSC clones derived from five independent experiments exclusively transcribed the WT *MeCP2* allele. Eight of these iPSC clones were generated by lentiviral infection.

(E) Sequencing analysis of two X-linked SNPs for nonaffected fibroblasts and a representative iPSC clone. All four iPSC clones showed the same XCI pattern.

(F) Analysis of an X-linked SNP for iPSCs derived from human fetal lung fibroblasts WI-38. The SNP is recognized and cleaved by the restriction enzyme MfeI (rs492933 CAA/GTTG). Representative WI-38-derived iPSC clones expresses the X_d allele. All 12 iPSC clones generated from three independent experiments showed the same XCI pattern. In (C)–(F), bracketed numbers indicate total number of established iPSC clones.

(G) Two models to account for the loss of mosaicism followed by reprogramming into iPSCs. In both models, cells expressing the X_d (blue marbles) are reprogrammed into iPSCs more efficiently than the other cells (red marbles). In model (i), reprogramming proceeds without XCR; in model (ii), cells undergo transient XCR (yellow marbles), but then revert to expression of the previously active X chromosome—a form of epigenetic memory.

respectively. Likewise, two separate X-linked SNPs (rs3269 and rs2805901) that we identified in the juvenile control fibroblasts allowed similar discrimination by sequencing. To distinguish between the two alleles in the well-established fetal lung fibroblast cell line WI-38 control, we identified an X-linked SNP (rs492933), which is recognized and cleaved by the restriction enzyme MfeI. We reprogrammed fibroblasts (passage 9–10) from patient-72 (Fib72), patient-80 (Fib80), and WI-38, and from the nonaffected, juvenile control donor, using established protocols (Park et al., 2008; Takahashi et al., 2007). At the start of the experiments, all donor fibroblast cultures showed balanced XCI (left panels in Figures 1C–1F). ES-like colony formation was monitored by morphological appearance and by using TRA-1-60 live staining (Chan et al., 2009) ~4 weeks post-infection (Figure 1B). Picked colonies were characterized according to published protocols (Park et al., 2008; Takahashi et al., 2007) and met all criteria for pluripotency (Figures S1 and S2 available online).

The XCI status of the various clones was then examined directly by taking advantage of the allelic polymorphisms described above. RNAs from 14 Fib72, 13 Fib80, 12 nonaffected WI-38, and 4 nonaffected juvenile control iPSC clones were analyzed for XCI. Representative results are shown in Figures 1C–1F. These results indicate that only one allele was expressed in any given iPSC clone, even when reprogrammed in more physiological (5% O_2) conditions (Figures S4C and S4D), as reported by Tchieu et al. (2010). Strikingly and unexpectedly, all the iPSC clones from each patient or control donors expressed the *same* X chromosome (Fib72, mutant *MeCP2*; Fib80, WT *MeCP2*; control, AGT and ACG polymorphisms; WI-38, AAT polymorphism) (Figures 1C–1F). We will refer to this X as the dominant X (X_d) and to the other X as the unfavored X (X_u).

Further corroboration of XCI was obtained by transcriptional analysis using *XIST* probes (*XIST* RNA is known to coat the inactive X) (Figure S3A), and immunohistological analysis of iPSC populations using antibodies recognizing histone H3 lysine 27 trimethylation (H3K27me3), a marker of repressive chromatin (Figure S3B). These results confirmed XCI in the vast majority of early-passage iPSCs. However, one clone (iPS-72-A) judged

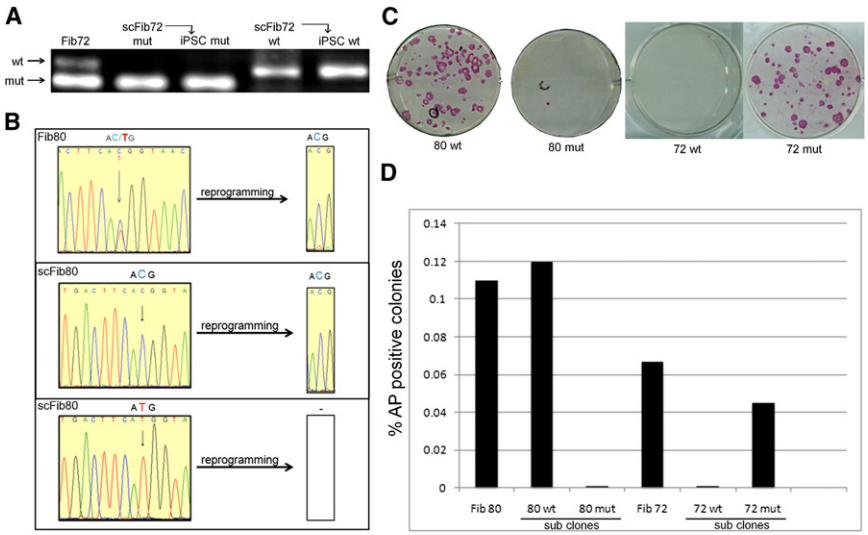

Figure 2. One X Chromosome Confers an Advantage during Reprogramming

(A) RT-PCR analysis of Fib72 subclones (scFib72), expressing either the mutant or WT *MeCP2* allele, and derived iPSC clones. iPSC clones exhibit the same XCI pattern as the original fibroblasts.

(B) Sequencing results for the original mixed population (AC/TG; upper panel), WT subclone (ACG; middle panel), and mutant subclones (ATG; bottom panel) of Fib80 and derived iPSC clones.

(C) Representative alkaline phosphatase (AP) staining of iPSCs derived from WT and mutant subclones of Fib72 and Fib80. AP assay was performed 4 weeks after retroviral infection.

(D) Summary of reprogramming efficiency of the original mixed populations as assessed by alkaline phosphatase staining (three to six independent experiments were quantified for each subclone).

to have undergone XCI by analysis of polymorphic transcripts (above) did not express *XIST* transcripts or exhibit H3K27me3 focus staining even upon differentiation (Figure S3; see asterisks in panels S3B and S3C). This could be explained by the observation that female hESCs that carry an inactive X tend to lose XCI markers during extensive passaging as a result of ''culture adaptation'' (Dvash et al., 2010; Shen et al., 2008; Silva et al., 2008). With extended passages (9 to 15 passages after infection), other iPSC clones lost XCI markers as well (data not shown). Overall, these results demonstrate that a marked skewing toward a population expressing a particular X (X_d) chromosome occurred during reprogramming. The most likely explanation for this reprogramming bias is that fibroblasts containing a particular active X chromosome are more efficiently reprogrammed into iPSCs. This hypothesis was confirmed by the following experiment.

Donor fibroblasts were subcloned to prepare populations that expressed one or the other X chromosome. These populations were then reprogrammed into iPSC clones and the transcriptional status of the X chromosome was examined. The resultant iPSC clones always exhibited the same XCI pattern as the original fibroblast population (Figures 2A and 2B). However, as before, no mutant Fib80 clones were obtained and while numerous mutant Fib72 colonies were seen, only three WT Fib72 clones were obtained, analyzed, and shown to have the same XCI pattern as the original fibroblast clones (Figure 2A); two of these clones (iPS-72-DL1 and iPS-72-DL2) were fully characterized and met all criteria for pluripotency (Figures S1 and S2). In order to provide a more quantitative reprogramming comparison, the experiment was repeated using alkaline phosphatase staining to quantify successful reprogramming. Dramatic differences in reprogramming efficiencies were seen between the two populations. For Fib80, clones expressing the WT allele were reprogrammed into iPSCs at a significantly higher efficiency (~0.1%, n = 5 experiments) than mutant fibroblast clones (~0.001%, n = 6 experiments) (Figures 2C and 2D). Similarly, mutant subclones from Fib72 were reprogrammed at higher efficiency (~0.05%, n = 3 experiments) than WT Fib72 clones (~0.001%, n = 4 experiments) (Figures 2C and 2D). In conclusion, our results indicate that not only do all cells in an iPSC colony express the same X allele, but nearly all iPSC clones made from the same individual donor also express that same X allele. This suggests that the expression status of the X chromosome endows a particular population of female cells with a dramatic advantage or disadvantage during cellular reprogramming.

Human Fibroblasts Skew toward the X_d upon Serial Passaging

Because the process that inactivates X chromosomes in females is random (Lyon, 1961; Ozbalkan et al., 2005), most females show a balanced mosaicism. Transcript analysis of fibroblasts from Fib72, Fib80, and fetal (IMR90 and WI-38) and juvenile controls showed biallelic expression of X-linked genes (Figures 3A–3F), indicating that the original populations were a balanced mosaic of cells that express either the paternal or the maternal allele. Consistent with these results, cloning of the RTT fibroblast populations yielded roughly equal numbers of WT or mutant subclones (data not shown). In contrast, transcript analysis of late passaged cultures of all these cells consistently exhibited homogeneous X-linked expression, indicating that loss of mosaicism occurred quite rapidly (6 to 27 cell divisions) during in vitro passaging (Figures 3A–3F). Strikingly, in numerous independent experiments, the skewing consistently occurred toward the same X allele (n = 8 experiments for Fib72; n = 6 for Fib80; n = 6 for nonaffected fibroblasts #1; n = 2 for IMR90; n = 3 for nonaffected fibroblasts #2; n = 5 for WI-38; n = 2 for nonaffected fibroblasts #3 [not shown]; a fourth juvenile control was already skewed when we obtained the cells [not shown]). These results demonstrate that the observed skewing is highly predictable and not random. Interestingly, in all cases tested, the XCI skewing of fibroblasts in vitro (Figures 3A–3C and 3F) precisely mirrored the pattern seen during reprogramming to iPSCs (Figures 1C–1F).

To further investigate the dynamics of the skewing, RNA was collected from Fib80, WI-38, and juvenile control fibroblast

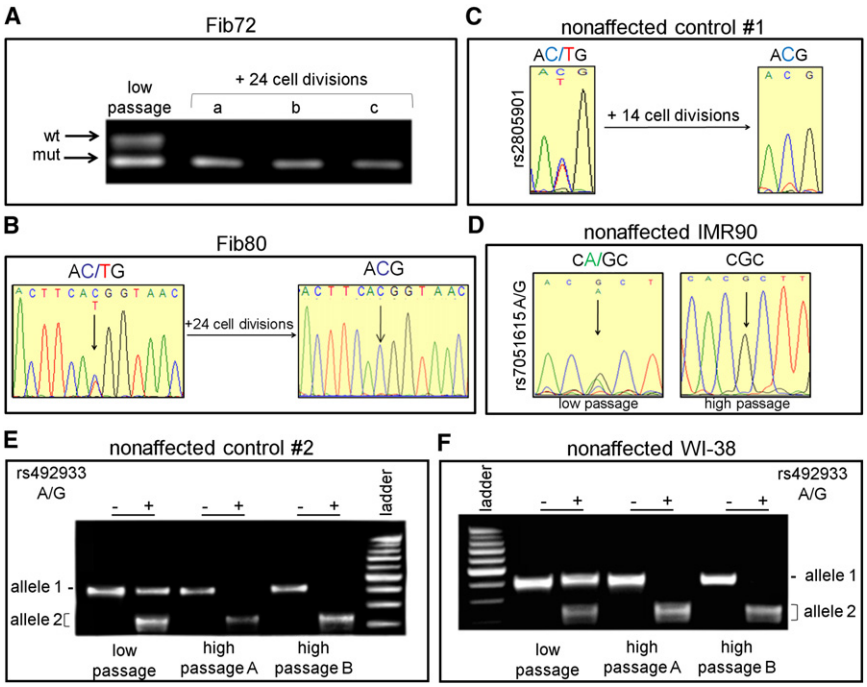

Figure 3. Nonrandom Skewing upon Serial Passaging of Female Fibroblasts

Fibroblasts were cultured and analyzed for X expression at different times: (A), Fib72; (B), Fib80; (C), nonaffected control #1; (D), IMR90; (E), nonaffected control #2; and (F), WI-38. Biallelic expression in low passage populations and monoallelic expression (loss of mosaicism) upon passaging is shown. One form of the SNP rs492933 (CAATTG but not CAGTTG) is recognized and cleaved by the restriction enzyme MfeI (E and F). All clones skew in favor of the same allele (n = 8 experiments for Fib72; n = 6 for Fib80; n = 5 for nonaffected fibroblasts #1; n = 2 for IMR90; n = 2 for nonaffected fibroblasts #2; n = 4 for WI-38) after ~27 cell divisions (D–F) or as indicated in the figure (A–C). See also Figure S4 for skewing in different growth conditions.

populations after approximately three to six cell divisions. Sequencing analysis showed a gradual, progressive shift toward a homogenous population that expressed the same X_d chromosome (Figures 4A–4C and Figure S4A). These results indicate that X-linked skewing occurs gradually during cellular proliferation.

The data above contrast divergent transcript levels to assess cellular skewing kinetics, but may not allow accurate estimates of the two cell populations if mutant and WT transcript stabilities differ. Fib72 cells carry a heterozygous deletion in the *MeCP2* gene (Figure 1A), which generates a truncated protein as a consequence of a shift in the reading frame. Due to the location of the epitope, antibodies raised against the MePC2 C terminus do not react with the mutant form of the protein (Marchetto et al., 2010). This allowed us to distinguish visually the two cell populations within a mixed culture (Figure S5B). Quantification of the ratio between the two cell populations approximately every six cell divisions showed a gradual skewing of 1%–2% in every single cell division (Figure 4D). This proliferative advantage of the mutant 72 cells was further confirmed by directly studying cells undergoing DNA synthesis. Early-passaged cultures were treated for 4 hr with the alkynyl nucleoside analog 5-ethynyl-2′-deoxyuridine (EdU). If the expression of a particular X endows a population with a proliferative advantage, we would expect more cells in that population to incorporate EdU during the short labeling period. Indeed, in Fib72, double staining for EdU/MeCP2 indicated that mutant cells have a proliferative advantage over the WT fibroblasts (Figures S5C and S5D) with 17% ± 3% of the mutant cells labeled as opposed to 9% ± 3% of the WT. Collectively, our results demonstrate that the mosaic nature of female fibroblasts is lost during extended passage in vitro as a consequence of proliferative advantage.

Skewing as described above has only been observed in vivo in certain disease states or in old age (see Discussion). This indicates that some aspect of in vitro culture leads to skewing. Many tissues in vivo, including fibroblasts, experience hypoxic conditions. Thus, we tested whether culture in 5% O_2 (rather than 21% O_2) with or without free radical scavengers had any impact on skewing. The results in Figures S4A and S4B show that skewing is unaffected by either of these modifications.

A possible explanation for the skewing could be that cells expressing the X_u are more prone to replicative stress and consequently acquire critically shortened telomeres and enter senescence earlier than cells expressing the X_d (Harley et al., 1990). The trigger for this process seems to be the increase in persistent DNA damage foci, which can be detected by immunostaining against 53BP (Schultz et al., 2000; d'Adda di Fagagna et al., 2003). Using 53BP staining as a measure of DNA damage, we observe that the disadvantaged fibroblast population expressing the WT *MeCP2* allele has far more DNA damage foci than the population expressing the mutant *MeCP2* allele (Figures 4E and 4F). We also find (via β-gal staining) many senescent cells in the late passage cells. Though it is very likely that these were predominantly MeCP2 WT, for technical reasons, we could not demonstrate this (data not shown). However, since DNA damage foci are a prelude to cell cycle exit either through senescence or apoptosis, it is tempting to conclude that this differentially imposed damage is responsible for the relative loss of cells expressing the X_u.

Exogenous Telomerase Can Prevent Skewing in Culture and during Reprogramming

Telomere shortening can be prevented by expression of telomerase (Bodnar et al., 1998). To investigate whether exogenous telomerase could prevent or delay the fibroblast skewing, we ectopically expressed either active or catalytically impaired human telomerase reverse transcriptase (hTERT) and tested their effects on skewing during subsequent in vitro proliferation (Counter et al., 1998; Hahn et al., 1999). Only populations that

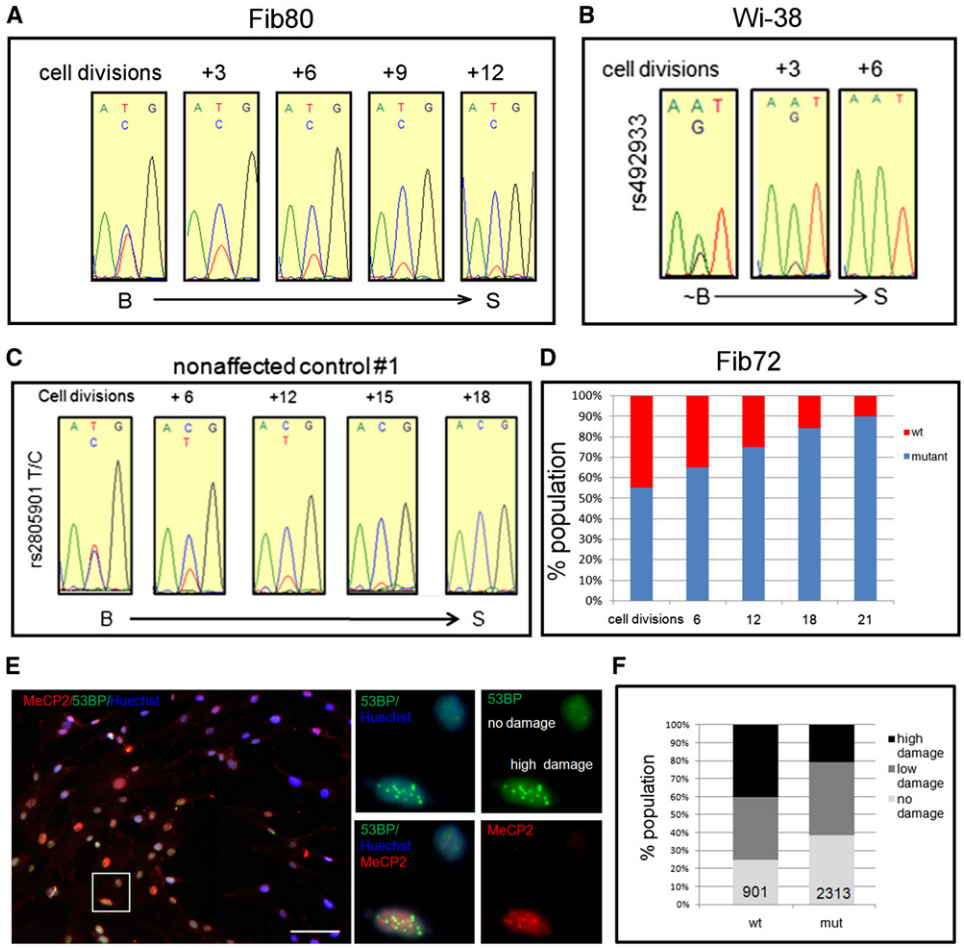

Figure 4. Skewing as a Result of Proliferative Advantage

Gradual skewing toward one allele occurs upon serial passaging: (A) Fib80; (B) WI-38; (C) nonaffected cells; and (D) Fib72. B, balanced; S, skewed. (D) Comparison of WT and mutant Fib72 by staining against MeCP2. A total of 7220 cells were counted. (E) Double staining of Fib72 against WT MeCP2 (red) and 53BP (green). Hoechst is shown in blue. Bars, 100 μm. (F) Quantification of low (1–2) and high (>2) DNA damage 53BP foci in WT and mutant Fib72. A total of 3214 cells were counted. See also Figure S5.

remained balanced after drug selection were further analyzed (Figure 5). Samples containing inactive telomerase skewed after ~12 (WI-38, Fib80) to ~36 (control #2) cell divisions (Figures 5A–5C), as expected. Strikingly, sister dishes from all three samples, expressing the active hTERT, stayed balanced during serial passaging in vitro. These results suggest that active telomerase can dramatically slow down or prevent skewing in female fibroblast cultures in vitro.

To test whether these immortalized (hTERT+) fibroblasts can generate both isogenic iPSC populations that clonally express either the paternal or the maternal X allele, we reprogrammed WI-38 cells, ectopically expressing either active or catalytically impaired hTERT. ES-like colony formation was monitored as described above (not shown) and colonies were collected ~4 weeks postinfection. Strikingly, both iPSC populations, expressing either the paternal or the maternal X, were obtained from the hTERT+ population (Figure 5E). In contrast, all nine iPSC clones, derived from hTERT− fibroblasts, expressed the same X_d allele. Finally, instead of reprogramming cells that already expressed exogenous hTERT, we added hTERT to the reprogramming mix and also found that we could easily generate an isogenic hiPSC pair from Fib72 (Figure 5D). These results suggest that hTERT expression rescues the disadvantage conferred by expression of the X_u chromosome, during both in vitro proliferation and reprogramming.

Restoring the Lost Population

Loss of mosaicism upon serial passaging poses an obstacle for in vitro disease modeling (see Discussion). The remedies described above would not work on a totally skewed population. To restore the disadvantaged (lost) population, we forcibly reactivated both X chromosomes by adapting protocols reported by Hanna et al. (2010). These researchers demonstrated that female human iPSCs can, under given circumstances, acquire morphological and functional features of murine iPSCs, including reactivation of the silenced X chromosome. XCR followed by subsequent differentiation should theoretically restore the mosaic nature of somatic lineages. To test this hypothesis, we reprogrammed Fib80 fibroblasts into iPSCs using a lentivirus carrying *Oct4*, *Nanog*, *Lin28*, *Sox2*, and a puromycin resistance

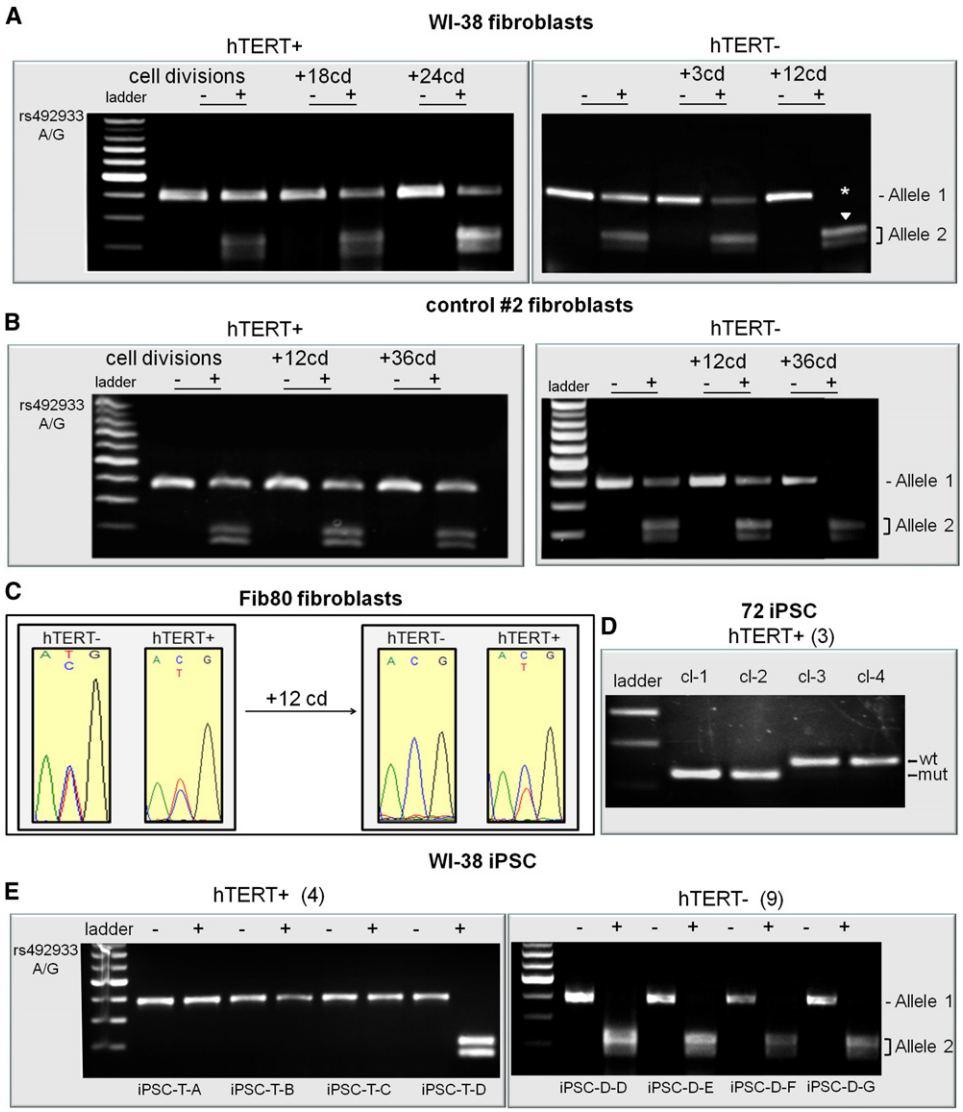

Figure 5. Ectopic Expression of Telomerase Prevents Skewing during Fibroblast Culture and Reprogramming

Analysis of allele-specific expression of SNPs from hTERT-modified fibroblasts during extended culture (A–C) and after reprogramming into iPSCs (D and E). (A and E), WI-38; (B), control #2, and (C), Fib80 cells, ectopically expressing telomerase (hTERT) or catalytically inactive telomerase (control) after selection and further propagation. (A) After ~24 cell divisions, telomerase$^+$ WI-38 cultures retain both alleles (two bands) whereas telomerase$^-$ cells, after ~12 cell divisions, lose allele 1 (*) and skew in favor of allele 2 (arrowhead). (B) Loss of mosaicism in telomerase$^-$, but not in telomerase$^+$, control #2 cells, as explained in (A). (C) Retention of both alleles in Fib80 telomerase$^+$, but not in telomerase$^-$, cells after ~12 cell divisions, as shown by sequencing analysis. (D and E) Isogenic iPSC clones, expressing either X_d or X_u are generated when reprogramming: (D) Fib72 fibroblasts with five factors (hTERT plus the four Yamanaka factors), or (E), hTERT+ WI-38 with the four Yamanaka factors. In brackets, total number of established iPSC clones is shown.

marker. As expected, we obtained iPSC clones that exclusively expressed the WT allele. After one passage, iPSCs were shifted to murine iPSC media containing human LIF with a combination of the ERK1/2 inhibitor PD0325901, the GSK3 inhibitor CHIR99021, and forskolin (Hanna et al., 2010) (Figure 6A). After 1 week of puromycin selection, we observed packed, dome-like, naive iPSC colonies (Figure 6B) as well as flattened, conventional hiPSCs (Figure 6C). Similar results were obtained when established iPSCs were reinfected with the lentiviruses (data not shown). All the human iPSC colonies expressed OCT4 (Figure 6D). Three passages after infection, most colonies were a mix of cells that expressed either TRA-1-60 or stage-specific embryonic antigen, SSEA-1, but not both (Figure 6E). Without continuous supplementation with LIF or the inhibitors, the dome-like iPSCs differentiated after one to three passages. We selected individual dome-like hiPSC clones, further passaged them with trypsin under puromycin selection, and investigated the status of both X chromosomes at different time points by analyzing WT and mutant *MeCP2* transcripts. Analysis of *MeCP2* transcripts after infection (passage 3) still showed homogeneous expression. However, at later passages (passage 10) both mutant and WT transcripts were observed, indicating that

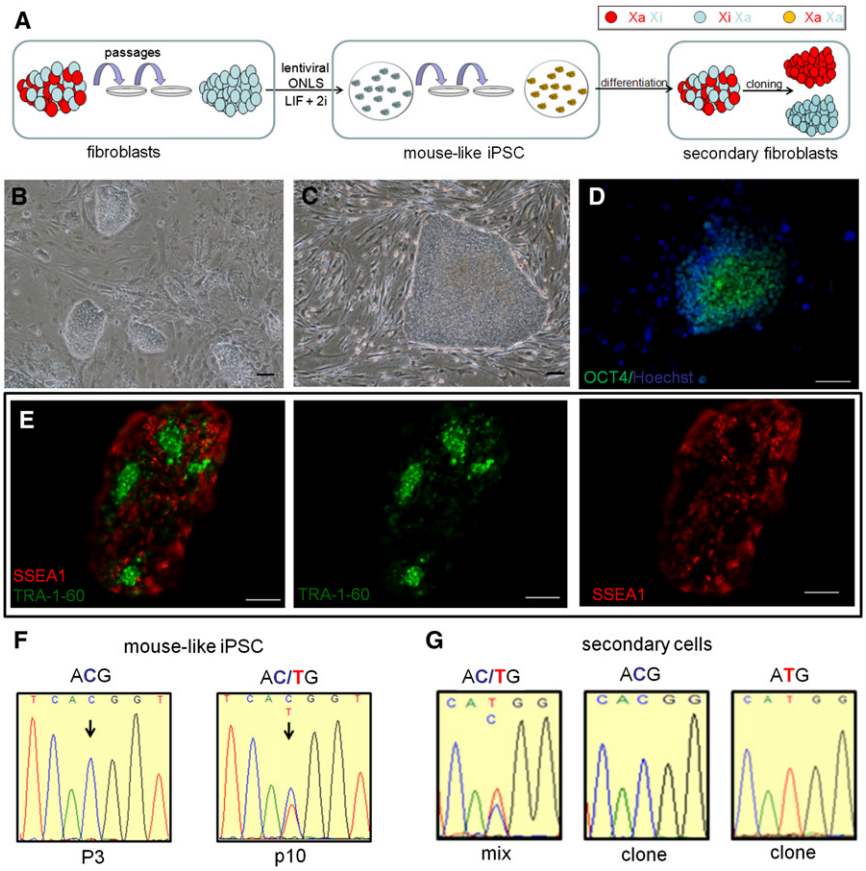

Figure 6. Reactivation of Both X Chromosomes in Human iPSCs

(A) Generation of the "lost" fibroblast population (blue) by XCR (orange) and subsequent differentiation. ONLS: Oct4, Nanog, Lin28, and Sox2. (B) Colony morphology of naive iPSCs and (C) normal human iPSCs. (D and E) Immunostaining of (D) Oct4 (green) and (E) SSEA-1 (red) + TRA-1-60 (green) in naive iPSC colonies, with Hoechst in blue. Sanger sequencing analysis of *MeCP2* transcripts in (F) naive iPSC colonies at different passages, and in (G) secondary cells before and after cloning is shown. Bars, 100 μm. See also Figure S6.

XCR occurred (Figure 6F). Accordingly, ~12% of the dome-like colonies lost H3K27me3 punctuate staining in pluripotent (OCT4+) cells, but not in spontaneously differentiated (OCT4−) cells (Figures S6A–S6E). Next, we differentiated the dome-like colonies. Although the differentiated cells obtained still expressed most of the exogeneously added genes (Figure S6F), we were able to obtain clones that expressed either WT or mutant *MeCP2* (Figure 6G), thereby successfully restoring the "lost" population of somatic cells (Figure 6A and schematic in Figure 7). These data support the conclusion that female human iPSCs, in contrast to their murine counterparts, do not normally reactivate X chromosomes during reprogramming, and further refute an alternate possibility that reactivation occurs but is unstable and quickly followed by silencing of the same X—a form of epigenetic memory (Figure 1G (ii)).

DISCUSSION

iPSC Reprogramming and XCR

During early human development, both X chromosomes remain active in females from the zygote stage until random XCI begins after the blastocyst stage (Okamoto et al., 2011). As a result human tissues are composed of a mosaic of cells that differ in the particular X that is inactivated. During reprogramming of mouse fibroblasts into iPSCs, XCR occurs, resulting in two active X chromosomes (Maherali et al., 2007). The status of the X chromosomes during reprogramming of human cells into iPSCs is less clear: Tchieu et al. (2010) reported that all cells in individual iPSC colonies retain the same inactive X chromosome and concluded that no XCR took place. Marchetto et al. (2010) presented evidence that iPSC colonies made from female RTT patients contained two active X chromosomes in most if not all the cells (Marchetto et al., 2010), suggesting that XCR occurs during reprogramming.

Here we have performed a detailed investigation of the X chromosome status in iPSC colonies derived from both RTT patients and numerous healthy controls and found, like Tchieu et al. (2010), that all cells in individual iPSC colonies retain an inactive X chromosome. This is in disagreement with Marchetto et al. (2010); however, their conclusion was based on the absence of specific histological marks of the inactive X (XIST RNA coating and H3K27me3 staining) with Fib72 with no direct transcriptional assays performed. We and others (Dvash et al., 2010; Shen et al., 2008; Silva et al., 2008) have found that some of these histological marks (but not X inactivation itself) can be lost during extended cell culture. In addition, our results concur with recent data from Cheung et al. (2011) who, like us, used transcriptional assays to show that cells in all examined RTT iPSC colonies express only one X chromosome. We conclude that XCR does not occur during hiPSC reprogramming. Reprogramming of human cells is therefore not as comprehensive as it is in murine cells.

Tchieu et al. (2010) concluded that no XCR occurs during somatic cell reprogramming; however, they could not rule out transient XCR followed by nonrandom XCI (see Figure 1G). To examine this possibility, we attempted to effect XCR during reprogramming by using hypoxic reprogramming conditions (see Lengner et al., 2010); however, no XCR was observed. We then deployed a modified version of the reprogramming protocol reported by Hanna et al. (2010): using forced expression of *Lin28*, *Sox2*, *Oct4*, and *Nanog*, we converted conventionally prepared iPSCs into transiently stable, naive iPSCs that exhibited XCR (Figure 6). Subsequent iPSC differentiation and subcloning of the progeny yielded cells that expressed the previously X_u. These results indicate that naive human iPSCs do not retain an epigenetic memory of the previously inactive X and

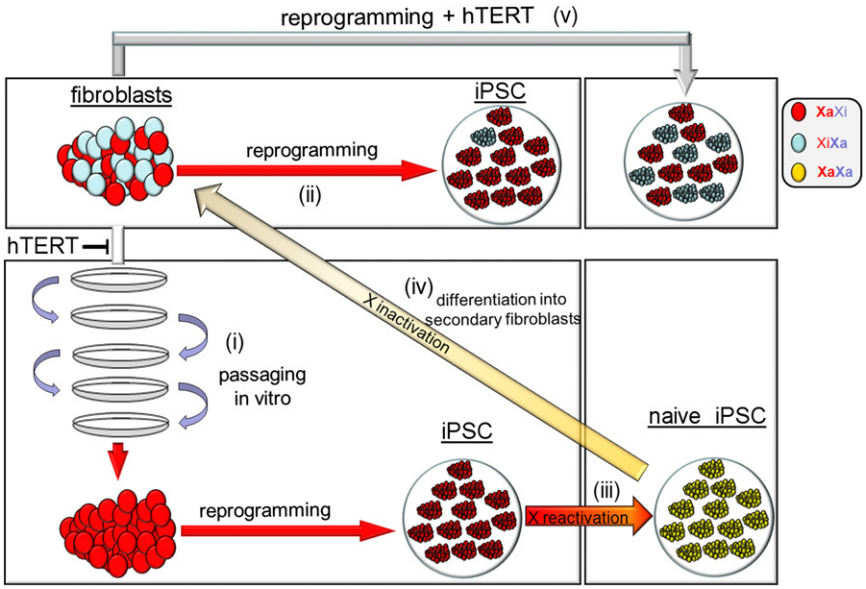

Figure 7. Model Describing the X Chromosome Dynamics In Vitro

In female fibroblasts, one cell population (red) has a growth advantage over the other (blue) and unless hTERT is ectopically expressed, mosaicism is gradually lost upon serial passages in vitro (i). The same disadvantaged population is reprogrammed into iPSCs at a lower efficiency (ii), and cannot be obtained once mosaicism is lost. Thus, loss of one fibroblast population during passages poses an obstacle for in vitro disease modeling. Skewing of both iPSCs (v) and fibroblasts can be alleviated by ectopic expression of hTERT. Mosaicism can be restored by forced XCR (yellow) (iii), followed by differentiation into secondary fibroblasts (i.e., random X inactivation) (iv).

strengthens the conclusion of Tchieu et al. (2010) that reprogramming of human fibroblasts is not accompanied by XCR.

X Chromosome Skewing during Cellular Reprogramming and In Vitro Culture

Here, we demonstrate that all cells in an individual iPSC colony express the same X chromosome and, strikingly, nearly all colonies generated from any particular donor express the same X chromosome. In every case, the starting fibroblast population showed a balanced mosaic of cells expressing each X chromosome. These observations were highly reproducible in many separate experiments using RTT and other nonaffected fibroblast populations (including the well-studied WI-38 fibroblasts). From these experiments we concluded that fibroblasts expressing the X_u were refractory to reprogramming.

To elucidate the reprogramming advantage conferred by a particular X in more detail, we investigated X chromosome dynamics during in vitro proliferation. Although starting populations of Fib72 and Fib80 fibroblasts are mosaic in the representation of each active X, this situation rapidly changes during extended culture, and after approximately 12 population doublings, one active X predominates. To date, we have demonstrated skewing in two RTT patients and five nonaffected fibroblast populations, including WI-38 (Hayflick and Moorhead, 1961) and IMR90 cells (Nichols et al., 1977). In other instances, we found that the populations were already skewed when we obtained the cells. The simplest explanation for the skewing in fibroblasts is that cells expressing the disadvantaged X enter a quiescent, senescent, or apoptotic state earlier than their isogenic relatives. We explored this possibility by distinguishing between cells expressing the favored or X_u using an anti-MeCP2 antibody that only stains cells expressing the WT gene, and demonstrate that Fib72 cells containing the X_u (expressing WT MeCP2) proliferated more slowly and accumulated more DNA damage than their isogenic siblings. To investigate whether the decreased proliferative capacity and increased DNA damage could be a consequence of critically shortened telomeres, we expressed hTERT and catalytically inactive TERT in fibroblasts and examined X chromosome dynamics during proliferation and reprogramming. We find that hTERT (but not catalytically inactive hTERT) alleviates skewing in proliferating fibroblasts during the examined time period. However, we cannot rule out that skewing might eventually occur even in cells expressing hTERT. Importantly, however, exogenous hTERT expression in fibroblasts (or addition of hTERT to the four reprogramming factors) overturned the observed reprogramming bias and yielded iPSC clones expressing either X. In conclusion, these results suggest that skewing can be alleviated by elongating telomeres in cells expressing the X_u, thereby preventing telomere-associated DNA damage and senescence. It has been shown that the efficiency of reprogramming correlates with telomere length (Marion et al., 2009). Our results further highlight the importance of telomere maintenance during cellular reprogramming.

Skewing In Vitro and In Vivo

Skewing has previously been defined as a deviation of $\geq$25% from balanced (50:50) inactivation of each parental X chromosome (Kubota et al., 1999; Minks et al., 2008). It has been shown to occur in X-linked diseases (Bretherick et al., 2005; Li et al., 2006; Lose et al., 2008; Ozbalkan et al., 2005) and ~10% of healthy females, mainly in tissues with high proliferation, such the hematopoietic lineage (Bolduc et al., 2008; Knudsen et al., 2007). However, skewing in dermal fibroblasts has not been reported except in females with structural abnormalities of one X chromosome (Carrel and Willard, 2005). We speculate that skewing in vivo is normally prevented by a combination of low proliferation rates and suppressive niche factors with the latter predominant during early development when cell proliferation is high.

We do not know why one X chromosome is superior to the other. The human X chromosome contains over 1000 genes, and mutations in some have been implicated in the disease-related skewing mentioned above. For example, DKC1, a telomerase subunit, is mutated in dyskeratosis congenital (Heiss et al., 1998), and FANC B encodes a component of a complex involved

in the DNA damage response, and if mutated, results in Fanconi's anemia (Meetei et al., 2004). Thus, X-linked polymorphisms in such genes or others with related functions could cause the observed skewing in vitro. Alternatively, the performance of the two Xs may be influenced by parental imprinting such that the X_d always comes from the mother or father. We are currently investigating this using fibroblasts from identified pedigrees.

Implications of Skewing on Disease Modeling

Disease-specific hiPSCs can theoretically differentiate into any cell type of the human body, and offer an unprecedented opportunity to examine disease states and explore novel drug development approaches (Colman and Dreesen, 2009). Heterozygous X-linked diseases might be particularly suitable for iPSC-based modeling because females patients are a mosaic of cells, and therefore, both affected (expressing the mutant allele) and WT control (expressing the WT allele) cell types can be generated from the same patient iPSCs. Furthermore, isogenic cell populations that solely express the WT allele could be exploited for cell replacement therapy. Here we show that upon reprogramming of a balanced mosaic fibroblast population, all iPSC colonies as well as differentiated fibroblasts exclusively express the same X. Furthermore, we demonstrate that the in vitro-imposed bias in reprogramming can be overcome in several ways: first, if the patient fibroblasts are low passage and the reprogramming experiments are large enough, iPSC clones containing the unfavored, active X should be obtained, and this outcome could be optimized if the fibroblasts are subcloned beforehand. This might explain why Tchieu et al. (2010) were able to obtain isogenic sibling iPSCs from one cell population. Second, skewing can be prevented by expression of hTERT: reprogramming TERT+ cells or adding hTERT to the reprogramming cocktail allows both types of isogenic iPSCs to be generated. Third, forced reactivation of both X chromosomes (by overexpression of Nanog, Lin28, Sox2, and Oct4) can generate naive iPSCs that, upon differentiation, generate both WT and mutant cell types.

In conclusion, our finding that a particular X chromosome confers an advantage during reprogramming highlights challenges for the in vitro modeling of X-linked disease. In addition, our data point to the absence of telomerase being a contributory factor to skewing in proliferating fibroblasts. Many other specialized cell types undergo replication in the absence of telomerase, and these too may display a skewing phenotype. Lastly, WI-38 and IMR90 fibroblasts have been used extensively for long-term culture studies in cancer and senescence. Our finding that the proportion of cells expressing a particular X chromosome could gradually shift over the course of a study introduces a hitherto unconsidered variable into any experimental interpretation and points to the more prudent choice of male or cloned female fibroblasts for future analyses.

EXPERIMENTAL PROCEDURES

Culture of Human Fibroblasts

RTT patient fibroblast cells (GM17880, GM11272) and WI-38 (GM06814) cells were purchased from Coriell Cell Repositories (http://ccr.coriell.org/). IMR90 cells were purchased from ATCC. Nonaffected juvenile female fibroblasts were a gift from Dr. Bruno Reversade. Fibroblasts were maintained in MEM supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 1% NEAA, and antibiotic (10 μg/ml penicillin and streptomycin).

For cell immortalization, cells were transfected with retroviral construct pBABE-Puro-DN-hTERT (catalytically inactive hTERT) or pBABE-hTERT-Neo (obtained from Addgene) and selected with puromycin and Neomycin G418, respectively (Counter et al., 1998; Hahn et al., 1999).

Culture of Human iPSCs and Naive iPSCs

Human iPSCs were generated according to published protocols (Takahashi et al., 2007) with slight modifications, using retroviral vectors encoding the human cDNAs of *KLF4*, *SOX2*, *OCT4*, and *C-MYC* (Addgene). Eight iPSC clones from patient-80 were also derived by using lentiviral vectors encoding the human cDNAs of *OCT4*, *SOX2*, *NANOG*, and *LIN28* (Addgene). Infected cells were left on the original plates in a hESC-medium (Knockout DMEM containing 20% knockout serum replacement, 2 mM L-glutamine, 1% NEAA, 0.1 mM β-mercaptoethanol, 1% NEAA, 0.1% antibiotic [10 μg/ml penicillin and streptomycin], and 4 ng/ml bFGF) that was conditioned for 24 hr by irradiated feeders. Medium was supplemented with 0.5 mM valproic acid (VPA, Sigma) for 3 weeks. iPSC colonies were manually picked after 3–4 weeks and cultured on irradiated MEF.

Naive iPSCs were produced by using a published protocol (Hanna et al., 2010) with several modifications. Briefly, human fibroblasts, or established iPSCs, were reprogrammed using lentiviral transgenes OSNL (*OCT4*, *SOX2*, *NANOG*, and *LIN28*). Colonies were picked after ~4 weeks and expanded by trypsin digestion on puromycin-resistant MEF feeders containing N2B27 medium (Hanna et al., 2010) with a combination of the ERK1/2 inhibitor PD0325901 (1 μM, Axon), the GSK3 inhibitor CHIR99021 (3 μM, Axon), and forskolin (10 μM, Tocris Bioscience). Puromycin was added to select for colonies that did not silence the transgenes.

RNA Isolation, Reverse Transcription, and SNP Analysis

Total RNA was extracted using the Nucleospin RNAII Kit (Macherey-Nagel) with DNase digestion. RNA was quantified using ND-1000 spectrophotometer (Biofrontier Technology) and first strand cDNA was produced with M-MulV reverse transcriptase (Biolabs) using 1 μg of total RNA input. PCR was performed using Supermix system (Invitrogen). Primer sequences are listed in Table S1 available online. For sequencing analysis, cDNA was sent to 1^{ST} Base. To identify SNP rs492933 in WI-38 and control #2, a region of *OPHN1* was amplified by PCR with primers given in Table S1 and analyzed by restriction digest with Mfe1.

Immunostaining

Cells were fixed in 4% paraformaldehyde in PBS at room temperature and blocked in 4% fetal calf serum with 0.1% Tween 20 for 60 min at room temperature. Cells were then stained with primary and secondary antibodies (Invitrogen and Alexa Fluor, respectively) according to standard protocols. Primary antibodies used were as follows: Oct4 (Santa Cruz, sc-5279, 1:200), MeCP2 (Sigma, m6818, 1:1000), and SSEA-1 (Santa Cruz, sc-21702, 1:500). StainAlive antibodies TRA-1-60 (Stemgent, 09-0068) were added directly to the culture dish for 30 min and imaged after two washes with PBS. Images were captured with a Zeiss axiovert 200 microscope. Images were enhanced using Paint Shop Pro software and processed evenly across the entire field using Paint Shop Pro software.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.stem.2011.06.004.

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