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Xenotropic retrovirus *Bxv1* in human pancreatic β cell lines

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It has been reported that endogenous retroviruses can contaminate human cell lines that have been passaged as xenotransplants in immunocompromised mice. We previously developed and described 2 human pancreatic β cell lines (EndoC-βH1 and EndoC-βH2) that were generated in this way. Here, we have shown that B10 xenotropic virus 1 (*Bxv1*), a xenotropic endogenous murine leukemia virus (MuLV), is present in these 2 recently described cell lines. We determined that Bxv1 was also present in SCID mice that were used for in vivo propagation of EndoC-βH1/2 cells, suggesting that contamination occurred during xenotransplantation. EndoC-βH1/2 cells released *Bxv1* particles that propagated to human 293T and *Mus dunni* cells. Mobilization assays demonstrated that *Bxv1* transcomplements defective MuLV-based retrovectors. In contrast, common rodent β cell lines, rat INS-1E and RIN-5F cells and mouse MIN6 and βTC3 cells, displayed either no or extremely weak xenotropic helper activity toward MuLV-based retrovectors, although xenotropic retrovirus sequences and transcripts were detected in both mouse cell lines. *Bxv1* propagation from EndoC-βH1/2 to 293T cells occurred only under optimized conditions and was overall poorly efficient. Thus, although our data imply that MuLV-based retrovectors should be cautiously used in EndoC-βH1/2 cells, our results indicate that an involuntary propagation of *Bxv1* from these cells can be easily avoided with good laboratory practices.

Introduction

Diabetes is caused by deficiency or malfunction of pancreatic β cells. Our understanding of the mechanisms underlying β cell maintenance and failure in humans has been hampered by the scarcity of material available for research. It is hence a major breakthrough that 2 functional human β cell lines, termed EndoC-βH1 and EndoC-βH2, have recently become available (1, 2).

EndoC-βH1/2 cell lines were developed from human fetal pancreatic buds. Pancreatic cells were transduced with lentivectors encoding SV40 T antigen and human telomerase and amplified through several passages as xenotransplants in SCID mice (1, 2). Cell lines developed by this method are at risk of infection by endogenous xenotropic murine leukemia viruses (X-MuLVs) (3). X-MuLVs are gammaretroviruses that infect proliferating cells from most mammalian species, including human and wild mice, through a receptor encoded by the *Xpr1* gene (4, 5). Most mouse laboratory strains are refractory to infection by X-MuLVs because they express a restrictive allele of *Xpr1* (*Xpr1*^{n}). They still harbor X-MuLVs as endogenous retroviruses, probably because the germ cells of their wild ancestors were infected before the acquisition of *Xpr1*^{n} (5). X-MuLVs were first isolated from NZB mice and then were found in most laboratory mouse strains (5). Production of infectious X-MuLV particles depends on the strain carrying the provirus. They are produced throughout life by NZB and F/St mice. In most other strains, viruses are barely (or not at all) detectable, although their production can be induced by immune stimuli such as xenotransplantation (5).

Contamination of xenografted human cells by endogenous X-MuLVs has been reported since the 1970s, but few studies have provided comprehensive data regarding origin, expression, titer, propagation, and transcomplementation properties of the invading virus. We report here that human EndoC-βH1/2 cells contain several copies of B10 xenotropic virus 1 (*Bxv1*), probably acquired during their expansion in SCID mice. Exposure of human cells to EndoC-βH1/2 cell–conditioned medium turns them into *Bxv1* infected and producing cells. However, this propagation is poorly efficient, possibly because *Bxv1* titers in EndoC-βH1/2–conditioned medium are relatively low. Finally, we show that *Bxv1* transcomplements MuLV-based retrovectors.

Results and Discussion

EndoC-βH1 cells express a xenotropic envelope protein. To generate new mAbs against human pancreatic β cell surface markers, we created a hybridoma library from mice immunized with cultured EndoC-βH1 cells (Kirkegaard et al., unpublished observations). One of these mAbs, termed 13F25, stained plasma membranes of EndoC-βH1, but not HepG2, cells, which are a human hepatoma–derived cell line (Figure 1A). The detected protein was concentrat-

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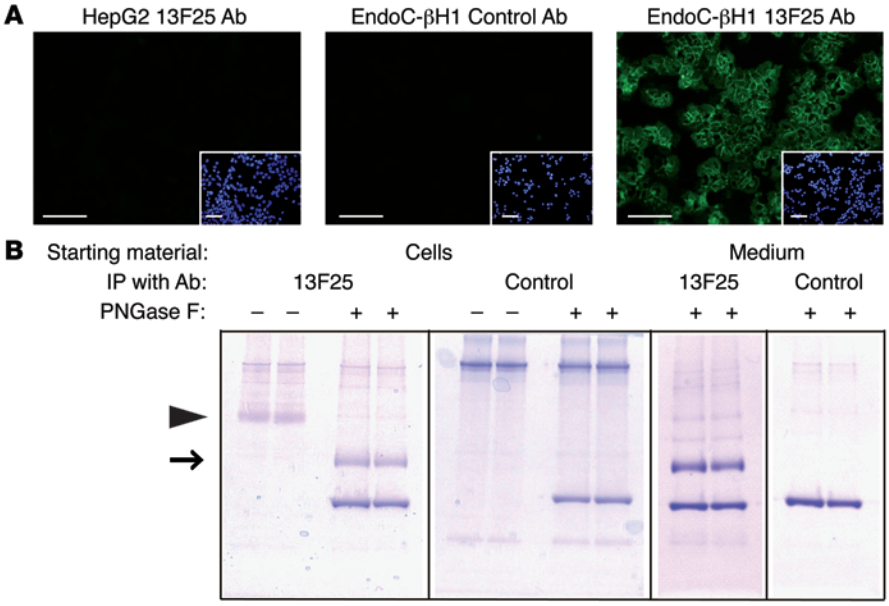


Figure 1. 13F25 identifies expression of a xenotropic envelope viral protein in EndoC-βH1 cells. (**A**) 13F25 mAb specifically binds to live EndoC-βH1 and not HepG2. Insets show DAPI staining. $n = 10$. Scale bars: 50 μM. (**B**) Coomassie gels showing the proteins purified by IP from EndoC-βH1 or conditioned medium using mAb 13F25 or the isotype control Ab. The proteins, indicated with arrowhead at approximately 65 kDa or arrow (after PNGase F treatment) at approximately 50 kDa, were excised for identification by MS ($n = 5$ and 2 for IP from cells and from medium, respectively). See complete unedited blots in the supplemental material.

ed at the plasma membrane, present in EndoC-βH1–conditioned medium, and glycosylated (Figure 1, A and B).

To identify the antigen recognized by 13F25, target proteins were purified by IP from both EndoC-βH1 lysates and conditioned medium. Isolated proteins were treated with PNGase F, separated by SDS-PAGE, and visualized by Coomassie staining. A protein of molecular weight around 65 kDa (Figure 1B), reduced to 50 kDa after PNGase F treatment and absent in the isotype control, was excised from the gel and subjected to in-gel digestion with trypsin prior to liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) analysis. The identified peptides were mapped to a group of envelope proteins from X-MuLVs (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI83573DS1). Thus, EndoC-βH1 cells express a X-MuLV envelope protein present at their plasma membrane and in the culture medium, which suggests that they harbor and possibly produce a xenotropic retrovirus. This prompted us to determine the identity of the retrovirus and whether it is produced by EndoC-βH1 cells.

Bxv1 provirus in the genome of EndoC-βH1/2 cells and SCID mice. The close similarity between the envelope proteins from X-MuLVs precluded their distinction based on a partial protein sequencing. To identify the virus encoding the 65-kDa protein, proviral X-MuLV sequences were searched by genomic PCR. We used primers that amplify a large proportion of the sequence coding the envelope protein from numerous X-MuLVs on DNA from HeLa, 293T, 22Rv1, EndoC-βH1, and EndoC-βH2 cells. 22Rv1 cells, our positive control, derive from a xenografted human prostatic cancer and produce high titers of xenotropic MuLV-related virus) (XMRV), a retrovirus closely related to X-MuLVs (6). The primers amplified a 1,453-bp product in 22Rv1, EndoC-βH1, and EndoC-βH2, but not in 293T or HeLa, cells (Figure 2A). Amplicon sequencing from EndoC-βH1 showed 100% identity with a sequence present on chromosome 1 of the C57BL/6J mouse genome. This location maps to an endogenous X-MuLV, *Bxv1* (or *Xmv43*) (5).

To test whether *Bxv1* is present in SCID mice used for xenotransplantation and determine whether EndoC-βH1/2 cells contain a complete proviral genome, we selected PCR primers to amplify the *Bxv1* genome. The 8 overlapping PCR fragments covered 7,605 bp out of the 8,662 bp of the *Bxv1* genome, excluding long terminal repeat (LTR) sequences (Figure 2B). When tested on genomic DNA, these primers amplified products of identical size in EndoC-βH1 and SCID mice, whereas no amplification occurred in 293T cells (Figure 2C). All PCR products were sequenced and revealed 100% sequence identity between SCID mice and EndoC-βH1 cells and only 1 single nucleotide mismatch with the published *Bxv1* sequence. These data strongly suggested that EndoC-βH1 cells contained at least 1 complete *Bxv1* genome and became infected during their passaging in SCID mice. Using quantitative PCR, EndoC-βH1 and EndoC-βH2 were shown to contain 22–32 and 6–7 (complete or partial) *Bxv1* genomes, respectively. Finally, we tested the 13F25 mAb antibody on lysates from EndoC-βH1, HepG2, and VCaP, a human prostate cancer cell line producing infectious *Bxv1* particles (7). 13F25 bound to several bands around 65 kDa in both EndoC-βH1 and VCaP lysates, but not in HepG2 (Figure 2D), confirming that 13F25 indeed detects *Bxv1* expression.

EndoC-βH1 cells produce replication-competent Bxv1 viruses. The presence of at least 1 complete *Bxv1* genome in EndoC-βH1 cells and detection of Env in their medium (Figure 1B) led us to examine whether they produce viral particles. Proliferating 293T cells were exposed to EndoC-βH1 conditioned medium in the presence of polybrene. After 13 days (and 2 passages), reverse-transcriptase PCR (RT-PCR) using *Bxv1 env* primers indicated that 293T cells expressed viral mRNAs (Figure 3, A and B). Thus, EndoC-βH1 cells produce replication-competent *Bxv1* particles.

Bxv1 is a gammaretrovirus, a group of retroviruses whose defective derivatives are widely used as retrovectors (hereafter referred to as MuLV-based retrovectors). Both XMRV and the X-MuLV NZB transcomplement MuLV-based retrovectors (8, 9). To test transcomplemention by *Bxv1*, we used as tester cells a 293T derivative, termed 293T-TVA-Hy, transduced with the

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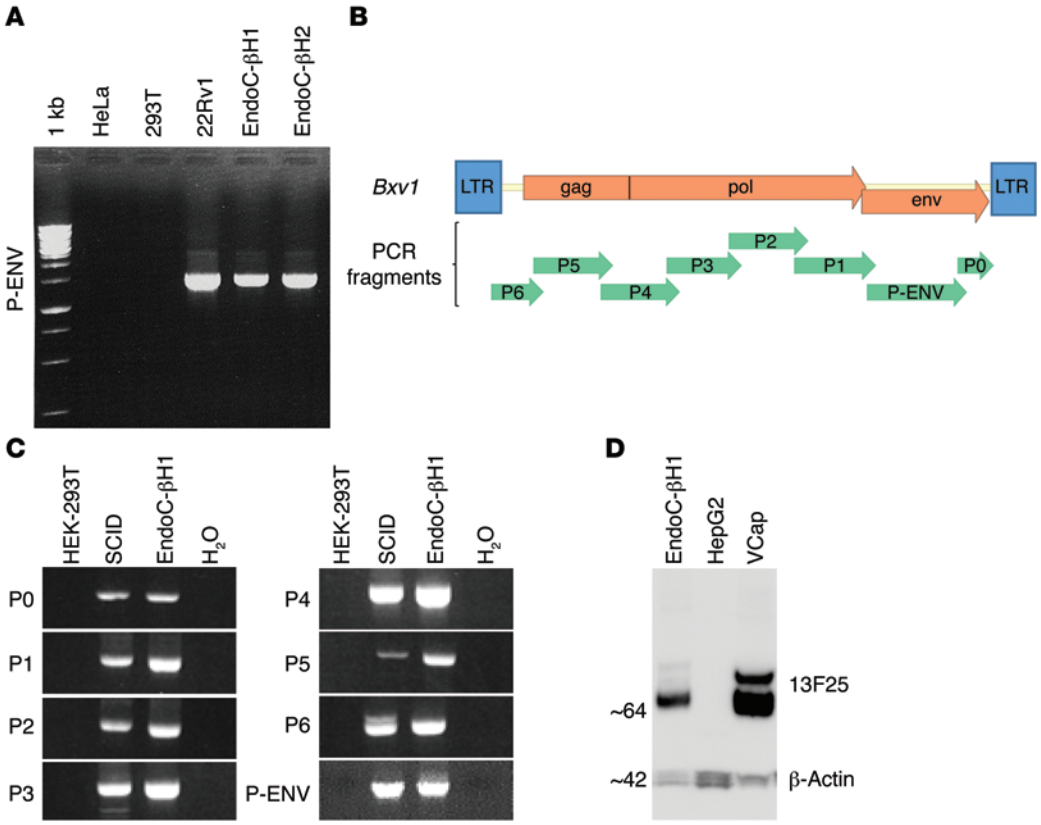


Figure 2. *Bxv1* genome is integrated in EndoC-βH1/2 cells and SCID mice. (**A**) The sequence encoding the envelope protein from a broad range of xenotropic MuLV was amplified from genomic DNA from the indicated cell lines to generate the envelope protein-encoding fragment (P-ENV) PCR product (see **B**). HeLa and 293T cells are negative controls. (**B**) Position of the 8 PCR products covering the *Bxv1* sequence. P0, primer set 0. (**C**) Amplification of *Bxv1* genome using the 8 overlapping PCR fragments showing amplification in EndoC-βH1 cells and in SCID mice. Results are representative of 3 independent experiments. (**D**) Western blot showing that VCaP and EndoC-βH1 lysates are positive for 13F25 ($n = 6$). See complete unedited blots in the supplemental material.

pPRIHy-TVA MuLV–based retrovector (10). pPRIHy-TVA encodes no retroviral protein, but encodes hygromycin resistance (as well as the avian TVA receptor). Hygromycin resistance was used as a recordable marker for retrovector mobilization. 293T-TVA-Hy cells were exposed to EndoC-βH1–conditioned medium, leading to 293T-TVA-Hy*. Four weeks later, the conditioned medium from 293T-TVA-Hy* cells was in turn added to naive 293T cells (Figure 3A). Upon hygromycin selection, numerous resistant cells (293T*) were detected, demonstrating pPRIHy-TVA mobilization in 293T-TVA-Hy* cells (Figure 3C). Importantly, 293T* cells (and, as expected, 293T-TVA-Hy* cells) were positive for the *Bxv1* genome and transcripts (Figure 3B and Supplemental Figure 2), indicating that secondary infected cells (293T-TVA-Hy*) not only produce all *Bxv1* proteins required for pPRIHy-TVA mobilization, but also infectious *Bxv1* particles. Naive 293T-TVA-Hy cells were negative for *Bxv1* genome and transcripts (Figure 3B and Supplemental Figure 2), and their conditioned medium did not transmit hygromycin resistance (Figure 3C). In summary, *Bxv1* transcomplements MuLV-based retrovectors and can be propagated from EndoC-βH1 to 293T cells, which hence turn into *Bxv1* producers. However, *Bxv1* expression is considerably lower in secondary infected cells compared with EndoC-βH1 cells (Figure 3B). Interestingly, *Bxv1* expression decreased 4-fold when EndoC-βH2 cells were withdrawn from the cell cycle upon stable expression of CRE recombinase (Figure 3B and ref. 2).

Bxv1 transcomplements defective MuLV-derived retrovectors in EndoC-βH1/2 cells. Results described above raised concerns regarding the use of MuLV-based retrovectors in EndoC-βH1/2 cells. We carefully examined this potential pitfall using an unpublished derivative of EndoC-βH2 transduced with pPRiHy-TVA (EndoC-βH2–TVA-Hy cells). 293T cells were exposed to the conditioned medium of EndoC-βH2–TVA-Hy cells. Selection of the resulting cells (293T-1, Figure 3A) showed that many of them had acquired hygromycin resistance (data not shown). Thus, the presence of *Bxv1* in EndoC-βH2 cells led to MuLV-based retrovector mobilization. Moreover, conditioned medium of 293T-1 cells transmitted hygromycin resistance to another batch of naive 293T cells, leading to 293T-2 cells whose conditioned medium again transmitted hygromycin resistance (leading to 293T-3 cells) (Figure 3, A and C). *Bxv1* genome and transcripts were readily detected in 293T-1, -2, and -3 cells (Figure 3B and Supplemental Figure 2). Thus, each step reconstituted the replicative helper virus (*Bxv1*)/defective retrovector (pPRIHy-TVA) tandem, meaning that EndoC-βH2 (or 293T) cells coinfected by *Bxv1* and a MuLV-based retrovector invariably released both types of viral particles. As expected, transient transfection of MuLV-based retrovectors in EndoC-βH1 cells also led to their transcomplementation, which was abrogated upon deletion of their packaging sequence (data not shown).

Amphotropic, polytropic, and xenotropic retroviruses can infect human cells while mouse NIH3T3 fibroblasts are susceptible to amphotropic and polytropic, but resistant to xenotropic, viruses (4, 5, 9). NIHT3T3 cells exposed to the conditioned medium of EndoC-βH2–TVA-Hy cells did not display any resistance to hygromycin, indicating that the retrovectors released by EndoC-βH2–TVA-Hy cells were not amphotropic or polytropic. Under the same conditions, NIH3T3 cells stably expressing human XPR1 (NIHT3T3-huXPR1) gave numerous hygromycin-resistant foci (Figure 3, A and C). Thus, transduced EndoC-βH2

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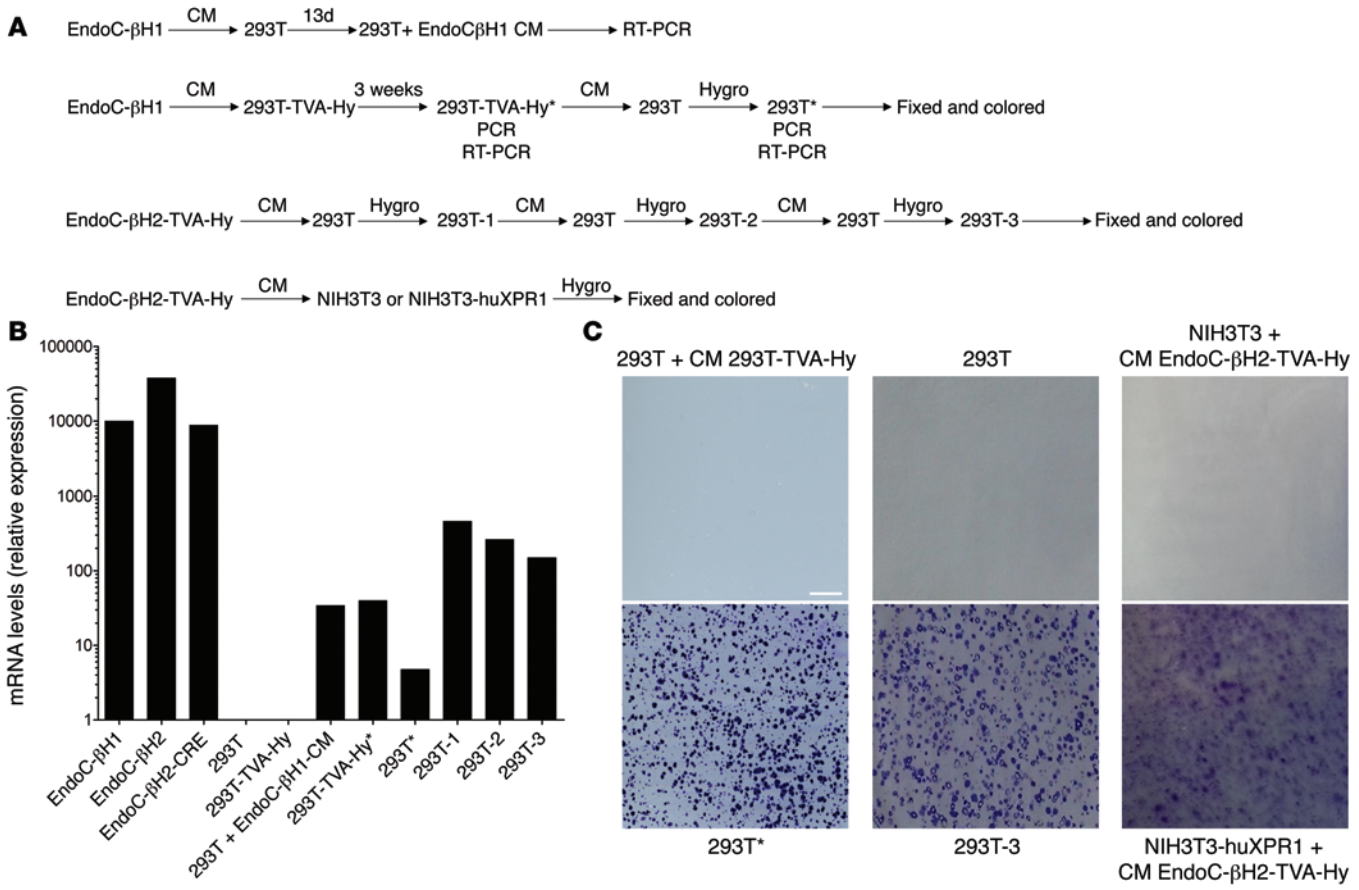


Figure 3. *Bxv1* infectious particles are produced by EndoC-βH1/2 cells and transcomplement MuLV-based retrovectors. (**A**) Procedure followed to study *Bxv1* propagation and transcomplementation properties. CM, conditioned medium. (**B**) *Bxv1* expression was analyzed by real time RT-PCR with primers for its *env* gene. Relative mRNA level for each population is given. (**C**) Left: control 293T cells exposed to the conditioned medium of 293T-TVA-Hy cells (upper panel) or 293T* (bottom panel) after selection in hygromycin, fixation, and coloration with crystal violet. Center: control 293T (upper panel) or 293T-3 cells (bottom panel). Right: parental NIH3T3 (upper panel) or NIH3T3-huXPR1 cells (bottom panel) exposed to the CM of EndoC-βH2–TVA-Hy cells. Propagation and transcomplementation properties of *Bxv1* were evidenced in 6 and 5 independent experiments, respectively. Scale bar: 1 cm. An identical area is shown for each dish.

cells released retrovectors pseudotyped with the xenotropic envelope, confirming that they arose as a result of transcomplementation by *Bxv1*.

Finally, we examined whether *Bxv1* (or *Bxv1* relatives) are also found in common rodent β cell lines. Both genomic sequences and transcripts from *Bxv1*, or closely related retroviruses, were detected in MIN6 and βTC3 cells (Supplemental Figure 3), consistent with the wide prevalence of endogenous xenotropic or polytropic retroviruses among mouse laboratory strains (5). In contrast, rat INS-1E and RIN-5F cells were negative, despite the latter having been passaged in immunocompromised mice, suggesting that rat cells are not easily infected by endogenous X-MuLVs or fail to induce their production in mice. Importantly, all rodent cell lines were negative in Western blot experiments using the 13F25 antibody and only βTC3 cells showed a detectable transcomplementation activity, albeit at an extremely low level compared with EndoC-βH2 cells (Supplemental Figure 3). Thus, even though X-MuLVs are detected in 2 mouse β cell lines, only EndoC-βH1/2 cells show a marked ability to mobilize MuLV-based retrovectors under our culture conditions.

Quantification of virus production by EndoC-βH1/2 cells. Production of replication-competent particles was next quantified in EndoC-βH1/2 cells using the classical PG4(S+L–) retrovirus infectivity test (11). The 22Rv1 cell line was included as a reference. Quantification was done after 7 days of culture using the 50% tissue culture infective dose ($TCID_{50}$) titration method (Table 1). We expressed virus titer as number of *Bxv1* particles per number of EndoC-βH1/2 cells. EndoC-βH1 and EndoC-βH2 cells produce 5.6 and 14 *Bxv1* particles per 100 cells, respectively, in agreement with the 4-fold higher expression of *Bxv1* transcripts in EndoC-βH2 compared with EndoC-βH1 (Figure 3B). This is more than 10-fold lower than the titer of XMRV in the conditioned medium of 22Rv1 cells (Table 1). These results confirmed that EndoC-βH1 and EndoC-βH2 produce replication-competent virus, although at relatively low titers.

Table 1. Virus quantification

Cell line	Titer $TCID_{50}$/ml	Particles produced/cell
EndoC-βH1	9.96×10^{4}	5.59×10^{-2}
EndoC-βH2	1.31×10^{5}	1.38×10^{-1}
22Rv1	2.42×10^{7}	2.23

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Overall, our data do not support concerns previously raised regarding the horizontal propagation of X-MuLVs (3, 12). Indeed, though the propagation of *Bxv1* from EndoC-βH1/2 cells to other human cells is feasible, it appears markedly inefficient: (a) *Bxv1* mRNA expression is much lower in secondary infected 293T cells than in EndoC-βH1/2 cells; (b) *Bxv1* mRNA expression is much higher when both *Bxv1* and a retrovector are used to generate and select secondary infected cells (Figure 3B), suggesting that the dissemination of *Bxv1* from cell to cell in a petri dish leaves many noninfected cells even after several weeks; (c) propagation was not observed under nonoptimized conditions, such as addition of half-diluted EndoC-βH1–conditioned medium to 293T cells without polybrene (data not shown). Although some cells may be more permissive than 293T to *Bxv1*, our data indicate that inadvertent propagation can be easily avoided by good laboratory practices.

The most concerning problem appears to be the transcomplementation of MuLV-based retrovectors by *Bxv1*. This means that transduction of EndoC-βH1/2 cells with such retrovectors led to their release in a xenotropic form, in addition to *Bxv1*, making possible an indefinite propagation of both viral particles in most mammalian proliferating cells, including those of human origin. This should be considered when potentially harmful transgenes are introduced into EndoC-βH1/2. In this case, other vectors should be preferred. Importantly, no SV40 T encoding lentiviral particles were detected in the conditioned medium of EndoC-βH1 cells (data not shown). Thus, self-inactivating (SIN) lentivectors appear safer than MuLV-based retrovectors for stably transferring potentially harmful sequences in EndoC-βH1/2 cells.

Methods

See Supplemental Methods.

Statistics. Viral titer determination was expressed as $TCID_{50}$ using the statistical Spearman-Kärber formula as previously described (13).

Author contributions

CR, JSK, OA, and PR designed and performed experiments, analyzed the data, and contributed to the writing of the manuscript. MG designed and performed experiments, analyzed the data, and provided reagents/materials. ODM designed experiments, analyzed the data, and contributed to the writing of the manuscript. RS designed experiments and analyzed data. SI performed experiments, analyzed data, and provided reagents/materials. EBN and MD performed experiments and analyzed data. TF analyzed the data and provided reagents/materials.

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