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Lack of Genotoxicity due to Foamy Virus Vector Integration in Human iPSCs

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Abstract

Integrating vectors can lead to the dysregulation of nearby chromosomal genes, with important consequences for clinical trials and cellular engineering. This includes the retroviral and lentiviral vectors commonly used for deriving induced pluripotent stem cells (iPSCs). We previously used integrating foamy virus (FV) vectors expressing *OCT4*, *SOX2*, *MYC*, and *KLF4* to reprogram osteogenesis imperfecta mesenchymal stem cells (MSCs). Here we have studied the effects of 10 FV vector proviruses on neighboring gene expression in four iPSC lines and their corresponding iPSC-derived mesenchymal stem cells (iMSCs). Gene expression profiles in these iPSC lines showed that none of the 38 genes within 300 kb up- or downstream of integrated proviruses had a significant difference in mRNA levels, including 5 genes with proviruses in their transcription units. In the iMSCs derived from these iPSCs, the same type of analysis showed a single dysregulated transcript out of 46 genes found near proviruses. This frequency of dysregulation was similar to that of genes lacking nearby proviruses, so it may have been due to interclonal variation and/or measurement inaccuracies. While the number of integration sites examined in this paper is limited, our results suggest that integrated FV proviruses do not impact the expression of chromosomal genes in pluripotent human stem cells or their differentiated derivatives. This interpretation is consistent with previous reports that FV vectors have minimal genotoxicity, even when integrating near or within genes.

Keywords

Foamy virus vectors; retroviral integration; genotoxicity; cellular reprogramming

Introduction

Insertional mutagenesis due to vector integration is a major concern for gene and cell therapy. Integrated proviruses containing strong promoters and enhancers have the capacity

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Conflict of Interest

The authors declare no conflict of interests.

to increase expression of nearby chromosomal genes, and activation of adjacent proto-oncogenes by gammaretroviral vectors can lead to clonal dominance and malignancies in human clinical trials[1–4]. Changes in vector design such as long terminal repeat (LTR) deletions (self-inactivating or SIN vectors) that lack enhancer activity and the use of internal promoters have been developed and may improve safety[5–7]. Nevertheless, integration of SIN vectors still results in the dysregulation of adjacent genes in hematopoietic stem cells and induced pluripotent stem cells (iPSCs)[8, 9]. SIN lentiviral (LV) vectors are less genotoxic than gammaretroviral vectors because in comparison they integrate away from transcription start sites and regulatory elements[10–12]. However, their integration within transcription units can still lead to dysregulation[13, 14]. In addition, it has been shown that the genotoxicity of SIN LV vector integration alone can give rise to iPSCs without reprogramming factors[15].

Foamy virus (FV) vectors are an alternative type of retroviral vector with a large packaging capacity[16], wide host range[17], and a cDNA genome[18, 19]. FV vectors may be less oncogenic than gammaretroviral and LV vectors because they do not preferentially integrate within genes or active transcription units[20], have less transcriptional read-through activity[21], and their deleted LTRs lack enhancer activity[21]. Also, the integration of FV vectors in canine hematopoietic stem cells did not lead to clonal expansion or malignancies after transplantation[22], even when near proto-oncogenes[23]. While these studies suggest that FV vectors may have minimal genotoxicity, the effects of integrated FV vector proviruses on neighboring gene expression have not been determined previously.

We have shown that FV vectors can be used to reprogram mesenchymal stem cells (MSCs) with efficiencies similar to other vector systems, especially when the reprogramming genes were expressed from an internal Moloney murine leukemia virus (MLV) LTR promoter[24]. FV-derived iPSCs were pluripotent and could be differentiated into bone-forming MSC-like cells (iMSCs). Here we examine the effects of these reprogramming vectors on chromosomal gene expression by determining their chromosomal locations and performing a global transcriptional analysis of FV-derived iPSC clones and their differentiated iMSC derivatives.

Results & Discussion

Four independent iPSC lines were chosen for this study. iPSC clones c1, c2, c3, and c4 were derived by transducing bone marrow MSCs from osteogenesis imperfecta patients with the polycistronic FV reprogramming vector ΔΦ53MOSKMETNW, which expresses *OCT4, SOX2, KLF4* and *MYC* as a single transcript separated by peptide cleavage signals under the control of an internal MLV LTR promoter (Figure 1a). These iPSC lines were shown to express pluripotency genes and had trilineage developmental potential by teratoma assays[24]. Cytogenetic analysis of two of these 4 iPSC lines showed that they had normal karyotypes[24]. Expression of the vector-encoded, polycistronic transgene cassette was assessed by RT-PCR and qRT-PCR and found to be absent in iPSC clones c2 and c3, at a low level in iPSC clone c1, and persistent in iPSC clone c4 (Figure 1b and 1d). The MLV LTR promoter present in the vector is known to be frequently silenced in pluripotent cells[25].

Each iPSC clone was differentiated into iMSCs by embryoid body formation, plating on gelatin-coated dishes, and serial passaging in the presence of fetal bovine serum as described[26]. Because reactivation of the viral transgenes can occur with differentiation, we evaluated the expression of the FV reprogramming cassette in these iMSC cultures, and observed reactivation in clones c1 – c3, and persistent expression in c4 (Figure 1c and 1d). These iMSCs expressed type I collagen and mesenchymal cell surface markers (Figure 1e and 1f), and formed bone in vitro and in vivo[24].

We identified a total of 10 FV integration sites in the four iPSC lines by Southern blot analysis (Figure 2a–b). Each of these was mapped unambiguously by inverse PCR (Figure 2c) to a unique location in the human genome that agreed with the restriction digest fragment sizes observed on Southern blots (Table 1). Six of the integrants were located inside transcription units and four were outside of genes (Figure 3). Two of these integrants were within 300 kb of the *EPHA5* or *ERBB4* proto-oncogenes associated with lung cancer[27] and multiple types of human tumors[28–30] respectively. Overall, the 10 integrants were located within 300 kb of 46 different measurable transcripts.

We determined the effects of FV proviruses on chromosomal gene expression by performing a global transcriptional analysis on undifferentiated cultures of iPSC clones c1, c3, and c4, as well as differentiated iMSC cultures of clones c1–c4 (undifferentiated iPSC clone c2 was not available for analysis). The Illumina Bead Chip v3 was used to measure the expression levels of 25,000 genes with 48,801 probes, and the levels obtained from each clone were compared to the average of the other clones for both undifferentiated iPSCs and differentiated iMSCs. The percentage of > 2-fold up- or down-regulated probe signals ranged from 0.02–0.51% between the iPSC clones and 0.17–0.68% between their iMSC cultures (Table 2). These findings demonstrate that nearly 1% of all probe signals may vary >2-fold between clones of iPSCs or iMSCs, and this represents the background variation level in our expression profiles.

We next evaluated the signal level of every microarray probe that detected a transcript located within a 300 kb window up- or downstream of an FV integration site. In the three iPSC clones studied, none of the 61 probes within this window had > 2-fold signal differences when comparing cells that contained the nearby provirus to those lacking the provirus (Supplemental Figure 1). These iPSC data must be interpreted cautiously, since the reprogramming vectors were silenced or expressed at a low level in 2 of these clones (c1 and c3).

In the four iMSC cultures studied, all of which expressed the reprogramming vector transcript, one of 74 probes assayed (1.4%) had a >2-fold signal increase (3-fold). This single probe detects the *EBF1* transcript. In clone c3, integrant number 3 is 14 kb away from the *EBF1* transcription start site and transcribed from the opposite strand (Figure 3). Although this places the probe 415 kb from the integration site and outside the ±300 kb window, it was included because it was the only probe that detected the *EBF1* gene. We confirmed these microarray data by quantitative RT-PCR of *EBF1* mRNA which showed a 5, 12, and 210-fold increase in clone c3 iMSCs when compared to clone c2, c1 and c4 iMSCs respectively. While it is possible that the enhancer activity of the internal MLV

promoter increased *EBF1* transcription, this observation may also have been unrelated to the provirus. The 1.4% of probes found near integration sites that were dysregulated (Table 2) was not statistically different than the 0.45% percent of all probes dysregulated in iMSC c3 (Fisher exact test, p value = 0.284). In addition, a power analysis showed that at least 2 neighboring probes would have to be dysregulated to achieve significance. This probe signal was also particularly inconsistent among iMSCs, since it varied 42-fold in the 3 iMSC cultures that lacked a nearby provirus.

Six of the proviruses we studied were located within the introns of transcription units, and none of these transcripts were dysregulated by provirus integration. In two cases, intron-embedded proviruses were in the same orientation as the transcription unit, and the signals of downstream probes were still not significantly altered. These data confirm that FV vector proviruses do not result in read-through transcription, as suggested by prior transfection-based assays[21]. They also suggest that integration within an intron is not genotoxic, presumably due to splicing out of the vector provirus, since nonsense-mediated decay or premature termination would otherwise have decreased transcript levels.

In summary, our analysis of 10 independent FV vector proviruses failed to demonstrate any dysregulation of nearby genes, although we cannot rule out that a larger sample size might have included a small percentage of dysregulated loci. Nevertheless, these results stand in contrast to those obtained with gammaretroviral and LV vectors, where dysregulation was noted in 3.2–20% or 3–13% of genes near integration sites respectively[14, 31–33]. Also, a study analyzing only intragenic integration sites in LV-derived iPSC clones showed that 11% of these genes were dysregulated[9], while in our study none of the genes containing FV vector proviruses were altered. Even when using SIN gammaretroviral and LV vectors, internal promoters with strong enhancer elements such as the MLV LTR increased the frequency of nearby gene dysregulation[14]. Notably, the FV vector used in our experiments contained the MLV promoter, but no dysregulation of nearby genes was observed. In addition, read-through transcription and aberrant splicing events from gammaretroviral and LV vectors can result in fusion transcripts that alter adjacent gene transcript levels[13, 21, 34–37], which we did not observe with FV vectors, suggesting that fusion transcripts were not produced at significant levels. In conclusion, we have found that FV vectors have minimal genotoxicity, supporting their use in gene therapy and cellular reprogramming.

Materials & Methods

Cell Culture

Osteogenesis imperfecta MSCs were established from discarded bone fragments of affected individuals undergoing corrective surgery with Institutional Review Board approval[38]. Both MSCs and iMSCs were cultured in Dulbecco’s modified Eagle's medium (DMEM) with low glucose containing 10% characterized fetal bovine serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, and supplemented with 2 mM L-glutamine. Human iPSCs were cultured on irradiated mouse embryonic fibroblasts (MEFs) derived from the progeny of DR-4 mice[39] crossed with CF-1 mice (Charles River Laboratories, Wilmington, MA) in DMEM/F12 medium supplemented with 20% Knockout

Serum Replacement (Invitrogen, Grand Island, NY), 1% nonessential amino acid solution, 1% sodium pyruvate, 0.1 mM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 ng/ml basic fibroblast growth factor as described[40], or on human embryonic stem cell (hESC)-qualified matrigel (BD Biosciences, Bedford, MA) in TeSR 2 medium (StemCell Technologies, Vancouver, BC) when expanded for RNA analysis. iPSCs were derived from MSCs with the FV vector ΔΦ53MOSKMETNW as described previously[24], where iPSC clones c1–c4 corresponded to iPSC clones iPSCe2-FVc1, iPSCe2-FVc2, iPSCe2-FVc3, and iPSCe2-FVc4. iMSCs were derived from iPSCs as described for hESCs[26].

Identification of FV integration sites

Genomic DNA was isolated from each iPSC line by using the Puregene DNA purification system (Gentra Systems, Minneapolis, MN). Southern blot analysis and restriction digests were performed according to standard protocols. Radiolabeled probes were synthesized by random priming using Rediprime II (GE Healthcare, Piscataway, NJ). For inverse PCR, two μg of genomic DNA was digested with 8 units of Nla III, Hae III, Aci I, Hha I, or Msp I restriction endonuclease (New England Biolabs, Beverly, MA) at 37°C for 2 hours, extracted with phenol/chloroform, and precipitated with ethanol. Nucleic acid pellets were resuspended in 355 μl of water, 40 μl of 10X ligase buffer, and 5 μl of T4 DNA ligase (New England Biolabs, Beverly, MA). Ligation reactions were incubated at 16°C for 16 hours. The ligase reactions were heat-inactivated, extracted with phenol-chloroform, precipitated with ethanol, and resuspended in 20 μl of 10 mM Tris (pH 8.0), 1 mM EDTA. One microliter was used as template for PCR amplification with oligonucleotides ik213f (5’-GGGTGATTGCAATGCTTTCT) and ik214r (5’-TGTCTCTCATCCCAGGTACG) or ik224f (5’-AGCCTTGCTAAGGGAGACATCTAGTG) and ik225r (5’-GTTCTTCACCTCCTTCCCTGTA). DNA fragments were excised from agarose gels and cloned using the TA cloning vector pGEM T-easy (Promega, Madison, WI). DNA sequences were obtained from these cloned PCR products.

Statistical Analysis

Statistics were performed with JMP 9.0 statistical analysis software (SAS Institute Inc., Cary, NC). Data was analyzed using the Fisher's exact test and two-sample proportions analysis. *P* values <0.05 were considered statistically significant. For power analyses, statistical power was set at 0.8.

RT-PCR and gene expression analysis

RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol from iMSCs or iPSCs grown on matrigel. cDNA synthesis was performed from total RNA using the Superscript III First-Strand Synthesis System, as per the manufacturer’s instructions (Invitrogen). FV vector transgene expression was detected with primers Foamy-f and Foamy-r by PCR as previously described[24]. Quantitative RT–PCR was performed using a StepOnePlus Real-Time PCR System and TaqMan Gene Expression Assay (Applied Biosystems, Calsbad, CA) or the Bio-Rad MyiQ Single Color Real-Time PCR Detection System and Bio-Rad iQ SYBR Green Supermix (Bio-Rad,

Hercules, CA) using the manufacturer's recommended conditions. Gene expression profiling by Illumina Bead Chip v3 was performed as described[24].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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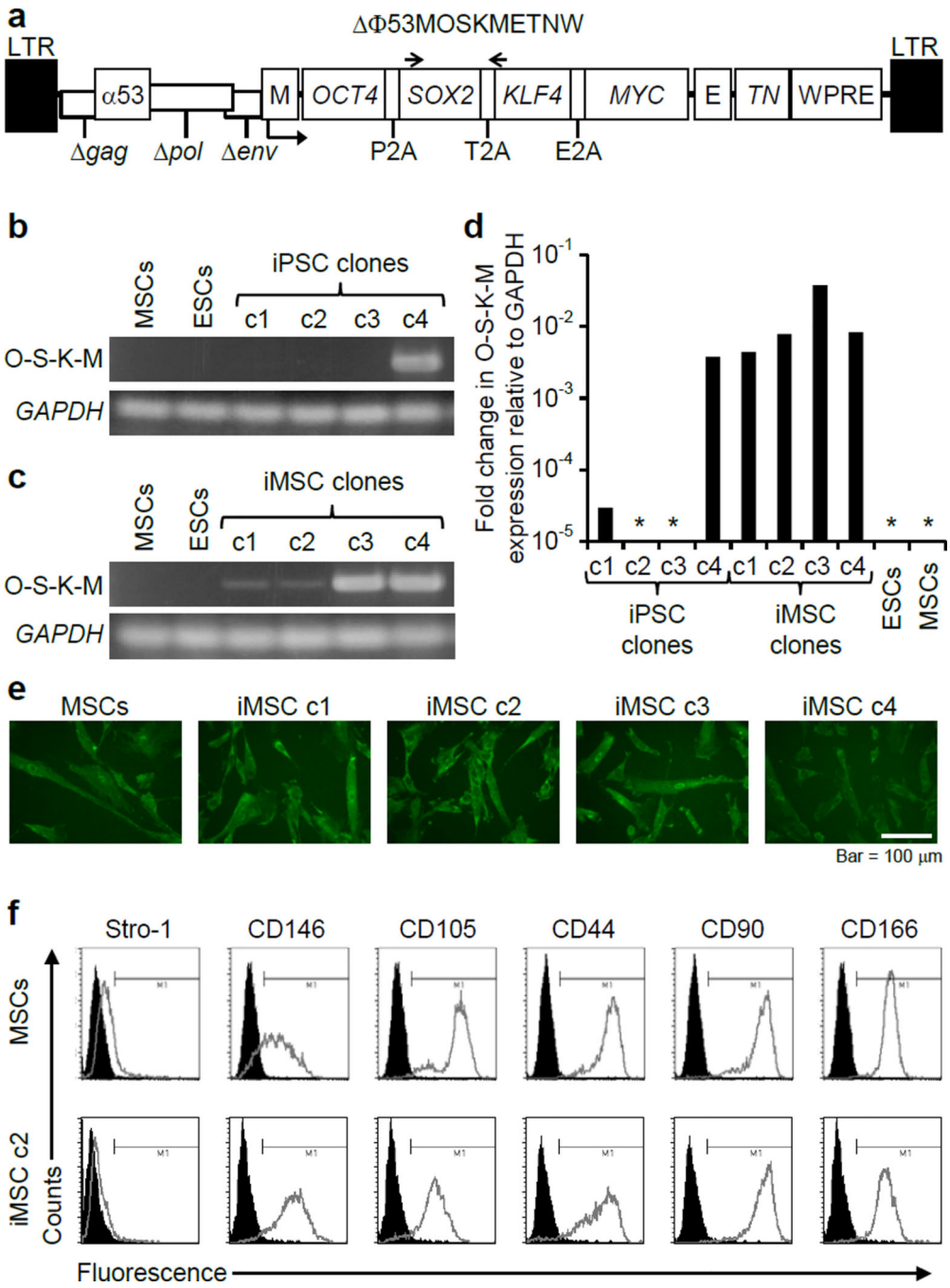

Figure 1. iPSC derivation and differentiation

(a) The FV vector ΔΦ53MOSKMETNW is shown containing a polycistronic 2A peptide-linked reprogramming cassette with *OCT4*, *SOX2*, *KLF4*, and *MYC* open reading frames. E, EF1α promoter; M, MLV promoter; *TN*, Thymidine kinase-neomycin fusion protein gene; α53, anti-p53 shRNA; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element. The locations of primers Foamy-f and Foamy-r are indicated by arrows. **(b)** RT-PCR analysis showed silencing of the FV polycistronic transcript in three of the four iPSC clones during reprogramming, with *GAPDH* transcript controls. O-S-K-M, reprogramming

vector transcript. **(c)** RT-PCR showing expression of the reprogramming vector after differentiation of iPSCs into iMSCs. **(d)** mRNA levels of the FV polycistronic transcript (O-S-K-M) as determined by qRT-PCR and shown as fold change relative to GAPDH. *No transcript detected. **(e)** Collagen expression detected by immunohistochemistry with anti-human α2 Type I procollagen antibody in MSCs and iMSCs. Bar = 100 μm. **(f)** Representative flow cytometry analysis of MSC surface markers produced by MSCs and iMSC c2.

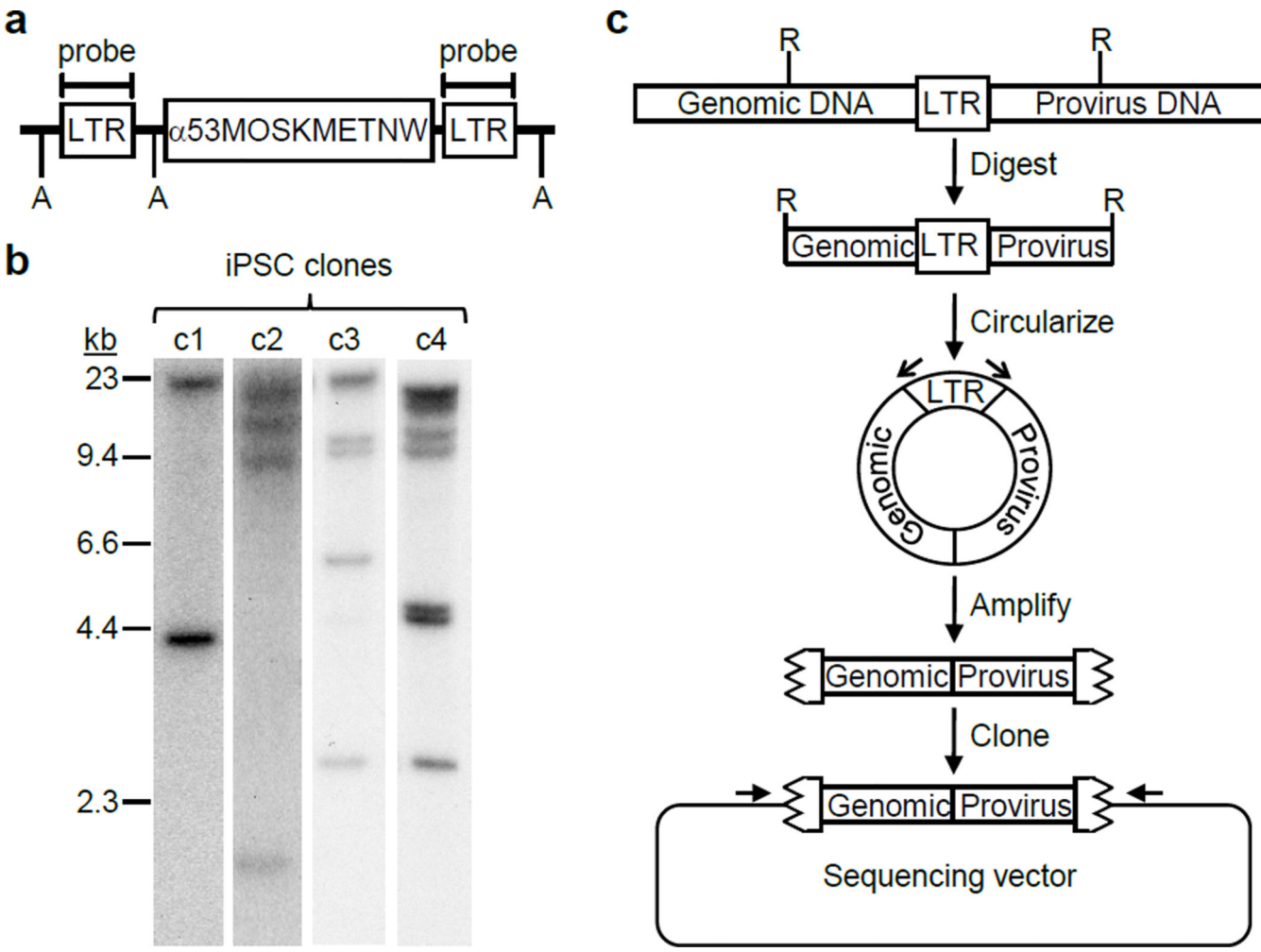

Figure 2. Identification of FV integration sites

(a) Diagram of an integrated reprogramming vector with locations of the LTR probe shown. A, *Avr* II sites. **(b)** Southern blot analysis of *Avr* II-digested genomic DNAs to determine the number of FV vector integration sites in each iPSC clone. Each integrant produces 2 LTR-hybridizing fragments. (**c**) Inverse PCR strategy for identifying chromosome-provirus junctions. R, restriction enzymes sites; open arrows, LTR-specific PCR primers; jagged box, LTR remnant; closed arrow, sequencing primers.

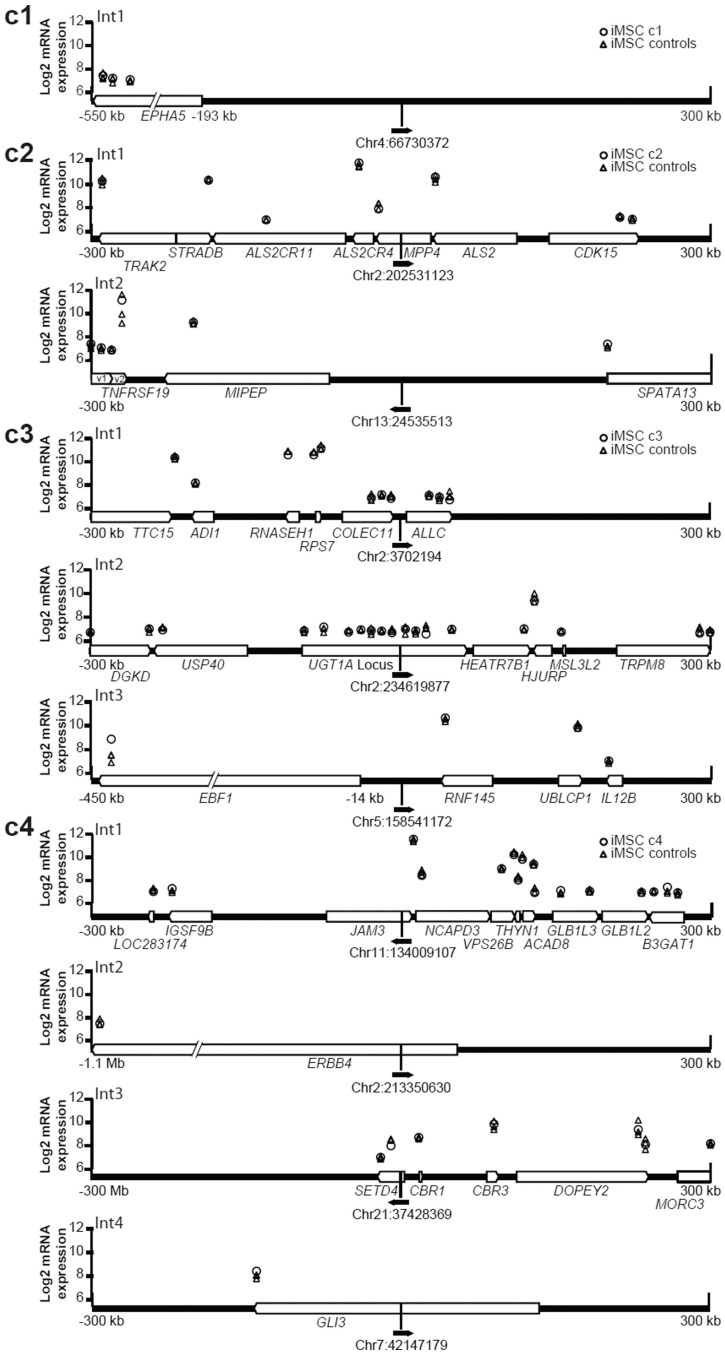

Figure 3. Provirus locations and neighboring gene expression in iMSCs

The vector proviruses found in each clone are shown with chromosomal location (February 2009 freeze of the human (hg19) genome) and a solid black arrow in the direction of vector transcription. Cellular genes within a 300 kb window up- or downstream of each provirus are shown as white block arrows in the direction of transcription. Micorarray probe signal levels are shown above their chromosomal positions as log2 values. Probe signals from each

iMSC clone containing the integrant (○) were compared to the remaining three iMSC clones as controls (△). Int1–Int4 identifies distinct integration sites within each clone.

Table 1

Predicted LTR fragment sizes after restriction digestion.

iPSC clone	Number of proviruses	LTR Fragment	Junction 1	Junction 2	Junction 3	Junction 4
c1	1	1	18874			
		2	4048			
c2	2	1	24771	12496		
		2	8676	1276		
c3	3	1	13996	15656	18215	
		2	6063	2713	9014	
c4	4	1	12431	28874	11901	21449
		2	3078	9216	4915	5555

Fragment sizes are in base pairs and based on the February 2009 freeze of the human (hg19) genome.

Table 2

mRNA probe signals in iPSCs and iMSCs.

Cell type	No. of probe ignals with >2-fold up or down variation	Percent of all probe signals with >2-fold differences	No. of probes for genes within 300 kb of an integrated provirus	No. of probe signals for genes within 300 kb of proviruses with >2-fold differences
iPSC c1	248	0.51	3	0
iPSC c3	12	0.02	35	0
iPSC c4	64	0.13	23	0
iMSC c1	81	0.17	3	0
iMSC c2	332	0.68	14	0
iMSC c3	219	0.45	34	1
iMSC c4	281	0.58	23	0