Induced pluripotent stem cells as a model for telomeric abnormalities in ICF type I syndrome

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Human telomeric regions are packaged as constitutive heterochromatin, characterized by extensive subtelomeric DNA methylation and specific histone modifications. ICF (immunodeficiency, centromeric instability, facial anomalies) type I patients carry mutations in DNA methyltransferase 3B (DNMT3B) that methylates de novo repetitive sequences during early embryonic development. ICF type I patient fibroblasts display hypomethylated subtelomeres, abnormally short telomeres and premature senescence. In order to study the molecular mechanism by which the failure to de novo methylate subtelomeres results in accelerated telomere shortening, we generated induced pluripotent stem cells (iPSCs) from 3 ICF type I patients. Telomeres were elongated in ICF-iPSCs during reprogramming, and the senescence phenotype was abolished despite sustained subtelomeric hypomethylation and high TERRA levels. Fibroblast-like cells (FLs) isolated from differentiated ICF-iPSCs maintained abnormally high TERRA levels, and telomeres in these cells shortened at an accelerated rate, leading to early senescence, thus recapitulating the telomeric phenotype of the parental fibroblasts. These findings demonstrate that the abnormal telomere phenotype associated with subtelomeric hypomethylation is overridden in cells expressing telomerase, therefore excluding telomerase inhibition by TERRA as a central mechanism responsible for telomere shortening in ICF syndrome. The data in the current study lend support to the use of ICF-iPSCs for modeling of phenotypic and molecular defects in ICF syndrome and for unraveling the mechanism whereby subtelomeric hypomethylation is linked to accelerated telomeric loss in this syndrome.

INTRODUCTION

Telomeres are nucleoprotein complexes present at the termini of eukaryotic chromosomes. In vertebrates, they consist of the hexameric TTAGGG repeat, and function in maintaining the stability and integrity of chromosome ends (1). Telomere length in humans varies between 5 and 15 kb, being longest at birth and shortening gradually every cell division partly because of the end replication problem (2). Telomere shortening to ∼3–5 kb triggers replicative senescence (3). In embryonic and adult stem cells, telomere length is maintained by telomerase, an RNA–protein enzymatic complex that elongates shortened telomeres by reverse transcription (4).

A non-coding RNA, TERRA (TElomeric Repeat containing RNA), is transcribed from telomeric regions, and its role in telomere function is still under debate (5–10). Among other functions, TERRA has been proposed to affect telomere length by directly inhibiting human telomerase (11,12) as well as by blocking the progression of the replication fork by binding to the single-stranded telomeric DNA of the leading strand (13). In yeast, in the absence of a telomere maintenance mechanism, an increase in TERRA transcription from even a single telomere may lead to telomere shortening in cis and trigger premature cellular senescence (14).

Similar to centromeric regions, human telomeric regions are packaged as constitutive heterochromatin, characterized by specific histone modifications, including tri-methylation of histone H3 at Lysine 9 and histone H4 at Lysine 20, low levels of H3 and H4 acetylation, and Heterochromatin Protein 1 alpha (HP1α) binding (15). Human subtelomeric regions, adjacent to the

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telomeres, are composed mainly of repetitive sequences and are rich in the dinucleotide CG (16–18). While in human sperm and oocytes subtelomeres are scarcely methylated, these regions undergo extensive de novo methylation during early development (19). Both DNA methyltransferases 3A and 3B (DNMT3A and DNMT3B) carry out de novo DNA methylation of promoter regions during mammalian embryonic development (20–23); however, repetitive DNA is mostly methylated by DNMT3B (20,22). Hypomorphic mutations in DNMT3A and DNMT3B carry out immune system of the patients (24,25). In addition to centromeric recessive disorder, often fatal in childhood due to the defective anomalies) syndrome type I (MIM 242860), a rare autosomal lead to ICF (immunodeficiency, centromeric instability, facial manifestations) syndrome type I (MIM 242860), a rare autosomal de novo initiated around the implantation stage when DNA methylation occurs (30). When considering appropriate models for this developmental stage, ICF syndrome-model mice are excluded owing to the significant differences in telomere biology between mouse and human (31,32). Alternatively, human induced pluripotent stem cells (hiPSCs) present an attractive model for the implantation stage (33). During generation of wild-type (WT) hiPSCs, telomerase is activated, telomeres elongate, subtelomeres are further methylated and TERRA levels are elevated (34–38). When WT hiPSCs differentiate into fibroblast-like cells (FLs) telomerase is downregulated, telomeres shorten and TERRA levels are reduced (36). Based on the foregoing, we generated ICF-iPSCs and characterized their telomeric phenotype. We demonstrate that ICF-iPSCs are indeed suitable for modeling the telomeric abnormalities in ICF syndrome and are a reliable platform for deciphering the molecular pathology linking aberrant subtelomeric methylation and accelerated telomere loss.

RESULTS

Generation of ICF-iPSCs from patient fibroblasts

iPSCs were generated from three ICF type I patient-derived fibroblasts: one male, designated pG (24), and two females, designated pR (24) and pW [patient 2 in 39]. DNMT3B mutations in patient cells were confirmed by resequencing, and their fibroblasts were demonstrated to enter premature replicative senescence (40) (Supplementary Material, Fig. S1A and B). iPSCs were generated using retroviral vectors encoding OCT4, SOX2, KLF4 and c-MYC, as described (33). Three iPSC clones from patient pG (pG 20, pG 23, pG 28), two iPSC clones from patient pR (pR 75, pR 76) and one iPSC clone from patient pW (pW 54) were characterized and validated as bona fide iPSCs (Fig. 1 and Supplementary Material, Figs S2–5). OCT4 and Nanog promoters were hypomethylated in the undifferentiated state and remethylated following differentiation (Fig. 1B), suggesting that DNMT3B is not required for remethylation of these promoters and that DNMT3A carried out this function. Pluripotency of the iPSCs was validated by both in vitro differentiation via embryoid bodies (Fig. 1C and Supplementary Material, Figs S3–5) and in vivo differentiation in teratomas (Fig. 1D and Supplementary Material, Figs S3–5); in both cases, the capacity to differentiate into representatives of all three embryonic germ layers was evident. All six iPSC clones were grown for 40–60 passages in culture, and displayed a normal karyotype at both early and late passages (Supplementary Material, Figs S2–5). Analyses of DNMT3B expression at the RNA and protein levels, in the undifferentiated and differentiated states, indicated normal splicing isoforms in both states, and the expected expression of the full-length protein isoform DNMT3B1, in addition to the DNMT3B3 protein isoform, in the undifferentiated state only, presumably from the allele carrying the missense mutation (40) (Fig. 1E and Supplementary Material, Fig. S2A). We conclude from the above findings that ICF-iPSCs display normal characteristics of hiPSCs and overcome the premature senescence phenotype present in the parental ICF fibroblasts.

Methylation analysis reveals that subtelomeres remain abnormally hypomethylated in ICF-iPSCs

DNA methylation levels are generally slightly elevated in hiPSCs in comparison to the parental fibroblast cells (41), and subtelomeric regions have been shown to follow that rule (36). To determine whether subtelomeric hypermethylation is necessary for reprogramming to pluripotency, we examined the subtelomeric methylation levels in ICF-iPSCs by two approaches. First, involving terminal restriction fragment (TRF) analysis with the isoschizomeric restriction enzymes HpaII (methylation sensitive) and MspI (methylation insensitive) (28) provides a global view of methylation levels at all subtelomeres. This analysis revealed no significant differences between TRF lengths produced by HpaII or MspI digestions in ICF fibroblasts, ICF-iPSCs at several passages (results not shown) and their derivative differentiated cells, indicating that subtelomeric regions in ICF cells remain stably hypomethylated after reprogramming and differentiation (Fig. 2A and B).

The second approach for subtelomeric methylation analysis utilized high-throughput sequencing of bisulfite-converted DNA, and provided a detailed view of methylation levels at specific subtelomeric regions. We used seven sets of primers that amplified eleven subtelomeric regions in fibroblasts, derived iPSCs and differentiated FLs (isolated from differentiated iPSCs) (36), from a normal (FSE) and an ICF (pG) individual. The regions of interest were amplified following bisulfite conversion, sequenced on the Ion Personal Genome Machine (PGM™) sequencer and analyzed using the BISMA and BiQ analyzer software (42,43) (Fig. 2C–E and Supplementary Material, Tables S1–5). Interestingly, this more detailed analysis indicated that, with the exception of subtelomere 5p, in the remaining six examined amplicons, undifferentiated ICF-iPSCs demonstrated a significant elevation ($P < 0.02$) in methylation levels compared with the original fibroblasts (Supplementary Material, Table S4), reflecting the residual activity of the defective DNMT3B. However, despite this elevation, subtelomeric methylation in ICF cells at all stages was still significantly reduced in comparison with their WT counterparts (Supplementary Material, Table S3). The degree of de novo methylation differed significantly among the subtelomeres assayed, implying
that subtelomeric regions vary in their affinity for de novo methylation. Pearson correlation and cluster analysis of the methylation patterns of all the subtelomeres grouped together (Fig. 2D and E) demonstrated that ICF (pG) versus WT (FSE) samples formed two distinct clusters, suggesting that the subtelomeric methylation status varies in a systematic manner between the ICF and the WT samples. In general, subtelomeric DNA methylation patterns showed high correlations between the different ICF undifferentiated and FL samples. In the WT samples, there was more similarity between the undifferentiated and differentiated states of a specific clone, as demonstrated clearly in the hierarchical cluster analysis (Fig. 2E). In summary, subtelomeric regions remain abnormally hypomethylated in ICF-iPSCs in comparison with WT-iPSCs, both in the undifferentiated and differentiated states.

Telomerase activity and telomere length dynamics during the process of ICF-iPSC generation and differentiation

We next proceeded to determine whether despite the subtelomeric hypomethylation, telomeres were elongated during reprogramming of ICF-iPSCs, a step crucial for ensuring the unlimited replicative capacity of stem cells. We first ascertained telomerase activation, which is necessary for normal elongation of telomeres during reprogramming (35, 37, 38, 44). Based on the results obtained from the TRAP assay (Fig. 3A and Supplementary Material, Fig. S6A), we concluded that telomerase was upregulated in all ICF-iPSCs, downregulated upon differentiation and totally silenced in derived FLs, similar to the situation during reprogramming of WT fibroblasts (36). No elevated activity of telomerase in ICF-iPSCs in comparison with WT-iPSC was evident by TRAP assay (Supplementary Material, Fig. S6A), suggesting that no such compensation was necessary during the reprogramming process. In addition, we tested whether the alternative (ALT) pathway of telomere elongation, which involves unequal telomere sister chromatid exchange (T-SCE) (45 –47), is active during reprogramming of ICF-iPSCs. Elevated rates of T-SCE at hypomethylated telomeres, concomitant with high levels of TERRA, have been reported in certain types of human cancers (48, 49). Utilizing the chromosome orientation

Figure 1. Generation and characterization of ICF type I human iPSCs. (A) ICF-iPSCs express pluripotent markers. ICF-iPSCs were stained with antibodies for pluripotent markers as described previously (70). Undifferentiated (UD) colonies of iPSC pG 20 display positive staining for NANOG, SSEA-4 and OCT4. The lower row displays nuclear staining by DAPI. The large nuclei surrounding the iPSC cells originate from the mouse embryonic fibroblasts that serve as a feeder layer, and are clearly not stained by any of the marker antibodies. Bar: 100 μm. (B) OCT4 and NANOG promoters are hypomethylated in UD ICF-iPSCs and remethylated in differentiated fibroblast-like cells (FLs). Analyzed DNA samples include pG fibroblasts (pG fib), iPSCs derived from pG fibroblasts, clone 20 (pG 20 UD) and FLs derived from pG 20 UD (pG 20 FL). (C) In vitro differentiated ICF-iPSCs express markers of all three embryonic germ layers. In vitro differentiation of ICF-iPSCs was carried out as described (36, 78) and immunostaining was performed for markers representing the three embryonic germ layers: α-fetoprotein (endoderm), Nestin (ectoderm) and Desmin (mesoderm) (79). Bar: 200 μm. The differentiated cells displayed in this figure are derived from pG 20 UD. (D) ICF-iPSCs generate teratomas containing all three embryonic germ layers. Tissue structures representing the three germ layers were evident as the following. Ectoderm: multilayered epithelium (ME), keratinocytes (KE); mesoderm: blood vessels (BV), cartilage (CA); endoderm: columnar epithelium (CE), glandular epithelium (GE). Bar: 200 μm. The teratoma analyzed in this figure originated from injected ICF-iPSC pG 20 UD. (E) The full-length isoform of DNMT3B, DNMT3B1, is transcribed in undifferentiated ICF-iPSCs. RT-PCR analysis of the catalytic domain of DNMT3B and of β-ACTIN (β-ACT) was performed on RNA derived from pG fibroblasts, three ICF-iPSC clones of pG, pG 20 UD, pG 23 UD and pG 28 UD, and differentiated derivatives of the three iPSC clones, pG 20 diff, pG 23 diff and pG 28 diff, as described previously (36). DNMT3B1 (3B1) and DNMT3B3 (3B3) are the two most common alternative transcripts of DNMT3B. The full-length transcript is expressed only in the pluripotent state.
Figure 2. Subtelomeric hypomethylation persists in ICF-iPSCs. (A) Terminal restriction fragment (TRF) methylation analysis was performed on DNA extracted from ICF fibroblasts (pG fib, pR fib, pW fib), five undifferentiated ICF-iPSC lines (pG 20 UD, pG 28 UD, pR 75 UD, pR 76 UD, pW 54 UD), their in vitro-derived differentiated cells (pG 20 diff, pG 28 diff, pR 75 diff, pR 76 diff, pW 54 diff) and fibroblast-like cells (FLs) (pG 20 FL, pG 28 FL, pW 54 FL). DNA was digested with the isoschizomeric restriction enzymes MspI (M) and HpaII (H). Size markers, in kb, appear on the left of the blots. (B) Mean telomere length (MTL), in kb, as determined by 2–4 repeats of TRF methylation analysis using the MATLAB-based software, MATELO. The MTLs obtained by HpaII or MspI digestions were not significantly different according to Student’s t-test, indicating hypomethylation of subtelomeric regions. (C) Methylation analysis of eleven subtelomeric regions by high-throughput sequencing of bisulfite-converted DNA. The heat map shows the relative methylation across the eleven indicated subtelomeric regions, in ICF fibroblasts (pG fib), two ICF-iPSCs (pG 20 UD, pG 28 UD), WT fibroblasts (FSE fib), two WT-iPSCs (FSE 15 UD, FSE 35 UD) and their FL derivatives (pG 20 FL, pG 28 FL, FSE 15 FL, FSE 35 FL). In this heat map, each subtelomeric region consists of several lines, each line representing a specific CG site analyzed in that subtelomeric region, in the 5′ to 3′ direction of the sequence. The subtelomeric average methylation percentage and the t-test P-values of comparisons between samples appear in Supplementary Material, Tables S2–5. P-values reflecting statistical significance were determined as $P < 0.05$. (D) Pearson correlation analysis and clustering of the high-throughput sequencing analysis of the samples described in (C) was performed using methylation data of all subtelomeric regions grouped together. Analysis was carried out using the BISMA software. The closer the values are to 1, the greater is the similarity between the two compared groups. (E) Hierarchical cluster analysis using methylation data of all subtelomeres grouped together. Analysis was carried out using BISMA software. The major dendrogram branches separate clearly between the WT samples and the ICF samples.
fluorescence in situ hybridization (CO-FISH) assay (50), we found no evidence for elevated T-SCE in any of the undifferentiated ICF-iPSCs analyzed (Supplementary Material, Figs S6B and C).

Following the observation that telomerase is activated in ICF-iPSCs, we examined telomere length dynamics during reprogramming and continuous growth in culture. TRF analysis of HinfI-digested DNA indicated that in all examined ICF-iPSCs, telomeres are normally elongated upon reprogramming of ICF fibroblasts to iPSCs. TRF analysis was performed on DNA extracted from ICF fibroblasts (pG fib, pR fib, pW fib), ICF-iPSCs [pG 20 UD at three passages (P), pR 75 UD (passage 24), pW 54 UD (passage 28)], their differentiated cells (pG 20 diff, pR 75 diff, pR 76 diff, pW 54 diff) and FLs (pW 54 FL). DNA was digested with HinfI. Telomeres were elongated when reprogrammed to pluripotency, and shortened when cells differentiated. Size markers, in kb, appear on the left of the blots. (C and D) Mean telomere length (MTL), in kb, as determined by 2–4 repeats of HinfI-TRF analysis, as shown in (B) and Supplementary Material, Fig. S6D. MTL was determined using the MATELO software. (E) Telomere-length elongation during ICF fibroblast reprogramming as demonstrated by telomere FISH. pG fibroblasts and pG iPSCs in the undifferentiated state (pG 20 UD, pG 23 UD, pG 28 UD) at early and late passages (P) were analyzed by telomere FISH for loss of hybridization signals from either one sister chromatid (STL) or from both chromatids (SFE). The y-axis indicates the percentage of STLs and SFEs detected in each sample. All ICF-iPSCs display a low percentage of telomere signal loss.

Figure 3. Telomeres are normally elongated in ICF-iPSCs. (A) Telomerase is normally activated in ICF-iPSCs upon reprogramming and downregulated after differentiation. Telomerase activity was detected by the TRAP assay in ICF fibroblasts (pG fib), ICF-iPSCs (pG 20 UD, pG 28 UD), differentiated iPSC cells (pG 20 diff, pG 28 diff) and fibroblast-like cells (FLs) derived from both iPSC clones (pG 20 FL, pG 28 FL). +/- refers to heat inactivation. (B) Telomeres elongate upon reprogramming of ICF fibroblasts to iPSCs. TRF analysis was performed on DNA extracted from ICF fibroblasts (pG fib, pR fib, pW fib), ICF-iPSCs [pG 20 UD at three passages (P), pR 75 UD (passage 24) pR 76 UD (passage 26), pW 54 UD (passage 28)], their differentiated cells (pG 20 diff, pR 75 diff, pR 76 diff, pW 54 diff) and FLs (pW 54 FL). DNA was digested with HinfI. Telomeres were elongated when reprogrammed to pluripotency, and shortened when cells differentiated. Size markers, in kb, appear on the left of the blots. (C and D) Mean telomere length (MTL), in kb, as determined by 2–4 repeats of HinfI-TRF analysis, as shown in (B) and Supplementary Material, Fig. S6D. MTL was determined using the MATELO software. (E) Telomere-length elongation during ICF fibroblast reprogramming as demonstrated by telomere FISH. pG fibroblasts and pG iPSCs in the undifferentiated state (pG 20 UD, pG 23 UD, pG 28 UD) at early and late passages (P) were analyzed by telomere FISH for loss of hybridization signals from either one sister chromatid (STL) or from both chromatids (SFE). The y-axis indicates the percentage of STLs and SFEs detected in each sample. All ICF-iPSCs display a low percentage of telomere signal loss.
telomeres were elongated during the reprogramming process (Fig. 3B–D). However, compared with the early passage cells, we found that during culturing for ~60 passages, the different clones displayed variable telomere length dynamics (Fig. 3B and C and Supplementary Material, Fig. S6D). Clone pG 20 maintained steady telomere length, clone pG 23 displayed telomere shortening during passaging and clone pG 28 continued to elongate over time. Notably, such clonal variability has been demonstrated previously for WT-iPSCs of the same genetic background and is not unique for ICF-iPSCs (35–38). In the case of patient pR, whose primary fibroblasts displayed a relatively high mean telomere length (MTL), the TRF analysis clearly showed that following reprogramming, the low-molecular-weight hybridization signals were eliminated, and a compacted high-molecular-weight telomere hybridization smear emerged (Fig. 3B).

Telomere elongation was also evaluated by FISH analysis (51,52). This method permits the study of individual telomere ends and thereby provides a detailed picture of telomere abnormalities at a single chromosome resolution, including chromosome ends with sister telomere loss (STL) and signal-free ends (SFE). We performed telomere FISH on primary fibroblasts of patient pG at the population doubling (PD) at which these cells were reprogrammed and on both early and late passage pG-iPSCs (Fig. 3E). Consistent with the TRF analysis data, we found that telomeres were sufficiently elongated during reprogramming to eliminate the STLs and SFEs observed in the primary fibroblasts, and that this situation was sustained for at least 60 passages. We were unable to obtain metaphases from pR and pW fibroblasts at the PD at which they were reprogrammed because of their extremely slow dividing rates and premature entry into senescence. However, in the undifferentiated state, iPSCs from both patients pR and pW maintained low levels of STLs and SFEs (results not shown). Collectively, our findings demonstrate that similar to previous studies (38,53–55), normal hiPSCs can be generated from cells bearing very short telomeres. Moreover, the reprogramming of ICF type I fibroblasts to normal undifferentiated iPSCs indicates that subtelomeric hypomethylation is not an obstacle in the reprogramming process; reprogramming overcomes the premature senescence phenotype observed in the primary fibroblasts derived from ICF patients, thereby enabling the cells to proliferate for at least 60 passages.

Abnormally high TERRA levels persist in ICF-iPSCs in both undifferentiated and differentiated states

TERRA is postulated to play a causative role in the generation of telomeric abnormalities in ICF syndrome (7,9,13,14,28,29,40). We therefore determined TERRA levels by northern analysis in ICF fibroblasts, ICF-iPSCs and in FLs derived from the latter cells and compared with normal iPSCs and FLs (Fig. 4A and B). As demonstrated previously (36), in WT-iPSCs, a significant elevation in TERRA levels was evident in comparison with the fibroblasts from which they were generated (Fig. 4A and B). In contrast, TERRA levels in ICF-iPSCs were similar or slightly altered in comparison with the parental pG fibroblasts; however, they were significantly higher in comparison with WT fibroblasts and iPSCs. In addition, there was a clear correlation between telomere length and TERRA length, as demonstrated previously (28,36). In conclusion, in both WT and ICF-iPSCs in the undifferentiated state, high TERRA levels do not restrict telomere lengthening and maintenance during continuous passage in culture.

TERRA was suggested to counteract the lengthening activity of telomerase during early development, suggesting that the balance between telomerase activity and TERRA levels may be crucial for normal telomere-length regulation (56). Northern analysis of TERRA in ICF-FLs of all patients indicated that even though the population of TERRA was shorter in length compared with undifferentiated iPSCs, TERRA levels were enhanced and reached levels similar to those observed in the original patient fibroblasts (Fig. 4A and B). This finding is in contrast to the situation in WT FLs, in which TERRA levels were reduced in comparison with the undifferentiated state (36) (and Fig. 4A and B). Thus, ICF-FLs recapitulate the situation in primary ICF fibroblasts, where high levels of TERRA are present in the absence of telomerase activity.

ICF-FLs display accelerated telomere loss and premature replicative senescence

The persistent high levels of TERRA in ICF-FLs prompted the question of whether telomeric abnormalities will be apparent
We infer from these findings that the molecular defect that persists, telomeres shorten and early senescence is triggered. We have demonstrated that despite the hypomethylated subtelomeres, high TERRA levels, telomeres and following differentiation, high TERRA levels, telomeres became visible on the TRF blots. While normal telomere shortening of cultured fibroblasts is ∼50 bp/PD (57), in pG 28 FL, the shortening was 111 bp/PD (between PD5.1 to PD10) and then 139 bp/PD (between PD10 to PD16.9). In pG 20 FL, the shortening was 78 bp/PD between PD11.8 and PD17.4 (Fig. 5C). At the point at which both FL lines reached senescence, the MTL length was ∼6–7 kb; however, the hybridization smear clearly extended below 3 kb. This suggests that all cells in the population contained at least several extremely short telomeres that were sufficient to trigger senescence (58). We conclude that following differentiation of the ICF-iPSCs, the derived FL cells recapitulate the telomeric phenotype of the original ICF fibroblasts, including hypomethylated subtelomeres, high levels of TERRA, accelerated telomere shortening and premature replicative senescence.

**DISCUSSION**

Human iPSCs are emerging as a potent in vitro model for studying biological processes that take place during human embryonic development and subsequent differentiation. Patient-specific iPSCs have been previously shown to be highly valuable for modeling telomeric, epigenetic and premature aging genetic diseases (53,55,59–62). The current study illustrates that ICF-iPSCs constitute likewise an authentic platform for modeling the epigenetic abnormalities at telomeric regions in ICF syndrome, taking advantage of the fact that hiPSCs mimic the developmental stage at which the molecular pathology of this disease initiates. We have demonstrated that despite the hypomethylated subtelomeres and high TERRA levels, telomeres are normally elongated and maintained at the iPSC-pluripotent state, and following differentiation, high TERRA levels persist, telomeres shorten and early senescence is triggered. We infer from these findings that the molecular defect that leads to accelerated telomere shortening is instigated only following differentiation. These findings suggest that if indeed TERRA is the culprit, the mechanism by which it exerts its effect is not mediated through telomerase inhibition, but in contrast, telomerase overrides the telomere-shortening phenotype. Hence, the consequences of subtelomeric hypomethylation will be apparent only after telomerase is downregulated and cannot compensate for the telomere shortening instigated by a yet unspecified mechanism. Similarly, the counteracting action...
of telomerase in cells containing high TERRA levels has been demonstrated by the ability of telomerase to rescue the short telomere phenotype in primary ICF fibroblasts (40), and additionally HCT116 cells knocked out for DNMT1 and DNMT3B efficiently elongate telomeres in the presence of high levels of TERRA (63). The continuous expression of telomerase throughout a major part of human embryonic development (64,65) may enable ICF syndrome embryos to reach birth and early childhood, albeit with short telomeres already at a very young age (40).

It should be noted that owing to their primed condition, the current iPSCs simultaneously encompass traits characteristic of both pre- and postimplantation stages (66). Future studies of naïve human iPSCs will provide information regarding the epigenetic state of telomeric regions prior to the wave of de novo methylation. However, based on previous studies (19,67), we suggest that during normal development, TERRA is being actively transcribed from hypomethylated subtelomeric regions in oocytes and sperm (Fig. 6A). This situation persists through zygote to blastocyst stages, consistent with suggested regulatory roles of TERRA in telomere length during the pluripotent state (5,8,12).

Following the occurrence of de novo subtelomeric methylation at implantation, TERRA promoters are still occupied by RNA polymerase II, and methylation per se is insufficient to silence transcription (68), thus TERRA transcription persists and levels remain high. We postulate that at later time points during differentiation, repressors and chromatin remodeling factors are recruited to the methylated subtelomeres. TERRA transcription is downregulated and telomeric regions acquire the silent heterochromatin state, including the characteristic histone modifications. Following this reasoning, the described elevation of subtelomeric methylation in WT-iPSCs (36) may mimic the attempt of DNMT3B to methylate these regions during normal development (Fig. 6B). We postulate that TERRA, which plays an important role in telomere-length regulation at the telomerase-positive pluripotent state, is expressed from early stages of WT-iPSC generation, prior to the occurrence of de novo methylation. When WT-iPSCs differentiate, TERRA is downregulated, as described during normal embryonic development (Fig. 6A). Accordingly, in ICF syndrome embryos, a disruption of this process occurs only after differentiation, as a consequence of the failure of the subtelomeric regions to undergo de novo methylation (Fig. 6C); The hypomethylated state of the subtelomeres is predicted to impair the recruitment of the relevant silencing factors to these regions, leading to heterochromatin TERRA expression in telomerase-negative somatic cells, resulting in the telomeric phenotype described in this syndrome. ICF-iPSCs mimic the in vivo situation in their failure to acquire de novo subtelomeric methylation and similar to the state in vivo, display the abnormal telomeric phenotype only in FL cells where telomerase activity is no longer present (Fig. 6D).

ICF syndrome type I is characterized by a multitude of phenotypic aspects, telomeric abnormalities comprising only one phenotypic facet. The entire pleiotropic range of abnormalities arises from the disruption of the fundamental process of de novo methylation at many genomic regions during embryonic development. ICF-iPSCs have already been shown to be valuable for deciphering phenotypic components of the syndrome related to disrupted methylation at protein coding regions (69). Here we conclude that ICF-iPSCs are also highly relevant for studying the molecular basis of the telomeric abnormalities evident in this syndrome, and should shed light on the general role that de novo DNA methylation plays during embryonic development at regions important for structural chromosomal integrity.

**MATERIALS AND METHODS**

**Cell culture of fibroblast cells**

Primary fibroblasts from three ICF type I patients, pG, pR (24) and pW [patient 2 in (39)], were grown in MEM media supplemented with 20% FCS, glutamine and antibiotics. Normal foreskin fibroblasts (FSE) were cultured as described previously (28). All fibroblasts were subcultured to maintain a continuous log phase growth, and the PD at each passage was determined (40).

**Generation of pluripotent stem cells from ICF fibroblasts**

Generation of the ICF-iPSCs was carried out according to Ref. (33). The previously described protocol (70) was followed, with the use of the c-MYC factor (Addgene plasmid 17220) in addition to OCT4, SOX2 and KLF4. Valproic acid, 0.9 mM, was added to the media as described previously (70); however, it was discontinued when colonies appeared within the first week of propagation.

**Identification of hiPSC colonies**

iPSC colonies were selected according to vital TRA-1-60 staining. iPSC colonies growing in 6-well plates were incubated without fixation with TRA-1-60 antibodies (sc-21705, Santa Cruz Biotechnology, Inc.) diluted 1 : 400, at 37°C for 1 h, and detected with a secondary antibody Cy3–antimouse IgM (#715-165-140, Jackson ImmunoResearch Laboratories, Inc.) at a 1 : 200 dilution, for an additional hour. Colonies whose morphology resembled that of hES and were TRA-1-60-positive, were isolated. These colonies were further expanded and assessed by molecular and functional parameters, as described (70). Teratoma generation and tissue processing were carried out as described previously (71).

**Differentiation of ICF-iPSCs**

ICF-iPSCs were induced to differentiate, as described previously (70). Following 7–10 days in suspension culture, EBs were plated on gelatin-coated 6-well culture dishes, as described (70). Differentiating cultures were grown for at least an additional two weeks. During this period, morphologically variable cell types, including FLs, became apparent in the culture dishes.

**Establishment of fibroblast-like cultures from differentiated ICF-hiPS cells**

FLs were isolated and cultivated as described previously (36).

**TRF analysis and DNA methylation studies by Southern analysis**

hiPSCs cultured for TRF analysis were grown as described previously (36,72). Genomic DNA purification, restriction analysis
and electrophoresis were performed as described (36). DNA was transferred to a MagnaGraph nylon transfer membrane (Water and Process Technologies) using a BIO-RAD vacuum blotter.

TRF analysis for length determination was performed following DNA digestion by HinfI, as described (28). Modified TRF analysis for subtelomere methylation status was performed on Figure 6. Subtelomere methylation and TERRA transcription in development and reprogramming of WT and ICF syndrome cells. A schematic model of the role of subtelomeric methylation during development and reprogramming. Lollipops represent CpG methylation and the wavy arrows represent TERRA transcripts. (A) Sperm and oocytes are hypomethylated at subtelomeres and express TERRA. This situation persists between fertilization until implantation at which subtelomeres are de novo methylated; however, they continue to express TERRA. When terminal differentiation occurs, repressors (ellipses) are recruited to the methylated subtelomeres and TERRA transcription is silenced. (B) When normal iPSCs are generated, required repressors are absent; therefore, TERRA is transcribed, despite subtelomeric methylation. After differentiation, repressors are expressed, bind to subtelomeres and silence TERRA transcription. (C) An embryo carrying two mutations in DNMT3B will behave similar to normal embryos until the implantation stage. At that point, DNMT3B will fail to properly methylate subtelomeres, and following differentiation, the necessary repressors will not be recruited to these regions, and TERRA expression will persist. (D) Similar to the situation in ICF embryonic development, ICF-iPSCs will not undergo de novo methylation, will continue to express TERRA following differentiation and will recapitulate the telomeric phenotype of ICF somatic cells.
Mspl and HpaII-DNA digested samples. Methylation at subtelomeric regions inhibits the digestion by HpaII and results in larger TRF sizes in comparison with the Mspl-digested samples. Telomere length was determined by TRF analysis following analysis with the MATELO software, freely available at http://md.technion.ac.il/pictures/storage/45/48.zip (last accessed on 18 February 2014).

Bisulfite sequencing

Bisulfite-converted DNA was sequenced using the Ion Torrent PGM high-throughput platform technology (73). Genomic DNA (1 μg) was bisulfite-converted with the Methylamp DNA Modification kit (EPIGENTEK, NY, USA) according to the manufacturer’s instructions. After bisulfite conversion, DNA was amplified using FastStart Taq polymerase (Roche) as follows: 95°C for 5 min; 4 cycles of 95°C for 1 min, 53°C for 3 min, 72°C for 3 min; 2 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 45 s; 40 cycles of 95°C for 30 s, 72°C for 1.5 min; 72°C for 10 min. Primers for amplification contained barcodes and adaptor sequences for downstream analysis of the sequenced material. Sequence and genomic location of primers appear in Supplementary Material, Table S1. PCR products were cleaned with the QIAquick PCR purification kit (#28104, Qiagen) and sub-jected to bisulfite high-throughput sequencing (73). Thousands of reads were generated for all analyzed amplicons, with the exception of subtelomeres 4p/4q, which gave only several hundreds of reads for each sample. Methylation analysis of subtelomeric regions was carried out using BISMA and BiQ analyzer software (42,43); the combination of the two increased the accuracy of the alignment process. Ion Torrent sequencer generates data files in a FASTQ format, which is incompatible with BISMA software. Therefore, prior to analysis with BISMA, all reads were converted to FASTA format. The BISMA default parameter values were the input for the analysis. As BISMA software uses up to 400 reads in the compilation step, the reads of each sample library were divided into sublibraries, each including 400 different reads. The methylation level of each library was computed as the average of the sublibrary methylation levels. In addition, a two-tailed t-test was used to statistically analyze the differences in methylation levels between different samples.

Methylation analysis of OCT4 and NANOG promoters by high-throughput sequencing of bisulfite converted DNA was carried out by calculating the average methylation percentage of all the CG sites of each amplicon. The number of reads for each sample varied between several hundred to several thousand.

RNA isolation, RT–PCR and northern analysis

ICF-iPSCs cultured for the purpose of RNA extraction were grown on fibronectin-coated plates with conditioned media, as described (36). RNA was extracted with TRI REAGENT (Molecular Research Center, Inc.) and treated with DNase I (#79254, Qiagen). For northern analysis, RNA was concentrated on columns (#74204, Qiagen). For RT–PCR analysis, 1 μg of DNase I-treated RNA was converted to cDNA using iScript synthesis kit (170-8890, BIO-RAD). Primers and amplification conditions for PCR of DNMT3B and β-ACT following RT appear in Ref. (36).

For northern analysis, 10 μg of RNA were subjected to electrophoresis on a 1.2% agarose gel and transferred to a MagnaGraph nylon transfer membrane. To detect transcripts from telomeric regions, membranes were hybridized to a 32P-p-ended (TAACCC) probe. To normalize signals arising from telomeric probes, the northern blots were rehybridized to a human β-actin probe (74). Hybridization signals were quantitated by phosphorimager analysis using the TotalLab software.

Western blot analysis

Samples containing 100–150 μg protein were electrophoresed in Tris–glycine–SDS running buffer on SDS–polyacrylamide gels (8%). DNMT3B was detected with goat anti-DNMT3B diluted 1 : 750 (T-16, sc-10236, Santa Cruz). To confirm equal protein loading, membranes were also reacted with a mouse anti-β-actin antibody diluted 1 : 1000 (Ab8224, Abcam).

Measurement of telomerase activity by TRAP assay

Telomerase activity was measured by TRAP assay, as described previously (36).

Telomere FISH

Metaphase chromosomes were prepared by standard techniques. Hybridization was performed with a peptide nucleic acid (CCCTAA)3 oligo conjugated to Cy3 as described (51,52,75). For CO–FISH analysis, cells growing in culture were subjected to 2.5 × 10−4 M BrdU for 18 h, and CO–FISH was performed as described previously (28).

Ten to twelve metaphases were analyzed for each sample. For telomere signal-free analysis, signals were scored for the presence of two (DS), one (STL) or absent (SFE) telomere hybridization signals. CO–FISH analysis was carried out as described previously (28).

Fluorescence microscopy

Fluorescence hybridization was visualized using a BX50 microscope (Olympus). Images were captured with an Olympus DP70 camera controlled by DP controller software (Olympus). Analysis and pseudocolor rendering were conducted using DP manager software (Olympus). DAPI was pseudocolored in red and CY3 in green.

Senescence-associated β-galactosidase (SA-β-Gal) assay

SA-β-Gal staining was carried out at pH 6 as previously described (76,77). Following staining, cells were photographed using a Nikon ECLIPSE Ti inverted microscope.

Immunofluorescence

Immunofluorescence was performed as described previously (40). Mouse anti-phosphorylated γH2AX (#05-636, Upstate) diluted 1 : 1000, was used to detect γH2AX foci.
SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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REFERENCES


