Loss of transcriptional control over endogenous retroelements during reprogramming to pluripotency

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Endogenous retroelements (EREs) account for about half of the mouse or human genome, and their potential as insertion mutations and transcriptional perturbators is suppressed by early embryonic epigenetic silencing. Here, we asked how ERE control is maintained during the generation of induced pluripotent stem cells (iPSCs), as this procedure involves profound epigenetic remodeling. We found that all EREs tested were markedly up-regulated during the reprogramming of either mouse embryonic fibroblasts, human CD34+ cells, or human primary hepatocytes. At the iPSC stage, EREs of some classes were repressed, whereas others remained highly expressed, yielding a pattern somewhat reminiscent of that recorded in embryonic stem cells. However, variability persisted between individual iPSC clones in the control of specific ERE integrants. Both during reprogramming and in iPSCs, the up-regulation of specific EREs significantly impacted on the transcription of nearby cellular genes. While transcription triggered by specific ERE integrants at highly precise developmental stages may be an essential step toward obtaining pluripotent cells, the broad and unspecific unleashing of the repetitive genome observed here may contribute to the inefficiency of the reprogramming process and to the phenotypic heterogeneity of iPSCs.

[Supplemental material is available for this article.]
by specific coopted ERE integrants that briefly evade repression seems critical to the identity of the pluripotent state both in mice (Macfarlan et al. 2012) and humans (Santoni et al. 2012; Lu et al. 2014). This layered repression process likely reflects at least in part the orderly action of cognate repressors, including KRAB-containing zinc finger proteins (KRAB-ZFPs), which together with their cofactor TRIM28 (also known as KAP1 or TIF1B) are key to the early embryonic control of a broad spectrum of retrotransposons (Wolf and Goff 2007; Matsui et al. 2010; Rowe et al. 2010). Interestingly, KRAB-ZFPs, encoded in the hundreds by the genomes of both mouse and human, are widely expressed in ESCs but subsequently adopt highly tissue-, stage-, and cell-specific patterns of expression (Barde et al. 2013; Corsinotti et al. 2013). Whether the same holds true for other yet-to-be-identified early embryonic controllers of EREs is unknown. However, it is likely that any lag between the de-repression of specific ERE integrants and the reactivation of their sequence- or class-specific repressors will open a window of opportunity for ERE-originating transcriptional perturbations and, at least for the small fraction of these elements still endowed with retrotransposition ability (Finnegan 2012), for insertional mutagenesis. Accordingly, the present study was undertaken to examine how the transcriptional control of EREs is maintained during the reprogramming of either murine or human somatic cells, and whether it is fully reestablished in induced pluripotent stem cells.

**Results**

**Global ERE de-repression during the reprogramming of mouse and human cells**

In order to assess the control of EREs during reprogramming to pluripotency, we transduced mouse embryonic fibroblasts (MEFs) harboring a Pou5f1-GFP transgene with a lentiviral vector expressing Pou5f1 (OCT4), Klf4, and Sox2 (hereafter called OKS) as a single polycistronic transcript from a spleen focus forming virus (SFFV) promoter (Supplemental Fig. S1A; Pasi et al. 2011). As expected, OKS-transduced MEFs formed GFP-positive colonies after ~12 d (Supplemental Fig. S1B) and silenced the OKS vector (Supplemental Fig. S1C). We picked and replated a series of iPSC clones and verified that they expressed Nanog at similar levels as ESCs (Supplemental Fig. S1D) and could be differentiated into embryoid bodies (EBs) (Pasi et al. 2011), indicating successful reprogramming. We then used real-time quantitative PCR (RT-QPCR) to measure the expression of families of EREs at various times of the reprogramming process and in the resulting iPSCs. We consistently observed marked increases in the transcript levels of all tested EREs in independent reprogramming experiments (Fig. 1A–D). The timing of the up-regulation was variable, usually initiating 6–10 d post-transduction with a burst of expression occasionally observed around day 2. In all iPSC clones tested, LINE1 and the ERV MusD remained highly expressed, whereas IAP (intracisternal A particle, another ERV) exhibited a repressed state comparable to that of ESCs, expressed a full set of pluripotency genes, induced the formation of teratomas when injected into immunodeficient mice, and were karyotypically euploid (Supplemental Fig. S1E–H). However, de-repression of all tested human ERE families was detected by RT-QPCR during the reprogramming process, with sharp increases around day 19 post-transduction across three independent experiments performed with cells from different donors (Fig. 1E,F; Supplemental Fig. S2). Similar to their murine counterparts, human LINE1s remained highly expressed in pluripotent cells, as did SVAs (Fig. 1E,F). In contrast, the LTR-containing HERVK, including its HERVK14ci strain, was silenced in iPSCs, mimicking the behavior of IAPs in the murine system (Fig. 1G,H).

To confirm and extend these observations, we performed RNA deep sequencing (RNA-seq) at multiple time points of an independent reprogramming experiment, including in our analysis the parental CD34+ cells, six of the resulting iPSC clones, and a human ESC clone (H1) (for RNA-seq data, see Supplemental Tables 1–3, and GEO accession number GSE57866). A comparison of the transcriptomes of cells harvested at day 19 post-transduction with that of the starting population confirmed the de-repression of multiple EREs, and further identified specific subclasses of dysregulated retrotransposons (Fig. 2A), with HERVH, HERVK, and their associated LTRs LTR7Y and LTR5-Hs displaying the most pronounced up-regulation (Fig. 2B). In iPSCs, some of these elements, such as HERVK and S71 (LTR6b), displayed a repressed state comparable to that found in ESCs, but others (e.g., HERVW, LTR17, LTR7Y, L1Hs) did not or did only partially (Fig. 2B,C). Of note, while significant expression of HERVH was detected in iPSCs and ESCs, as previously reported (Santoni et al. 2012), close to a third of the HERVH integrants up-regulated at day 19 were not among those ultimately detected in these cells (data not shown). Finally, in addition to the d19 peak of expression, we occasionally observed a much earlier burst around d2–d4 for some retroelements (e.g., HERVH and HERVK) (Fig. 2C). We further RNA-sequenced eight independent human ES cell lines (UCLA1-6 [Díaz Perez et al. 2012], H19, and an independent H1 sample) to verify that the elevated ERE expression levels detected in individual iPSC clones were not a general feature of pluripotent cells. We found very little heterogeneity between all tested human ES cell lines, which sharply contrasted with the inappropriate control of EREs in iPSCs and even more with their marked up-regulation during the reprogramming process (Fig. 2C).

To ascertain that the observed unleashing of EREs during reprogramming is not restricted to fibroblasts or blood cells, we assessed ERE expression during reprogramming of human liver cells. We thus reprogrammed primary human hepatocytes with OKS or with a vector that also included MYC (OKSM) (Sommer et al. 2010) and measured ERE-specific transcription by real time quantitative PCR. Confiming the generality of this phenomenon, all ERE classes re-capitulated the burst of expression during reprogramming previously observed in MEIIs and CD34+ cells (Fig. 2D).

**Deregulation of ERE-close gene transcripts during reprogramming**

We next asked whether the up-regulation of ERVs that occurred during reprogramming impacted the expression of nearby genes.
Figure 1. Global up-regulation of EREs during reprogramming. qPCR quantification of transcripts from indicated murine (A–D) and human (E–H) EREs during reprogramming of MEFs and cord blood CD34+ cells, respectively. Expression levels are indicated for parental cells (gray dots), cells transduced with an SFFV-GFP control vector (green dots), OKS-induced reprogramming time points (blue dots), individual IPS clones (orange dots), and ES cells (red dots). For the smoothing pattern across OKS-induced reprogramming time points, we computed a locally weighted polynomial regression (LOESS) with a 95% confidence interval.
Figure 2. (Legend on next page)
Heterogeneity of human iPS cells in repression of specific EREs and induction of prototypic ERE controllers

Importantly, when we compared levels of expression of individual EREs between iPS cell clones derived from a single donor and issued from the same reprogramming experiment, we noticed striking differences, notably for HERV-K, HERV-V, and L1Hs (e.g., cf. clone 6 and clone 43 or cf. clone 2 and clone 45) (Figs. 2C, 4A). HERV-K, for instance, was fivefold to ninefold more expressed in iP52 and iP56 compared with iP54, iP545, and human ES cells (Fig. 4A, top), whereas L1Hs was threefold more expressed in iP52 than in iP514. Interestingly, several members of the KRAB-ZFP gene family (e.g., ZNF492, ZNF649, ZNF208) exhibited marked differences in expression between iPS cell clones (Supplemental Figs. S3A, S4C). A direct comparison of the transcriptomes of two iPS cell clones confirmed that specific EREs and some KRAB-ZFPs were among the most discordant transcripts, suggesting that a failure to reactivate sequence-specific repressors during reprogramming might account for the lack of silencing of their target EREs in iPS cells (Supplemental Fig. S3B). Consistent with this hypothesis, Trim28 expression increased gradually during reprogramming, and Trim28 knockout MEFs failed to reprogram (data not shown). Noteworthy, known post-transcriptional controllers of retroelements (e.g., APOBEC3A and SAMHD1) (Bogerd et al. 2006; Hreekla et al. 2011; Lagouette et al. 2011) were transiently induced during reprogramming (Supplemental Fig. S3A), indicating the activation of at least some genome defense mechanisms along this process.

Incomplete control of specific EREs activates the transcription of nearby genes in iPS cells

Upon scoring the expression of specific ERE integrants, we again detected considerable heterogeneity between iPS cell clones (Fig. 4A, bottom). For example, a HERVH located on chromosome 8 was four-to fivefold more expressed in iP56 compared with other iPS cell clones and to human ES, while a HERV inserted on chromosome 22 was four-to eightfold more expressed in clones 2, 6, and 14 compared with clones 43, 45, and human ESCs (Fig. 4A, bottom). EREs can epigenetically affect the local genomic landscape owing to their content in a variety of cis-acting regulatory sequences (Burge et al. 2008; Kunarso et al. 2010; Rebollo et al. 2012; Schmidt et al. 2012; Chuong et al. 2013; Rowe et al. 2013). Correspondingly, we found numerous instances where lack of repression of a specific ERE was accompanied by up-regulation of the adjacent gene (Fig. 4B,C; Supplemental Fig. S4A,B). For example, the HIH1A1 gene, situated next to the above-mentioned chromosome-8 HERV-K, was significantly expressed only in iP56 (Spearman correlation 1 = 0.93, P < 0.007), while expression of the PRODH gene correlated perfectly with that of the adjacent chromosome-22 HERV-K among iPS cell clones (Spearman correlation 1 = 0.96, P < 0.003) (Fig. 4B,C). Likewise, expression of KLLK1 and C9orf129 paralleled that of HERV-K situated nearby (Supplemental Fig. S4A,B). Interestingly, with the exception of the HERV near PRODH (for which mapping of ChIP-seq reads was not possible), all these EREs were previously identified as bound by TRIM28 and adorned with the H3K9me3 repressive mark in human ESCs (Fig. 4B,C; Supplemental Fig. S4A,B; data not shown). Noteworthy, when differentiated to EBs or neural committed, some ERE de-repressed iPS clones regained control of specific integrants while others remained uncontrolled (Supplemental Fig. S5). For example, the ERE near PRODH in iP56 was still locked in the highly expressed state after neural commitment even though expression of PRODH itself was reduced about threefold (Supplemental Fig. S5).

To further explore this phenomenon, we examined the state of the chromatin at several of these loci by chromatin immuno-precipitation following by quantitative PCR (ChIP-qPCR). We found histone marks typical of active enhancers (H3K4me1 and H3K27ac) near the LTRs of the HERV-K situated next to HIH1A1 and PRODH in iPS cells exhibiting an up-regulation of the corresponding ERE–gene pairs, but not in clones where these units were fully repressed (Fig. 4D). Conversely, H3K9me3, a repressive mark that is a hallmark of KRAB/TRIM28-mediated silencing (Schultz et al. 2002), displayed the opposite pattern at these loci.

Discussion

These data demonstrate that the reprogramming of somatic cells to induced pluripotent stem cells is accompanied by a marked derepression of endogenous retroelements from all known classes. It has recently been suggested that transcription from EREs is important to drive ES-specific transcripts, in particular MERVL in...
murine cells (Macfarlan et al. 2012) and HERVH in human cells (Santoni et al. 2012; Lu et al. 2014). However, while we indeed found numerous HERVH integrants up-regulated in human ES cells compared with cord blood CD34+ cells, we also noted that more than a third of the members of this group found to be activated at day 19 of reprogramming were fully repressed in ESCs or iPSCs. Thus the possible requirement of HERVH-mediated transcription during IPS reprogramming and to maintain the pluripotent state comes at a price, as control is broadly released on this family of elements. Furthermore, HERVKs, including HERVK14ci, were markedly up-regulated during reprogramming but fully repressed in pluripotent cells. For most EREs, activation reached its peak shortly before the emergence of fully reprogrammed iPSCs. Our results suggest that this phenomenon might be due at least partly to a lack of synchronization between erasure of repressive chromatin marks at EREs on the one hand and reactivation of sequence-specific trans-repressors, for instance, KRAB-ZFPs, on the other hand. Importantly, at that stage, genes situated near up-regulated EREs had a greater chance of being themselves induced, consistent with ERE-based promoter or enhancer effects (Rowe et al. 2013). It could be that this contributes to the inefficiency of reprogramming, if it results in the stochastic activation of genes affecting the path to pluripotency (Polo et al. 2012). It will be interesting to determine whether ERE activation also occurs when reprogramming efficiency is increased by depletion of the MBD3 repressor (deterministic reprogramming) (Rais et al. 2013), by coexpression of CEBPA (Di Stefano et al. 2014), or following nuclear transfer. These faster reprogramming methods may be accompanied by timely reactivation of cognate KRAB-ZFPs, which may minimize aberrant ERE reactivation. ERE de-repression is predicted to result only rarely in mutagenic transposition events, owing both to the paucity of retroelements still endowed with transposition capacity (Quinlan et al. 2011; Finnegan 2012) and to the presence of restriction factors blocking their spread at a post-transcriptional level (Wolf and Goff 2008). However, the transcriptional perturbation of ERE-close genes may confer iPSCs or their progeny with phenotypic anomalies difficult to detect through conventional assays, such as blockade of differentiation to particular lineages, predisposition to oncogenic changes, aberrant release of bioactive molecules, or altered immunogenicity. Supporting this model, a recent comparative analysis of 49 iPSC lines derived from several human tissues detected an aberrant up-regulation of some LTR7/HERVH transcripts and neighboring genes, including HHLA1, in several differentiation-defective clones (Koyanagi-Aoi et al. 2013). Furthermore, the potential for more distal phenotypic anomalies resulting from inappropriate ERE-induced gene activation is illustrated by the recent observation that the schizophrenia-linked PRODH gene (Kempf et al. 2008) is controlled by the nearby HERVK (Suntsova et al. 2013), which we found deregulated in some iPSC clones. Our findings thus warrant an in-depth survey of the genomic, transcriptional, and epigenetic state of the repetitive genome of iPSC clones, whether these are to be used for basic research or are envisioned for clinical applications.

Figure 3. Up-regulation of ERE-close genes during reprogramming. (A) Using a twofold change cutoff and an adjusted P-value of <0.05, 3703 genes were found up-regulated in CD34+ cells at day 19 post-OKS transduction, compared with untransduced cells. (Left) Relative expression of HERVH (top) or HERVK (bottom) integrants situated close (<40 kb) or far (>80 kb) from these genes. No significant difference was detected (Wilcoxon test \( P = 0.14 \) for HERVK, 0.11 for HERVH). (Right) Reverse analysis, revealing that HERVH-close transcripts (n = 365 genes, or 695 transcripts) were more likely to be up-regulated than HERVH-distant transcripts (n = 18145 genes, or 30,053 transcripts). (NA) Not available due to insufficient numbers. (B) Expression levels of intragenic vs. intergenic HERVH or HERVKs at d19 of CD34+ cells reprogramming showing no bias toward intragenic HERVs. In fact, the reverse trend was observed, with more intergenic than intragenic HERVs up-regulated at d19 of reprogramming.
Figure 4. Clonal variability of ERE control in iPSCs. (A) Comparative expression of indicated ERE families (top) in parental CD34+ cells (average of three samples), in six iPSC clones simultaneously derived from their reprogramming, and in control H1 ES cells. (Bottom) Expression levels of individual HERV integrants in same cells as A (top). (B,C) Two examples of dysregulated HERV integrants and expression level of closest genes, HHLA1 for a HERVH on chromosome 8 (B) and PRODH for a HERVK on chromosome 22 (C). (Left) Expression level tracks, as well as hES H3K9me3 and TRIM28 binding data (obtained by ChIP-seq). Note that for the PRODH locus, ChIP-seq reads could not be mapped due to the high redundancy of this region, preventing binding site calling at this location. Red arrowheads indicate HERV orientation. (Right) Expression levels of ERE–gene pairs, with Spearman correlation calculated. (D) Activation (H3K4me1 and H3K27ac) and repressive (H3K9me3) histone marks at HERVs situated close to HHLA1 and PRODH and controls (activation mark negative controls, ZNF180 and ZNF420; positive controls, POU5F1 [OCT4] and NANOG promoters; H3K9me3 negative controls, EVX1 and GAPDH; positive controls, ZNF180 and ZNF420) depicted as a heatmap from least (blue) to most (brown) enriched. Two qPCR assays (e.g., ERE PRODH 5’ and ERE PRODH 5’ #2) were designed on the 5’ and 3’ of each ERE. Note active enhancer marks on the ERE near PRODH in iPS clones 2, 6, and 14 compared with iPS clones 43 and 45, and on the ERE near HHLA1 in iPS clone 6 compared with other clones. Up-regulated ERE–gene pairs are highlighted with a red box for appropriate iPSC clones.
Methods

Reprogramming

MEFs: Primary Pou5f1-GFP MEFS were prepared from E12 embryos (http://jaxmice.jax.org/strain/008214.html) and reprogrammed by transduction with OKS using four HCT116-transducing units (HC-TU) per cell as previously described (Pasi et al. 2011). CD34+ cells from human cord blood were obtained and prepared as previously described (Barde et al. 2013), before transducing 250,000 cells with OKS or OKSM using 20 HC-TU per cell. After 5 d, cells were switched to mTeSR1 medium (Stemcell Technologies no. 05859) and grown on a mouse fibroblast feeder layer until reprogrammed colonies emerged (~21 d). Individual human iPSC clones were then picked and expanded. Primary human hepatocytes were isolated from liver biopsies as previously described (Birraux et al. 2009) and plated on Matrigel or collagen before being transduced with OKS or OKSM using 20 HC-TU per cell. After 5 d, cells were switched to mTeSR1 medium (Stemcell Technologies no. 05859) and grown until reprogrammed colonies emerged (~25 d).

qPCR

Total RNA from cells at different reprogramming time points, iPSC clones, and ES cells was extracted with TRIzol reagent (Life Technologies no. 15596-018) and PurElink micro-to-midi total RNA Purification System (Life Technologies no.12183018). cDNA was prepared with SuperScript II reverse transcriptase and real-time PCR quantification was performed with FastStart Universal SYBR Green Master (Roche no. 4913914001). Normalization was done to two or three housekeeping genes (mouse: Gapdh, Cox6a1, Tfrc; human: TFRC, B2M).

Chromatin immunoprecipitation (ChIP)-PCR

Ten million iPSCs or ESCs were immunoprecipitated as previously described (Barde et al. 2013), with TRIM28- (Abcam ab10483), H3K9me3- (Diagenode pAb-056-050), H3K27ac- (Abcam ab7429), or H3K4me1- (Diagenode pAb-037-050) specific antibodies. SYBR green qPCR was performed to quantify enrichment at specific loci.

RNA-seq

RNA-seq was performed on an Illumina HiSeq 2000 sequencer (single-read 100-cycles assay). The library was generated from 250 ng total RNA using the TruSeq RNA Sample Preparation v2 kit (Illumina). Raw reads (100-bp single-end) were mapped to the human transcriptome (RefSeq), to the human genome (hg19 assembly), and to Repbase consensus sequences using the Bowtie aligner (Langmead et al. 2009) and allowing up to three mismatches, and counts were normalized to the transcript length and to the total number of reads (RPKM). Differentially expressed RefSeq transcripts and ERs were defined using the DESeq Bioconductor package (Anders and Huber 2010).

Data access

The RNA-seq data from this study have been submitted to the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE57866.

Acknowledgments

We thank S. Offner, S. Verp, and C. Raclot for technical assistance; the staff of the Lausanne University Hospital delivery room for help in obtaining cord blood; the University of Lausanne Genomics core facility for sequencing; the EPII histology core facility, Alessandra Pierisgilli, and Anne-Laure Rougemont-Pidoux for histological sections and analyses; and Vital-IT for computing. This work was financed through grants from the Swiss National Science Foundation to D.T., from the Louis Jeantet Foundation and the Novartis Consumer Health Foundation to B.E.W., from Fondazione Telethon to L.N., and from the European Research Council to D.T and L.N. (ERC 268721, KRAB’n’KAP, and ERC 249845, TARGETINGGENETHERAPY, respectively).

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Received January 21, 2014; accepted in revised form May 23, 2014.