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Original Article

No Evidence for Infection of Human Embryonic Stem Cells by Feeder Cell–Derived Murine Leukemia Viruses

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ABSTRACT

Until recently, culture and expansion of nondifferentiated human embryonic stem cells (hESCs) depended on coculture with murine embryonic fibroblasts. Because mice are known to harbor a variety of pathogens, such culture conditions implicate the risk of xenozoonoses. Among these pathogens, endogenous retroviruses, including murine leukemia viruses (MuLVs), are of special importance. It is well known that some strains cause pathogenic (e.g., leukemic) effects and that xenotropic, polytropic, and amphotropic MuLVs are able to infect human cells.

In view of potential clinical applications of hESC lines, it is therefore imperative to investigate potential infection of hESCs by mouse feeder cell–derived viruses. As a first step towards a comprehensive infection risk assessment, we have analyzed embryonic fibroblasts derived from different mouse strains for expression and release of xenotropic, polytropic, and amphotropic MuLVs. Moreover, several hESC lines have been investigated for expression of specific receptors for xenotropic/polytropic MuLVs, as well as for MuLV infection and expression.

Evidence for expression of humantropic MuLVs was found in cultures of mouse embryonic fibroblasts (MEFs). Moreover, expression of specific receptors for xenotropic/polytropic MuLV on human HEK293 and hESC lines and infection after coculture with an MuLV-producing mink cell line could be demonstrated. In contrast, no evidence of MuLV transmission from MEFs to human HEK293 cells or to the hESC lines I-3, I-6, I-8, and H-9 has been obtained.

Our results suggest that recently established hESC lines are free of MuLV infections despite long-term close contact with MEFs. STEM CELLS *2005;23:761–771*

INTRODUCTION

Novel technologies of cell transplantation and tissue engineering will help to overcome current therapeutic limits and may enable improved treatment of life-threatening diseases such as Huntington's disease or myocardial infarction. For those upcoming technologies, one major prerequisite is suitable cell sources such as human embryonic stem cells (hESCs).

Until recently, it was not possible to isolate and culture hESCs without support of so-called feeder cells, usually mouse embryonic fibroblasts (MEFs). These mouse-derived embryonic cells are necessary to supply factors that support undifferentiated growth and expansion of hESCs. Although it is now known that certain human cell types, including fetal and neonatal fibroblasts, support undifferentiated proliferation of hESCs [1–3] and activa-

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tion of the canonical Wnt-pathway has been proposed to maintain self-renewal of hESCs [4], most hESC lines that have been registered (http://stemcells.nih.gov/registry/index.asp) have been in long-term contact with murine feeder cells.

The continuing close contact of hESCs to MEFs poses the risk of transmission of pathogens from one species to another, and clinical application of such cells may result in xenozoonoses. Mice harbor a variety of parasites, bacteria, and viruses potentially pathogenic for humans. Special attention should be paid to endogenous retroviruses in the mouse genome. Similar to all other vertebrates, mice contain a variety of retroviral elements in their genome. Most of these retroviral elements are defective and do not lead to release of infectious retroviral particles. Nevertheless, it is well known that a variety of mouse strains contain functional intact viruses. These viruses cannot be eliminated by simple specified pathogen-free animal housing or gnotobiotic breeding.

Some endogenous retroviruses are able to infect foreign species, and some cause diseases, including leukemias, neuropathological effects, and immunodeficiencies, in mice and immunosuppressed primates [5–11]. Murine endogenous retroviruses, especially mouse mammary tumor virus–related B-type retroviruses and C-type retroviruses of the murine leukemia virus (MuLV) group, are the most extensively investigated endogenous viruses.

Because of the host range and potential pathogenicity of MuLVs, we focused on the analysis of potential MuLV transmission to hESCs. The dissemination of retroviral elements of the MuLV type in mice has been investigated extensively, in particular in a variety of inbreed strains. Most inbreed strains contain 40 to 60 copies per genome [12]. Based on the receptor and host specificity, MuLVs can be arranged within at least six groups [13, 14]: MDEV (Mus dunni endogenous virus), 10A1, ecotropic MuLV (not able to infect other cells than murine), amphotropic MuLV (capable of infecting many species, including mice), xenotropic MuLV (able to infect foreign species but not mice), and polytropic MuLV (able to infect a limited number of species only). Potentially humantropic and therefore relevant for this study are xenotropic, polytropic, and amphotropic MuLVs. Moreover, one has to keep in mind that other endogenous retrovirus-like elements, which are not humantropic or not completely functional, can be transmitted to human cells via humantropic MuLV virions [15, 16].

Xenotropic MuLVs are present in different mouse strains [17–21]. Xenotropic proviruses have been detected in all strains of Swiss mice analyzed so far [20, 21]. Swiss mice also include ICR (Institute of Cancer Research) mice, which are commonly used to isolate feeder cells. Whether this strain releases infectious xenotropic MuLVs has not been analyzed. At least in some animals of an ICR/Ha colony, an ecotropic, leukemia-inducing MuLV clone designated 334C leukemia virus has been isolated [22, 23].

Polytropic MuLVs have been isolated and described somewhat later as their xenotropic relatives [13]; their dissemination has been analyzed in less detail, as in the case of xenotropic MuLVs.

Amphotropic strains have been isolated initially from wild Californian mice [24] and are not naturally found in common laboratory strains. However, exogenous infections of the mice strains used to prepare feeder cells cannot be excluded completely. Modern retroviral vectors are based on amphotropic MuLVs.

Whereas MuLV usually replicate efficiently in differentiated somatic cells, there is some evidence that MuLV expression in embryonic cells is depressed [25–28]. So far, it has not been investigated whether MuLV expression is inhibited in embryonic/fetal fibroblasts.

The MuLV types described above differ particularly in the sequence of the envelope protein env, which is responsible for binding to the specific cellular receptor [11, 29]. Human receptors for amphotropic MuLVs [30] and xenotropic/polytropic [31] MuLVs recently have been cloned from HL-60 cells and T lymphocytes. Many adult and fetal tissues express the receptor for xenotropic/polytropic MuLV, at least at the mRNA level [31], whereas high levels of the amphotropic receptor can be found on different hematopoietic stem cells [32]. Whether these receptors are expressed on hESCs has not been analyzed hitherto.

The aim of this study was to determine whether embryonic fibroblasts of mouse strains, which are usually used to prepare feeder cells, release xenotropic, polytropic, or amphotropic MuLVs and whether hESCs, which have been grown for extended time on MEFs, have been infected by humantropic MuLV.

Materials and Methods

Cell Culture

DG75, a human B-cell line, was cultivated in RPMI 1640 supplemented with 10% fetal calf serum (Invitrogen Corporation, Carlsbad, CA, http://www.invitrogen.com), 2 mmol/L L-glutamine (Invitrogen Life Technologies), penicillin (100 U/ml; PAA, Linz, Austria, http;//www.paa.at), and streptomycin (100 mg/ml, PAA).

Human embryonic kidney (HEK) 293 and 293T cells, MCF13 cells, MLV-X cells, and MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen Life Technologies), 2 mmol/L L-glutamine (Invitrogen Life Technologies), penicillin (100 U/ml; PAA), and streptomycin (100 mg/ml; PAA). For culture of MCF13 cells, Nut Mix F-12 (Invitrogen Life Technologies) was added. For culture of MEFs, medium was supplemented with 1% nonessential amino acids (Invitrogen Life Technologies). For mitotic inactivation of MEFs and MLV-X, 8 μg/ml mitomycin C was used (Sigma-Aldrich, Steinheim, Germany, http://www.sigmaaldrich.com).

hESC lines I-3, I-4, I-6, I-8 [33], and H-9 [34] were cultured with mitotically inactivated MEFs (40,000 per cells/cm^2). The culture medium consisted of 85% knockout (ko)–DMEM, supplemented with 15% serum replacement, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acid stock, and 4 ng/ml basic fibroblast growth factor (all from Invitrogen Corporation). The cells were passaged every 4–6 days using 1 mg/ml type IV collagenase (Invitrogen Corporation). The same culture methods were applied when using human foreskin fibroblasts (huF10) instead of MEFs. The foreskin fibroblasts were derived and cultured as previously described [2].

When cultured in a feeder layer–free environment [35], the culture medium was supplemented with 1,000 U/ml leukemia inhibitory factor and 0.12 ng/ml transforming growth factor β_1 (Chemicon International, Temecula, CA, http://www.chemicon.com, and R&D Systems Inc, Minneapolis, http://www.rndsystems.com, respectively). Matrigel and MEF matrix (BD Biosciences, Bedford, MA, http://www.bdbiosciences.com, and Sigma-Aldrich, respectively) were used as culture matrix. The matrigel was prepared according to the manufacturers' instructions; the dilution was 1:20 in ko-DMEM medium. For MEF matrix production, mitotically inactivated MEF-covered plates (40,000 cells per cm^2) were used. MEFs were incubated for 5 minutes with lysis buffer consisting of 0.5% (vol/vol) Triton X-100 and 0.035% (vol/vol) ammonium hydroxide solution (29.5%) in phosphate-buffered saline (PBS) (from Sigma-Aldrich and Invitrogen Corporation, respectively), followed by three washes with PBS. Similarly to the culture with MEFs, hESCs were passaged every 4 days using 1 mg/ml type IV collagenase (Invitrogen Corporation).

hESCs were cultured for different numbers of passages on MEFs before analysis or transfer to MEF-free culture conditions, as follows: I-3, 89 passages; I-4, 45 passages; I-6, 71 passages; I-8, 34 passages; H-9, 45 passages. When MEF-free culture conditions were applied, hESCs were cultured for at least five passages without MEFs before collecting conditioned medium, DNA, or RNA samples for analysis. DNA and RNA samples were collected from confluent undifferentiated cultures of one well of a six-well plate (10 cm^2) each.

In Vitro Infection Assay Using Human HEK293 Cells or hESCs

Approximately 4×10^5 MEFs or MLV-X cells, respectively, were mitotically inactivated with mitomycin C. To eliminate nonhuman cells/DNA, 1×10^5 human HEK293 cells/HEK293T cells were mixed with the treated MEFs/MLV-X and cultured for eight passages.

For coculture with hESCs, approximately 2.5×10^5 MLV-X cells were mixed with 2.5×10^5 MEFs and treated with mitomycin C as described above. A total of 7×10^5 hESCs was cultured for 5 days on these cells and was analyzed after an additional seven to eight passages on human feeder cells.

Detection of MuLV Provirus or MuLV-RNA in Murine Feeder Cells by Conventional Polymerase Chain Reaction/Reverse Transcription–Polymerase Chain Reaction

For conventional polymerase chain reaction (PCR), 1×10^6 cells were lysed in 100 µl of 200 µg/ml proteinase K in PCR buffer for 3 hours at 56°C followed by 10 minutes of inactivation at 95°C. A total of 3.5 µl of these crude extracts served as template for PCR.

Preparation of total RNA was performed using Tri-Reagent (Sigma-Aldrich), according to the manufacturer's instructions. DNAse treatment and subsequent reverse transcription were essentially performed as described previously [36]. As template for subsequent conventional PCR reactions, 2.5% of the total amount of cDNA of each sample was used, as described below.

Feeder cells derived from the following mouse strains were analyzed: ICR, ICRx129neo (a transgenic neomycin-resistant 129 strain), and ICRxDR4 (crossbreed of 129/SvJae, 129/OlaHsd, C57BL/6, BALB/c).

PCRs for analysis of MEFs were performed with primers specific for MuLV *pol* (5'-ACC CCT CGA CAA CTA AGG GAG TT-3'; 5'-GGC TTA GTC AAG TCT GGC AAT CC-3'; 34 cycles, 60°C annealing), MuLV xenotropic *env* (5'-CAG CCA TAC ATA CAG ACC TTG G-3'; 5'-CAC CCT TCT CCT GAT TCG-3'; 34 cycles, 60°C annealing), MuLV polytropic *env* (5'-AGG GCA GGA GTA TCA GTA CAA CAT-3'; 5'-CAT CCC ART CGT CCC CTA TT-3'; R = A or G; 34 cycles, 60°C annealing), or MuLV amphotropic *env* (5'-ACC TGG AGA GTC ACC AAC C-3'; 5'-TAC TTT GGA GAG GTC GTA GC-3'; 34 cycles, 60°C annealing). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)–specific primers (5'-GGC CAA GGT CAT CCA TGA-3', 5'-TCA GTG TAG CCC AGG ATG-3'; 34 cycles, 55°C annealing) were used for internal positive controls (data not shown).

MuLV *pol*–specific primers are homologous to conserved regions of the *pol* gene of different classes of MuLVs, whereas primers for xenotropic MuLV were constructed to perfectly match sequences of available xenotropic MuLV strains but not polytropic or amphotropic MuLVs. Primers specific for polytropic MuLV perfectly match different polytropic MuLV strains but not xenotropic or amphotropic MuLV, and primers specific for available amphotropic MuLV sequences do not match polytropic and xenotropic MuLV.

For positive control, pBlueAmpho, a plasmid containing an amphotropic *env* sequence, DG75, a human B-cell line infected with a xenotropic MuLV, and MCF13, a mink cell line infected with polytropic MuLV, were used.

PCR sensitivity was determined for mouse and MuLV-specific PCRs using murine ICR MEFs; different quantities of lysed MEFs were mixed with lysed human cells. The sensitivity of the applied MuLV-specific primer pairs was 1 MEF in a background of 10^5 to 10^6 human cells for MuLV *pol*. Mouse cytochrome oxidase II (CyOII)-specific primers allowed the detection of 1 MEF

in a background of 10^6 to 10^7 human cells. All samples were tested at least three times.

Detection of MuLV-Receptor RNA in Human Cells by Conventional RT-PCR

Preparation of total RNA, DNAse treatment, and subsequent RT were performed as described above. Xenotropic/polytropic MuLV receptor–specific PCR was performed with the following primers and cycle conditions: 5'- GTT GAA ACC GGA CAT TGG TTT TTA-3'; 5'-TTA CTG TTA TCA CCT TCA TTG CAC A-3'; 34 cycles, 60°C annealing. GAPDH-specific primers (5'-GGC CAA GGT CAT CCA TGA-3', 5'-TCA GTG TAG CCC AGG ATG-3'; 34 cycles, 55°C annealing) were used for internal positive controls as described above (data not shown).

Detection of MuLV-RNA in Cell Culture Supernatants of Murine Feeder Cells

Viral RNA from cell culture supernatants was prepared using the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany, http://www.qiagen.com). cDNA synthesis and subsequent PCR were performed as described above.

Detection of MuLV Provirus and Mouse CyOII DNA in Cocultures of Human and Murine Cells by Means of Quantitative Real-Time PCR

For analysis of cocultures with HEK293 cells, cell lysates and cDNA were prepared as described above. DNA for PCR analysis of hESC cultures was prepared from cell cultures using the QIAamp kit (Qiagen) or the Wizard kit (Promega, Madison, WI, http://www.promega.com) according to manufacturers' instructions. The primers and probes used are detailed below. MuLV *pol*–specific primers are homologous to conserved regions of the *pol* gene of different classes of MuLVs. Real-time quantitative PCR with TaqMan probes specific for MuLV *pol* (5'-CTG AGG CCA GAA AAG AGA CTG-3'; 5'-ATC CAG AGG CGA CAG AAG C-3'; 5'-FAM-AAG ACC CCT CGA CAA CTA AGG GAG TTC CTA-TAMRA-3') and mouse mitochondrial CyOII (5'-CAC AAG AAG TTG AAA CCA TT-3'; 5'-TAA TAC GGG GTT GTT GAT T-3'; 5'-FAM-TAA TTG CTC TCC CCT CTC TAC GC-TAMRA-3') was performed on 60–500 ng of DNA, as depicted in Table 1, in a 25-µl reaction mix (qPCR Core Kit, Eurogentec, Seraing, Belgium, http://www.eurogentec.be) essentially according to manufacturer's instructions. GAPDH-specific primers and probe (5'-CCT GCA CCA CCA ACT GCT TA-3', 5'-CAT GAG TCC TTC CAC GAT ACC A-3', 5'-FAM-CCT GGC CAA GGT CAT CCA TGA CAA C-TAMRA-3') were used for internal positive controls. Differing from the manufacturer's instructions, final concentrations of 3.5 mM $MgCl_2$ and 320 nM probe were used. All PCRs were performed in an iCycler (Bio-Rad Laboratories, Hercules, CA, http://bio-rad.com). The MuLV *pol*–specific PCR was cycled at 95°C for 3 minutes, followed by 40 cycles at 95°C for 15 seconds, 50°C for 30 seconds, and, finally, 4°C until analysis by agarose gel electrophoresis. The murine CyOII-specific PCR was cycled at 95°C for 3 minutes, followed by 40 cycles at 95°C for 15 seconds, 55°C for 30 seconds, and, finally, 4°C until gel analysis.

The GAPDH-specific PCR was cycled at 95°C for 3 minutes, followed by 40 cycles at 95°C for 15 seconds and 52°C for 30 seconds. In addition to real-time recording of fluorescence signals, all PCR reactions were controlled by agarose gel electrophoresis.

Evaluation of Real-Time PCR Data with Regard to MEF-Derived Amplification of MuLV Sequences Versus Potential Amplification of MuLV Sequences from Infected Human Cells

Because the genomes of all mouse strains contain proviral MEF sequences, contaminations of the analyzed human cells with MEFs or with MEF-DNA could lead to false-positive results during PCR-based screening for MuLV infection. To decide whether amplification of MuLV sequences from hESCs during real-time PCR was based on MEF-derived proviral sequences or on infected hESCs, a principle, already used to analyze potential infection by porcine endogenous retroviruses [37, 38], was used. Because real-time PCR, in contrast to conventional PCR, is not based on end-point quantification but on quantification during logarithmic increase of the amplification product, actual quantitative measurement of template copies is possible. Accurate quantification is achieved using the so-called threshold cycle, designating the amplification cycle when a certain sample reaches a given threshold of fluorescence.

Serial dilutions of ICR feeder cells have been tested within each PCR experiment to receive a feeder cell–specific relation of the MuLV *pol* template copy number and the murine mitochondrial CyOII template copy number. Figure 1 shows typical standard curves resulting from real-time PCR on a serial dilution of feeder cells. Such a specific relation allows us to determine whether *pol* amplification signals of the human cell samples to be tested are derived from contaminating murine cells or DNA (relation of MuLV *pol* template and mouse CyOII template should be approximately the same as for murine feeder cells) or whether an infection has occurred (relation should be significantly higher than for feeder cells). Because absolute copy numbers of MuLV *pol* and CyOII sequences have not been determined, the following equations based on corresponding threshold cycles (C_T) of the real-time PCR have been derived:

$$C_{Tpol} = a \lg x + m$$
$$C_{TCyOII} = b \lg x + n$$

in which C_{Tpol} indicates the threshold cycle MuLV *pol*–specific PCR, C_{TCyOII} indicates the threshold cycle murine cytochrome oxidase II–specific PCR, *a* and *b* indicate the slope of the regression line, *m* and *n* indicate the y-intercept of the regression line,

and *x* indicates the number of feeder cells or amount of feeder cell DNA in the sample, respectively.

Because *x* is the same for both equations in a given sample independently of whether feeder cells of the standard curve or noninfected human cells contaminated with a certain amount of feeder cells/feeder cell DNA are tested, the equations can be merged as follows:

$$(C_{Tpol} - m)/a = (C_{TCyOII} - n)/b = \lg x; C_{Tpol} = (C_{TCyOII} - n)\, a/b + m.$$

The [actual C_{Tpol}/calculated C_{Tpol}] should be approximately 1 if the MuLV *pol*–specific amplification is based on contaminating MEFs/MEF-DNA only. In this case, an infection of the human cells can be excluded. If, for a certain sample, the [actual

Table 1. No evidence for MuLV infection of hESCs obtained by MuLV *pol*–specific real-time polymerase chain reaction

Samples tested	Amount of template DNA	Actual C_{TCyOII}	Calculated T_{Cpol} $C_{Tpol} = (C_{TCyOII} - n)$ a/b + m	Actual C_{Tpol}	Actual C_{Tpol}/ calculated C_{Tpol}
ICR-MEFs	3.5 cells/sample	33.57 (± 2.22)	41.44	37.03 (± 0.06)	0.89
ICR-MEFs	3,500 cells/ sample	27.94 (± 1.12)	34.12	28.91 (± 0.69)	0.85
gDNA I3 on MEFs, sample I	300 ng	23.08 (± 0.70)	28.25	30.02 (± 1.04)	1.06
gDNA I3 on MEFs, sample I	100 ng	24.98 (± 0.09)	30.27	31.79 (± 0.74)	1.05
gDNA I3 on MEFs, sample II	300 ng	24.15 (± 0.40)	29.19	28.78 (± 0.43)	0.99
gDNA I3 on MEFs, sample II	100 ng	24.84 (± 0.36)	30.09	31.84 (± 0.91)	1.06
gDNA I3 on MEF-matrix, sample I	500 ng	29.06 (± 0.09)	35.58	BDL	>0.89
gDNA I3 on MEF-matrix, sample II	500 ng	29.70 (± 0.19)	36.41	BDL	>0.87
gDNA I3 on matrigel, sample I	300 ng	33.25 (± 0.58)	41.02	BDL	>0.77
gDNA I3 on matrigel, sample I	100 ng	34.62 (± 0.55)	42.80	BDL	>0.74
gDNA I3 on matrigel, sample II	300 ng	33.76 (± 0.18)	41.69	BDL	>0.76
gDNA I3 on matrigel, sample II	100 ng	34.15 (± 0.25)	42.19	BDL	>0.75
gDNA I6 on MEFs, sample I	300 ng	23.73 (± 0.47)	28.65	30.65 (± 0.12)	1.07
gDNA I6 on MEFs, sample I	100 ng	24.95 (± 1.64)	30.23	31.79 (± 0.74)	1.05
gDNA I6 on MEFs, sample II	300 ng	23.34 (± 0.02)	28.14	28.78 (± 0.43)	1.02
gDNA I6 on MEFs, sample II	100 ng	25.22 (± 1.63)	30.59	31.85 (± 0.91)	1.04
gDNA I6 on MEF matrix, sample I	60 ng	27.76 (± 0.34)	33.89	BDL	>0.94
gDNA I6 on MEF matrix, sample II	265 ng	27.43 (± 0.31)	33.46	BDL	>0.95
gDNA I3 on huFeeders	100 ng	BDL	NC	BDL	NC
gDNA I8 on huFeeders	100 ng	33.17 (± 0.34)	30.00	29.59 (± 0.66)	0.99
gDNA I6 on huFeeders	100 ng	36.09 (± 0.24)	32.87	32.59 (± 0.28)	0.99
gDNA H9 on huFeeders	100 ng	33.97 (± 0.30)	30.80	30.07 (± 0.79)	0.98

For all tested hESC samples, the ratio of actual C_{Tpol}/calculated C_{Tpol} was in the range of 1, supplying evidence for the absence of MuLV infection and MuLV replication in the tested hESCs. A ratio of approximately 1 is expected to result from contaminating MEF DNA. In the case of a productive infection of the human cells, the ratio of actual C_{Tpol}/calculated C_{Tpol} would be significantly lower than 1. Slope and y-intercept of the linear regression line of the corresponding dilution row of embryonic feeder cells were as follows: slope MuLV *pol* (a) = -2.63; slope CyOII (b) = -2.02; y-intercept MuLV *pol* (m) = 41.05; y-intercept CyOII (n) = 33.27; calculated $C_{Tpol} = (C_{TCyOII} - 33.27)\ (-2.63/-2.02) + 41.05 = (C_{TCyOII} - 33.27)\ 1.3 + 41.05$.

Abbreviations: BDL, below detection limit; gDNA, genomic DNA; hESC, human embryonic stem cell; MEF, mouse embryonic fibroblast; MuLV, murine leukemia virus; NC, not calculable.

C_{Tpol}/calculated C_{Tpol}] is significantly lower than 1, this is evidence for an infection of the tested cells (a lower [actual C_{Tpol}/calculated C_{Tpol}] corresponds with a higher number of MuLV *pol* template sequences, as to be expected for the amount of contaminating murine DNA).

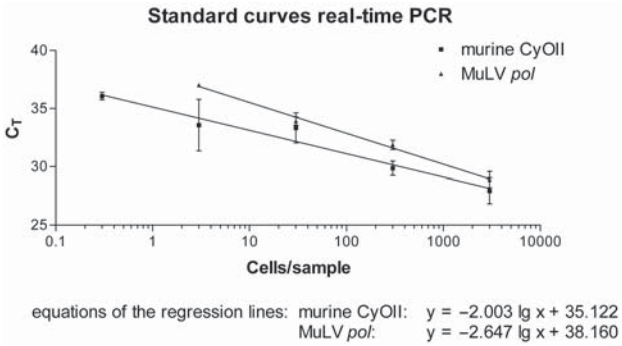

Figure 1. Standard curves of MuLV *pol*–specific and murine CyO-specific real-time PCRs. Typical graphs created from the threshold cycles of MuLV *pol*–specific and murine CyO-specific real-time PCRs. Threshold cycles (y-axis) are correlated to the number of cell equivalents serving as template in one PCR reaction on the x-axis. The resulting equations of the regression lines are depicted below the graph. Underlying values resulted from real-time PCR of a dilution row of mouse embryonic fibroblasts isolated from ICR mice. Abbreviations: CYO, cytochrome oxidase; MuLV, murine leukemia virus; PCR, polymerase chain reaction.

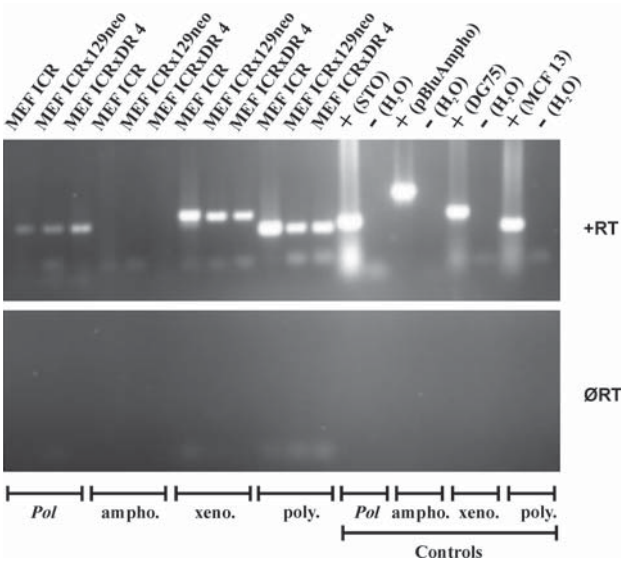

Figure 2. Xenotropic and polytropic MuLVs are expressed on the mRNA level in MEFs. Reverse transcription–polymerase chain reaction specific for xenotropic (xeno.) and polytropic (poly.) MuLV was performed on RNA of embryonic fibroblasts of different mouse strains frequently used to produce MEFs for human embryonic stem cell culture. pBlueAmpho, DG75 cells, and MCF13 cells were positive controls. Internal controls without RT excluded false-positive results due to contaminating genomic DNA. Abbreviations: ampho., amphotropic; MEF, mouse embryonic fibroblast; MuLV, murine leukemia virus; RT, reverse transcriptase.

RT-PCR–Based Assay for Reverse Transcriptase Activity in Cell Culture Supernatant of MEFs

Measurement of reverse transcriptase (RT) activity in the supernatant of MEFs was performed as described previously [39, 40].

Measurement of Reverse Transcriptase Activity in Cell Culture Supernatant of hESCs

Measurement of RT activity in the supernatant of hESCs was performed as described previously [41] using a commercial assay (Cavidi Tech AB, Uppsala, Sweden, http://www.cavidi.com) according to the manufacturer's instructions.

Results

MEF Cells Express Xenotropic and Polytropic MuLVs

RT-PCRs specific for MuLV *pol* as well as for xenotropic, polytropic, and amphotropic *env* genes of MuLVs were performed using total RNA of MEFs isolated from different mouse strains. Analysis of ecotropic MuLV expression has not been included, because those viruses are not able to infect human cells. Because most groups working with hESCs use feeder cells isolated from ICR mice or during genetic modification of embryonic stem (ES) cells, also from ICR mice crossbred with neomycin-resistant 129neo or fourfold-resistant DR4 mice, we analyzed those strains for MuLV expression. As depicted in Figure 2, strong RNA expression of xenotropic and polytropic MuLV was detected in MEFs, whereas no expression of amphotropic MuLV could be demonstrated. As expected, also no proviral sequences for amphotropic MuLV could be detected in the tested MEF cultures by PCR (data not shown).

Because cellular MuLV RNA expression does not necessarily imply release of viral particles, RNA was also prepared from cell culture supernatants. RT-PCR performed on these samples confirmed the results of RT-PCRs performed on cellular RNA (Fig. 3). Further evidence for release of MuLV particles was obtained by an enzymatic assay capable of detecting RT activity as a retroviral marker in cell culture supernatants. In all tested cell supernatants of cultured MEFs, RT activity was detected (Fig. 4).

Expression of Receptors for Xenotropic/Polytropic MuLVs on hESCs

Besides release of infectious virions, expression of specific receptors is a second prerequisite for productive MuLV infection of human cells in coculture settings. We have therefore analyzed mRNA expression of human receptors for xenotropic and polytropic MuLVs on human HEK293T cells as positive control and on several hESC lines. Human HEK293T cells expressed mRNA for the receptor for xenotropic/polytropic MuLV. A strong mRNA expression of this receptor was also found on all tested hESC lines (H9, I4, and I6) (Fig. 5).

MuLV Infection of HEK293T Cells and hESCs After Coculture with MLV-X

Because receptor expression is not ample evidence for susceptibility, infection experiments using the mink cell line MLV-X, which releases infectious xenotropic MuLV, were performed. To facilitate analysis, MLV-X was pretreated with mitomycin C. After initial culture with mitomycin-treated MLV-X, HEK293 cells were cultivated for an additional eight passages to eliminate MLV-X cells and DNA. In cases of hESCs, human cells were cultured on a 1:1 mixture of mitotically inactivated MEFs and MLV-X before being cultured for eight passages on human feeder cells, which was necessary to exclude false-positive results based on MEF/MLV-X contaminations. Eight passages after contact with MLV-X cells, MuLV proviral sequences could be demonstrated in HEK293 cells and in hESCs (Fig. 6). Moreover, robust MuLV mRNA expression was detected in hESCs after coculture with MLV-X, suggesting that in contrast to murine embryonic carcinoma cells [25–28], MuLV expression is not significantly repressed in hESCs on the transcription level (Fig. 6).

Coculture of HEK293T Cells with MEFs Does Not Result in MuLV Infection

Although productive MuLV infection of hESC could be demon-

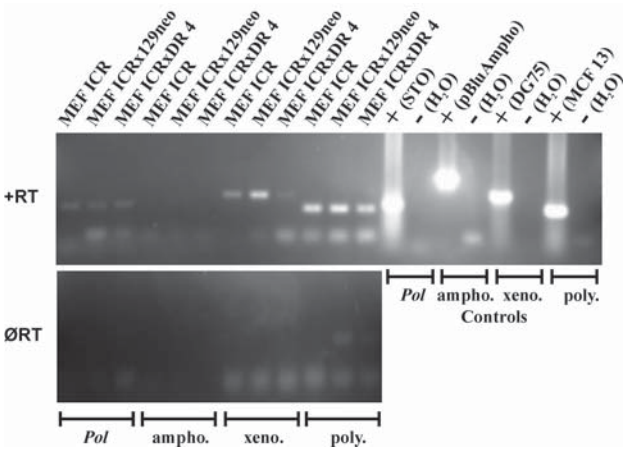

Figure 3. RNA of xenotropic and polytropic MuLV as a marker for MuLV particles can be detected in cell culture supernatants of MEFs. Reverse transcription–polymerase chain reaction specific for xenotropic (xeno.) and polytropic (poly.) MuLV was performed on RNA isolated from cell culture supernatants of embryonic fibroblasts of different mouse strains. pBlueAmpho, DG75 cells, and MCF13 cells were positive controls. Internal controls without RT excluded false-positive results due to contaminating genomic DNA. Abbreviations: ampho., amphotropic; MEF, mouse embryonic fibroblast; MuLV, murine leukemia virus; RT, reverse transcriptase.

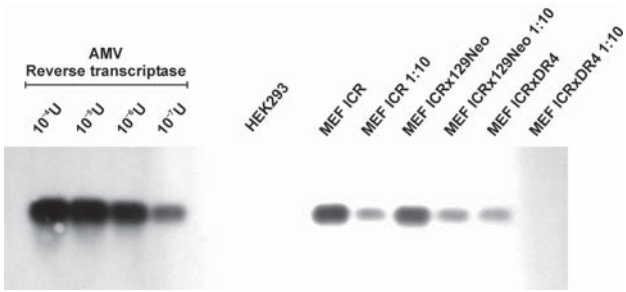

Figure 4. Detection of reverse transcriptase activity in cell culture supernatants of mouse embryonic fibroblasts (MEFs) as a marker for retroviral particles. Detection of reverse transcriptase activity was performed using a polymerase chain reaction–based reverse transcriptase assay. Avian myeloblastosis virus (AMV) reverse transcriptase was used as positive control; HEK293 cells were used as a negative control.

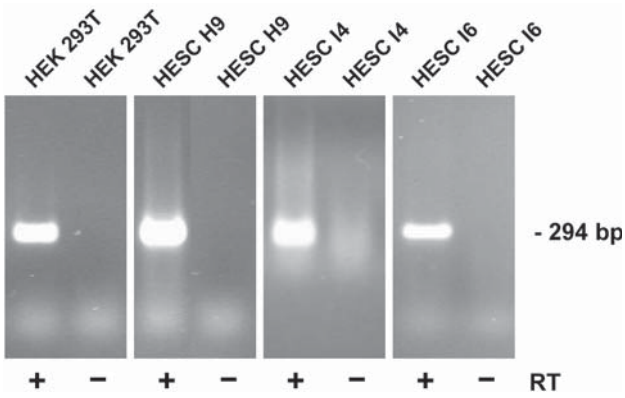

Figure 5. The receptor for xenotropic and polytropic MuLV is expressed on human HEK293T cells and hESCs. Total RNA of human HEK293 cells and different hESC lines was tested by reverse transcription–polymerase chain reaction specific for the human receptor for xenotropic and polytropic MuLV. Internal controls without RT excluded false-positive results due to contaminating genomic DNA. Abbreviations: hESC, human embryonic stem cell; MuLV, murine leukemia virus; RT, reverse transcriptase.

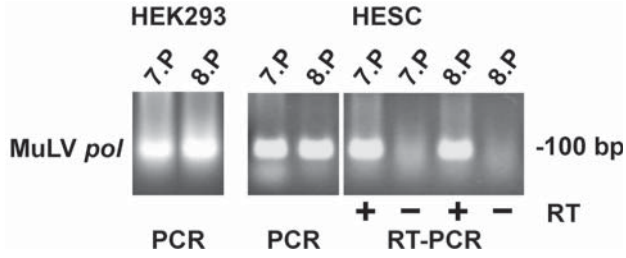

Figure 6. Productive infection of human HEK293 cells and hESCs after coculture with a mink cell line releasing infectious xenotropic MuLV. HEK293/hESCs, which had been cocultured for one passage with mitomycin-treated MLV-X, a mink cell line releasing infectious xenotropic MuLV, have been cultured for at least seven passages without further contact to mouse embryonic fibroblasts or MLV-X before preparation of genomic DNA or total RNA. Results of MuLV *pol*–specific PCR and, in case of hESCs, RT-PCR are depicted. Internal controls without RT excluded false-positive results due to contaminating genomic DNA. Abbreviations: hESC, human embryonic stem cell; MuLV, murine leukemia virus; RT-PCR, reverse transcriptase–polymerase chain reaction.

strated after coculture with MLV-X, and although in the culture supernatant of MEFs, MuLV-RNA and RT activity as markers for virion release could be demonstrated, only coculture with susceptible human cells can prove the release of significant titers of replication-competent humantropic MuLV. To investigate potential productive infection of susceptible human cells, mitomycin-treated MEFs were cocultured with human HEK293T (Fig. 7) as well as with HEK293 cells (data not shown). Cocultures were continued until no murine DNA could be detected (passage 7). Infection of the human cells by MEF-derived MuLV was not observed: MuLV proviral sequences and MuLV RNA were not detected at passage 7 or 8 (Fig. 7).

No Evidence for MuLV Infection of hESC Lines After Sustained Contact with MEFs

Four hESC lines were analyzed for proviral MuLV sequences by quantitative real-time PCR. To minimize contaminations with murine cells or murine DNA, not only hESCs on MEFs but also hESCs, which had been cultivated for at least five passages in the absence of MEFs, were analyzed. As demonstrated in Table 1, cultivation on MEF matrix, and even more on matrigel or on human feeders, indeed strongly reduced contaminating murine DNA, resulting in significantly increased threshold cycles during real-time PCR. All samples tested resulted in ratios of [actual $C_{T pol}$/calculated $C_{T pol}$] of approximately 1. For several samples, no ratio was obtained because the amount of MuLV *pol* template was below the detection limit. Provided that the actual $C_{T pol}$ was at least 31.84 for these samples, the obtained ratios would still be in a range of 1 or greater than 1. Because ratios of approximately 1 or greater than 1 exclude productive MuLV infection, our data clearly demonstrate that none of the tested hESC lines has been productively infected by MEF-derived MuLV. Further evidence for the absence of a productive MuLV infection has been obtained by analyzing MuLV expression and release in hESC cultures. Neither cellular MuLV RNA (Fig. 8) nor RT activity in culture supernatants (Fig. 9) was detected in the hESC lines I3, I6, I8, and H9 after several passages on human feeder cells.

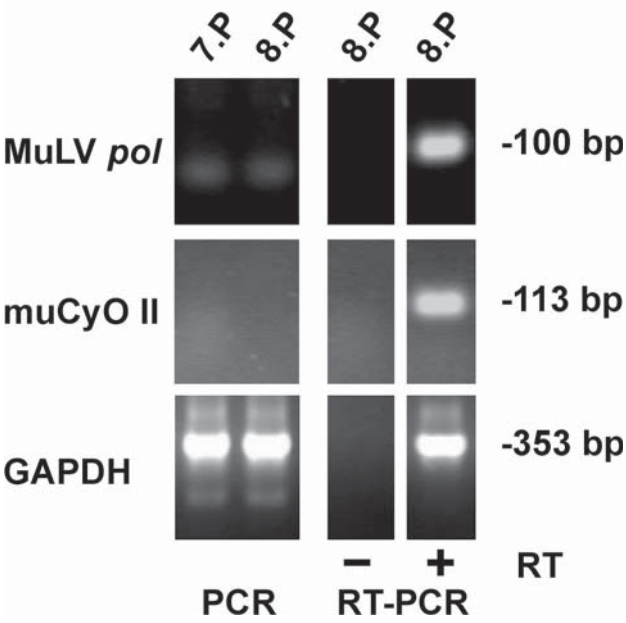

Figure 7. No infection of human HEK293T cells after coculture with MEFs. Mitomycin-treated MEFs were cocultured with human HEK293T cells. Cocultures were tested after seven or eight passages by MuLV *pol*–specific PCR/RT-PCR, which did not result in any evidence for MuLV infection. False-positive results due to contaminating murine DNA/RNA were excluded by murine mtDNA (CyO II)–specific PCR. Internal controls without RT excluded false-positive results due to contaminating genomic DNA. GAPDH was an internal positive control. MEFs served as positive control for all PCRs. Noninfected HEK293T cells were negative control. Faint bands visible in the upper left panel have a size below 100 bp and most likely represent surplus primers. PCR sensitivity: MuLV *pol*, detection of one MEF cell within 10^5 to 10^6 human cells; murine mtDNA: detection of one MEF cell within 10^6 to 10^7 human cells. Abbreviations: CyO, cytochrome oxidase; MEF, mouse embryonic fibroblast; MuLV, murine leukemia virus; RT-PCR, reverse transcriptase–polymerase chain reaction.

Discussion

hESCs may represent an outstanding cell source for future cell therapeutic applications. So far, most existing hESC lines have been established and continuously cultivated on MEF cells. At present, it is questionable whether those cell lines are suitable for clinical studies, because interspecies transmission of murine pathogens from MEFs to hESCs cannot be excluded. Of major importance may be the question of potential infection of hESCs by murine endogenous retroviruses. Although the risk of interspecies transmission of porcine endogenous retroviruses during xeno-

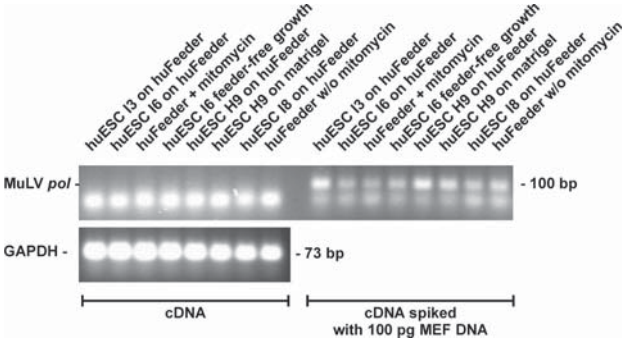

Figure 8. No evidence for productive MuLV infection of hESCs after sustained culture on murine feeder cells by MuLV *pol*–specific RT-PCR. RT-PCR specific for MuLV *pol* was performed on RNA of hESCs cultured for at least seven passages on human foreskin fibroblasts. The obtained results confirm the results of the quantitative real-time PCR assay. GAPDH was used as positive control. In parallel, all samples were spiked with MEF DNA for internal positive control. Abbreviations: hESC, human embryonic stem cell; MEF, mouse embryonic fibroblast; MuLV, murine leukemia virus; RT-PCR, reverse transcriptase–polymerase chain reaction.

transplantation has been addressed by a variety of studies [42], potential trans-species transmission of murine endogenous retroviruses from MEFs to hESC lines has not been investigated so far.

We have analyzed potential transmission of MuLV from feeder cells to hESCs. As a first step, MEFs derived from different mouse strains typically used to prepare feeder cells were analyzed for expression of humantropic MuLV strains. RT-PCR analyses demonstrated that all tested MEFs express polytropic and xenotropic MuLV. As expected, no amphotropic MuLV could be detected (Fig. 2). Evidence for release of virions carrying polytropic or xenotropic MuLV genomes was obtained by the detection of corresponding RNA as well as RT activity in cell culture supernatants (Figs. 3, 4).

The second prerequisite for infection of hESCs by MEF-derived xenotropic/polytropic virions is the expression of the specific receptor, which is believed to represent a phosphate transporter [31]. Indeed, mRNA expression of this receptor in different hESC lines was clearly visible (Fig. 5). Additional evidence for the susceptibility of hESCs for productive MuLV infection was obtained by coculture with mitotically inactivated MLV-X, a mink cell line known to release infectious titers of xenotropic MuLV. We were able to show the presence of MuLV provirus and robust MuLV mRNA expression in hESCs after up to eight passages on human feeder cells (Fig. 6).

Because the above data on MuLV expression and RT activity in MEF cultures do not prove the release of infectious titers of replication-competent humantropic virions, cocultures of ICR-derived MEFs and HEK293T cells, which express the receptor for xenotropic/polytropic MuLV (Fig. 5), and which are highly susceptible towards retroviral transduction in general, have been performed. For these experiments, experimental conditions were chosen that resulted in transmission of pig endogenous retroviruses from porcine cells to human HEK293 cells in previous experiments [36]. To mirror hESC culture as closely as possible, and to prevent further proliferation of the MEFs, mitomycin treatment was applied. After eight passages, contaminating MEFs or murine DNA had been eliminated from the cocultures as determined by murine CyOII-specific PCR. Despite at least 4–5 days of lasting intensive contact to murine feeder cells, no MuLV proviral sequences could be detected after eight passages, as depicted in Figure 7. Although we cannot exclude that single virions had entered the human cells, we can definitely exclude productive MuLV infection of the cocultured HEK293 cells. As described above, similar coculture experiments of HEK293T cells with MLV-X cells resulted in infection of 293 cells, proving that the design of the infection experiments was appropriate to allow virus transmission in general (Fig. 6).

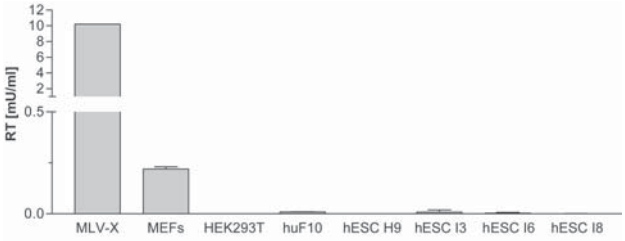

Figure 9. No release of RT activity in culture supernatants of hESCs. C-type RT activity in supernatants of hESCs, cultured for at least seven passages on human foreskin fibroblasts (huF10), has been determined using a commercial enzyme-linked immunoabsorbent assay–based assay. Culture supernatants of MEFs, HEK293T, and huF10 have been used as controls. Abbreviations: hESC, human embryonic stem cell; MEF, mouse embryonic fibroblast; RT, reverse transcriptase.

Finally, we investigated potential transmission of MEF-derived MuLV to different hESC lines. The analyzed hESCs had been in close contact to mitomycin-treated MEFs for a prolonged period compared with the human HEK293 cells. Because analysis of hESCs on MEFs was complicated by contaminating murine DNA, which contains a multitude of proviral MuLV elements, a novel real-time PCR-based assay has been developed. Simultaneous determination of the copy number of a mouse-specific gene and of a MuLV-specific sequence allowed us to discriminate whether detection of MuLV proviral sequences was based on amplification of the MuLV sequence from contaminating murine DNA only or also from infected hESCs. The mitochondrial CyOII gene was chosen as the murine target gene because, due to the high copy number within the cell, an even higher sensitivity than for the already very sensitive MuLV *pol*–specific PCR can be achieved.

If the human cells to be tested have not been infected by MuLV, the ratio of the copy number of MuLV template molecules/CyOII template molecules should be similar to the ratio obtained for MEFs, because amplification of both templates in the human cell sample is based on contaminating MEF-DNA only. In case of a MuLV infection of human cells, the ratio of the copy number of MuLV template molecules to CyOII template molecules should be clearly higher than determined for mouse feeder cells. Because it was not aim of the analysis to determine exact copy numbers of CyOII and MuLV *pol* templates, the ratios were calculated based not on absolute template numbers but on the corresponding threshold cycles, as described in Materials and Methods.

Overall, 14 cell samples of four different hESC lines were tested. As expected, mouse CyOII-specific real-time PCR demonstrated that ESC cultures on MEF were highly contaminated with murine DNA, whereas ESCs, which had been cultivated on MEF matrix, matrigel, or human feeders for at least five passages, contained much less mouse DNA (Table 1). In accordance with the cocultivation experiments using HEK293T cells, we did not obtain evidence for MuLV infection in any of the analyzed hESC samples (Table 1). Additional evidence for the absence of infection was obtained, because no cellular MuLV RNA expression (Fig. 8) or RT activity in culture supernatants of hESC was detectable after culture on human feeder cells (Fig. 9).

The fact that neither human HEK293 cells nor hESCs had been infected by MEF-derived MuLV and that productive infection of hESCs was achieved after coculture with MLV-X strongly argues against a specific inhibition of MuLV expression and replication in hESCs as the underlying reason, like previously described for murine ESCs and embryonic carcinoma cells [25–28]. Although our experiments supplied evidence for humantropic MuLV mRNA expression and virion release, it is much more likely that ICR-derived feeder cells do not release infectious and replication-competent humantropic virions, or at least not to a degree capable to infect and replicate.

Our study represents a first step towards an infection risk assessment on hESC lines isolated and/or grown on murine feeder cells. Although expression and release of xenotropic and polytropic MuLV from MEFs were demonstrated, and although hESCs have been shown susceptible towards MuLV infection in general, no transmission of MEF-derived MuLV to human HEK293 cells or to hESC lines could be detected. In conclusion, it can be assumed that the tested MEFs do not release infectious (and replication-competent) humantropic MuLV particles and that the titer of infectious (and replication-competent) humantropic MuLV is too low to result in productive viral infection.

Although it was not analyzed, whether other potentially pathogenic endogenous retrovirus-like elements, which are not humantropic or even not completely functional [16], can be transmitted to human cells via humantropic MuLV virions, this seems unlikely, especially if MEFs do not release MuLV virions able to enter the target cell. Nevertheless, further analysis of potential infection of hESCs by other murine pathogens (e.g., endogenous mouse mammary tumor viruses) will be necessary to assess whether NIH-registered hESC lines grown on murine feeder cells can be applied clinically without posing significant infection risks for the recipient.

Acknowledgments

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