C3H strain of mouse mammary tumour virus, like GR strain, infects human mammary epithelial cells, albeit less efficiently than murine mammary epithelial cells

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Mouse mammary tumour virus (MMTV) is a member of the genus Betaretrovirus, infects rodent cells and uses mouse tranferrin receptor 1 for cell entry. Several MMTV strains have been shown to productively infect, in addition to murine cells, various heterologous cell lines including those of human origin, albeit less efficiently than murine cells. Here, we analysed whether MMTV from C3H mice [MMTV(C3H)], reported previously to be incapable of infecting human cells, could productively infect human cells. Using a recently described high-titre MMTV-based vector carrying MMTV(C3H) envelope protein (Env), we successfully transduced cells of human origin. Furthermore, WT MMTV(C3H) was able to infect human cells, albeit less efficiently than mouse cells. The established infection was, however, sufficient to enable virus spread to every cell in culture. The infectivity of WT MMTV(C3H) and MMTV-based vectors carrying MMTV(C3H)Env was blocked by heat inactivation, an inhibitor of reverse transcription (3′-azido-3′-deoxythymidine) and pre-incubation with neutralizing anti-MMTV antibodies that did not neutralize vectors pseudotyped with amphotropic murine leukemia virus Env, providing evidence for an authentic, receptor-mediated and reverse transcriptase-dependent infection process. Persistently infected human Hs578T cells produced infectious virions capable of infecting naive human breast cells in culture, the infectivity of which could also be blocked by neutralizing anti-MMTV antibodies, demonstrating that virus particles released by the persistently infected Hs578T cells were related antigenically to the virus produced from murine cells. Taken together, our results show that MMTV(C3H), like MMTV(GR) and MMTV(RIII), is able not only to infect but also to replicate in cultured human breast cells.

INTRODUCTION

Virus cell entry requires the interaction of the viral envelope proteins with a specific cellular receptor(s), which determines the specificity of a virus for a particular host. Transferrin receptor 1 (TfR1), the cell-surface receptor for iron-loaded transferrin required for iron uptake into cells (Aisen, 2004), is known to serve as the primary cellular receptor for viruses belonging to three unrelated families: Paroviridae (canine and feline paroviruses), Arenaviridae (several rodent and human arenaviruses) and Retroviridae [the betaretrovirus mouse mammary tumour virus (MMTV)] (Abraham et al., 2009; Flanagan et al., 2008; Parker et al., 2001; Radoshitzky et al., 2008; Ross et al., 2002).

Canine parvovirus represents an important model for disease emergence in the wild. This virus emerged in dogs in the late 1970s when it was passed to dogs as a host-range variant of a feline parvovirus. Interestingly, it now appears that the transmission from cats to dogs and the subsequent emergence of the canine parvovirus was an indirect event involving passage through another carnivore species, racoon (Allison et al., 2012). The cross-species transmission was accompanied by the acquisition of only a few mutations that allowed efficient binding of the virus not only to the feline but also to the canine TfR1 orthologue (Hueffer et al., 2003).

New World clade B arenaviruses typically circulate in rodent species found in South America. Several of these viruses (e.g. Machupo virus, Junin virus, Guanarito virus and Sabia virus) are, however, emerging in human populations, where they cause hemorrhagic fever and lethal disease (Charrel & de Lamballerie, 2003; Choe et al., 2011). The ability to use the human TfR1 (hTfR1) in addition to their native rodent TfR1 correlates with their zoonotic potential (Choe et al., 2011). Whereas clade B arenaviruses that do not cause human disease (e.g. Amapari virus and Tacaribe virus) cannot utilize hTfR1 for cell entry, the...
pathogenic clade B arenaviruses effectively recognize the human orthologue, leading to more efficient infection of human cells and, in turn, zoonotic transmission (Abraham et al., 2009; Flanagan et al., 2008; Radoshitzky et al., 2008). Studies have shown that changes in as little as 1 aa of hTfR1 were sufficient for non-pathogenic arenaviruses to gain use of the receptor for cell entry (Abraham et al., 2009). Similarly, mutation of only a few amino acid residues enabled the conversion of the mouse TfR1 (mTfR1) to a compatible cell-entry receptor for pathogenic arenaviruses (Radoshitzky et al., 2008). At present, there is limited information about the determinants of receptor binding within the arenavirus entry glycoprotein, GP1. However, based on the aforementioned studies, it can be inferred that only minor changes in the receptor-binding domain of GP1 may dramatically change arenavirus tropism.

Thus, in both canine parvoviruses and New World clade B arenaviruses, the major cellular barrier preventing cross-species transmission and zoonosis appears to be the incompatibility of TfR1 with the viral TfR1-binding domain. The barrier is, however, rather weak, with only a small number of point mutations in either the viral TfR1-binding domain or TfR1 itself allowing expansion of virus host range. Indeed, reconstruction of TfR1 evolution has provided strong evidence for repeated cross-species transmission of paroviruses and arenaviruses and an ongoing host–virus arms race shaping their genomes (Demogines et al., 2013; Kaelber et al., 2012).

MMTV, the prototypic betaretrovirus causing mouse mammary epithelial cell tumours, also utilizes its host species TfR1 for cell entry (Ross et al., 2002). In the wild, the virus specifically infects rodents of the species Mus musculus. However, it appears that MMTV-like viruses once circulated more widely among rodents and other mammalian species. This belief is based on the presence of remnants of ancient infections, endogenous betaretroviral sequences, in the genomes of several mammalian species including rodents, bats, primates, ruminants and marsupials (Demogines et al., 2013; Hayward et al., 2013). More common occurrences of MMTV-like viruses can also be inferred from the recent observation that the amino acid residues corresponding to the MMTV envelope protein (Env)-binding site of mTfR1 were subjected to positive selection pressure (Demogines et al., 2013). This indicates that rodent species, currently devoid of infectious MMTV, have, during evolution, adapted (through the acquisition of mutations within TfR1) to block MMTV-like virus entry. Collectively, these findings suggest that betaretroviruses may be particularly adept at evading host defences and that the cross-species transmission of betaretroviruses has commonly occurred.

There have been conflicting reports regarding the ability of MMTV to infect human cells. Historically, this may stem from difficulties in working with the prototypic betaretrovirus due to the low infectivity observed for MMTV in cell culture, as well as the cumbersome construction of a functional molecular clone for the production of recombinant virus. Nevertheless, WT virus (i.e. harvested from tissue-culture fluids of MMTV-induced mouse mammary carcinoma cell lines or milk collected from nursing mice) originating from GR and RIII mice has been shown to productively infect, in addition to murine cells, various heterologous cell lines including those of human origin, albeit less efficiently than mouse cells (Crandell et al., 1973; Howard et al., 1977; Hsu et al., 2010; Indik et al., 2005a; Indik et al., 2007; Lasfargues et al., 1979). Importantly, the infectivity of both WT and egfp-marked virus was neutralized by specific anti-MMTV antiserum (Indik et al., 2005a; Indik et al., 2007). The infectivity of the egfp-marked MMTV virus was also abolished when Env was mutated, providing evidence for specific Env-mediated transfer of MMTV to the target cells (Indik et al., 2005a). Furthermore, the initially established infection of human Hs578T cells with WT MMTV(GR) virus was sufficient for virus spread, ultimately leading to the infection of every cell in culture (Indik et al., 2007).

In contrast to MMTV(GR) and MMTV(RIII), WT MMTV from C3H mice [MMTV(C3H)], has been reported to be incapable of infecting human cells, despite its relatively efficient binding to hTfR1 (Ross et al., 2002; Wang et al., 2008). Virus entry was reported to be blocked at the post-attachment phase, due to lack of internalization of MMTV-bound hTfR1 and subsequent trafficking to the late endosome, where membrane fusion occurs (Wang et al., 2008). Thus, it is reasonable to hypothesize that MMTV(C3H) may represent a host-range variant incapable of entering non-murine cells. Alternatively, the observed discrepancy between the various MMTV strains may be due to differences in the sensitivity of techniques used to detect virus infection. Most of the studies with MMTV(C3H) were performed using a surrogate infection/transduction system based on murine leukemia virus (MLV) cores pseudotyped with MMTV Env, which, in our hands, exhibits compromised infectivity compared with the WT MMTV(GR). It is thus conceivable that a lower efficiency of the MLV[MMTV Env] pseudoparticles was sufficient to establish infection of murine cells but was not sufficient for infection of the less-sensitive human cells. Here, we used our novel, highly sensitive MMTV-based vector production system (Konstantoulas & Indik, 2014) for transduction of cells of various origins, to determine whether MMTV(C3H) could infect heterologous cells. Additionally, we analysed whether MMTV(C3H) was capable of productively infecting human cells, and finally we directly compared the infectivity of MMTV(C3H) with that of MMTV(GR).

RESULTS

Infection/transduction of human cells with WT MMTV(C3H) and egfp-containing MMTV-based vectors carrying MMTV(C3H)Env

To investigate whether MMTV(C3H) infects non-murine cells, single-round transduction experiments were carried...
out initially using MMTV-based vector particles carrying MMTV Env (GR or C3H), ecotropic MLV Env or amphotropic 4070A MLV Env (generated using our recently described novel high-title MMTV-based vector production system (Konstantoulas & Indik, 2014)). Prior to transduction, virus particle titres were determined by real-time TaqMan reverse transcriptase (RT)-PCR targeting the egfp gene and normalized virus levels were used for the transduction of human (Hs578T) and murine (NMuMG) mammary epithelial cells.

As expected, the human cells were transduced with MMTV recombinant particles pseudotyped with the MLV 4070A Env (Fig. 1d) but not with the particles carrying the Env derived from the ecotropic MLV (Fig. 1e), which, however, successfully transduced cultured murine cells (Fig. 1a). In agreement with previously published data, Hs578T cells were susceptible to transduction with MMTV cores carrying the MMTV(GR) Env (MMTV[GR Env]) (Fig. 1b). Importantly, MMTV cores carrying the MMTV(C3H)Env (MMTV[C3H Env]) also successfully transduced Hs578T cells (Fig. 1c). The sensitivity being Hs578T cells to MMTV[GR Env] and MMTV[C3H Env] particles was only approximately two- to threefold lower than the sensitivity exhibited by NMuMG cells (Fig. 1a–c).

The transduction efficiency of the MMTV[C3H Env] particles on Hs578T cells was slightly reduced (~30% reduction) compared with the efficiency obtained with MMTV[GR Env] particles (Fig. 1b, c). The infectivity of both MMTV[C3H Env] and MMTV[GR Env] particles was completely blocked after pre-incubation with neutralizing goat anti-MMTV antibodies (Fig. 1b, c). In contrast, pre-treatment of the virions with non-specific serum had no effect on the infectivity of the WT viruses (Fig. 2c, d), confirming the specificity of the interaction between the virus and its cellular receptor. Heat inactivation of the virus eliminated the infection, as no PCR product was detected in Hs578T cells infected with heat-treated viruses (60 °C, 10 min) produced from either Mm5MT [MMTV(C3H)] (Fig. 2d) or GR [MMTV(GR)] (Fig. 2c) cells. Virus handled in a similar fashion without the heat inactivation retained its ability to infect target cells (Figs 2c, d), showing that proviral DNA transmission is temperature sensitive as expected for retroviral infection but not for gDNA transfer. Finally, the addition of AZT to the culture medium of the infected cells inhibited viral infection in a dose-dependent manner. Viral infection was completely abolished when ≥25 μM AZT was added to the culture medium of the infected cells, as illustrated by the absence of the MMTV-specific signal in PCR analysis of DNA from MMTV-infected Hs578T cells [MMTV(C3H)] (Fig. 2f) or [MMTV(GR)] (Fig. 2e).

Taken together, the results obtained with WT MMTV(C3H) and MMTV-based vectors carrying MMTV(C3H)Env demonstrated that MMTV(C3H), like MMTV(GR), has the capability to establish infection of human cells and that the infection is mediated by MMTV Env. The observation that the infectivity of both the WT virus and the vector was blocked by heat treatment, an inhibitor of reverse transcription (AZT), and neutralizing anti-MMTV antibodies (which did not show a non-specific antiviral activity; the infectivity of vectors carrying heterologous Env were not affected), together with the fact that virus harvested from supernatants of producer cells was filtered (0.45 μm) prior to infection, provide several lines of evidence supporting the concept of authentic infection rather than non-specific events, such as carry-over of proviral DNA from the producer cells, infection with virions pseudotyped with envelopes from other viruses, transfer of producer cells or non-specific virus uptake. Moreover, the infected cells were unequivocally of human origin (Fig. S1, available in the online Supplementary Material). Integration sites hosting MMTV(C3H) provirus, determined using a ligation-mediated (LM)-PCR protocol optimized for the amplification of virus-host junction sequences (Indik et al., 2007), were clearly identified as human sequences and could be
MMTV(C3H) infects human mammary epithelial cells

Fig. 1. Representative example of FACS analysis of MMTV–EGFP-transduced cells. NMuMG (a) and Hs578T (b–e) cells were transduced with MMTV vectors carrying MMTV(GR)Env (b), MMTV(C3H)Env (c), amphotropic 4070A MLV Env (d) or ecotropic MLV Env (e). For neutralization, virus was pre-incubated (4 °C, 30 min) with either anti-MMTV neutralizing antibody or non-specific serum (diluted 1 : 500). For heat inactivation, virus was incubated at 60 °C for 10 min. For 3′-azido-2′-deoxythymidine (AZT) treatment, AZT (37 μM) was added to the culture medium of transduced Hs578T cells. Transduction of target cells was detected by FACS analysis 3 days post-infection. The experiments were performed at least three times with similar results.
unambiguously mapped (≥ 99.8% nucleotide identity starting ≤ 1 nt from the proviral end) to loci in the human genome (Table 1), providing further evidence of infection of human cells.

**MMTV(C3H) productively infects human breast cells in culture**

The ability of MMTV(C3H) to replicate in cultured human mammary cells (Hs578T) was addressed in subsequent time-course experiments. MMTV(GR) was used as a positive control (Indik et al., 2007). Prior to infection, virus particle titres were determined by real-time TaqMan RT-PCR targeting the 5’ end of env, and normalized virus levels were used for infection of human cells. Quantification of proviral DNA at 1 week post-infection (p.i.), using real-time TaqMan PCR, showed approximately equivalent proviral loads in the infected cells. Next, the cells were cultured in the presence or absence of dexamethasone (DEX; 10^-6 M) and gDNA was extracted at regular time points. We reasoned that if human cells are able to support MMTV(C3H) replication, increasing levels of proviral DNA should be observed upon cultivation of these cells with DEX. As expected, increasing levels of proviral DNA were detected within Hs578T cells infected with MMTV(GR) over time (Fig. 3a, e), as assessed by semi-quantitative (targeting the

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**Fig. 2.** Infection of human cells with WT MMTV(C3H). (a, b) Genomic DNA was isolated from target cells (HEK293, Hs578T, HeLa and CrFK) infected with either MMTV(GR) (a) or MMTV(C3H) (b) at 1 week post-infection and analysed by PCR for the presence of MMTV sequences. (c, d) For neutralization, virus [MMTV(GR) (c) or MMTV(C3H) (d)] was pre-incubated (4 °C, 30 min) with either anti-MMTV neutralizing antibody or non-specific serum (1:500). For heat inactivation, virus [MMTV(GR) (e) or MMTV(C3H) (f)] was incubated at 60 °C for 10 min. (e, f) For AZT treatment, increasing amounts of AZT (10–50 μM) were added to the culture medium of infected Hs578T cells. –ve, Non-transduced Hs578T cells; +ve, producer cells. Equal DNA loading was controlled using glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific primers (bottom panels). The experiments were performed three times with similar results; a representative experiment is shown in each panel. Ab, antibody.
MMTV LTR– gag region and quantitative (targeting the 5’ end of the env-encoding region) PCR. Importantly, an increase in proviral DNA levels was also detected for cells infected with MMTV(C3H) (Fig. 3c, f). This increase was, however, observed only when cells were cultivated with DEX (Fig. 3c–f). Virus replication in human cells was further demonstrated by increased viral RNA (vRNA) levels at later cultivation time points in the culture supernatants of cells cultured in the DEX-containing medium (Fig. 3i, j).

Interestingly, although similar levels of proviral DNA were detected at early cultivation time points in cells infected with the two viral strains, a delayed increase in proviral copy number was observed for MMTV(C3H) (Fig. 3a, b). Based on the quantitative PCR data, it can be inferred that all Hs578T cells infected with MMTV(GR) carried at least one copy of the proviral DNA (Fig. 3f). Moreover, MMTV(C3H)-infected Hs578T cells needed to be cultured for at least 80 days before all cells carried at least one copy of the proviral DNA (Fig. 3f).

Hs578T cells infected with MMTV(C3H) or MMTV(GR) were subsequently cultured in DEX-containing medium (10–6 M) in the presence or absence of AZT (37 μM), to determine whether the increased proviral DNA and vRNA levels were due to ongoing retrovirus replication, which depends on reverse transcription. Importantly, no noticeable increase in proviral DNA levels was detected in cells cultured in the AZT-supplemented medium (Fig. 4). Thus, the elevated proviral copy numbers at later time points were due to ongoing replication of the betaretroviruses rather than a clonal expansion of initially infected cells.

To determine whether the infected cells expressed MMTV proteins and to quantify the infected cells by a non-PCR-based technique, indirect immunofluorescence staining with monospecific anti-MMTV-CA polyclonal serum was performed. A noticeable difference in the numbers of MMTV capsid (CA) protein-expressing cells was detected in the time-course experiment (Fig. 5). Whereas only a small number of MMTV-positive cells were detected in Hs578T cells shortly (1 week) after infection with MMTV(GR) (Fig. 5b), by week 6 the majority of cells expressed MMTV antigen (Fig. 5d). For Hs578T cells infected with MMTV(C3H), again only a small number of MMTV-positive cells were observed shortly after infection (Fig. 5f).

In contrast to MMTV(GR)-infected Hs578T cells, and similar to what was observed with the quantitative PCR data, only a modest proportion of the MMTV(C3H)-infected cells expressed the MMTV antigen by week 6 (Fig. 5h). The majority of MMTV(C3H)-infected Hs578T cells did, however, express the MMTV CA protein by week 14 (Fig. 5j). The increase was strictly DEX dependent, as no increase in the number of CA-positive cells could be observed upon cultivation of cells in DEX-free medium (Fig. 5c, g, i). These data demonstrated that the infected cells expressed viral structural proteins in a DEX-dependent manner and further support our quantitative PCR results showing that, at later cultivation times, all human cells became infected with MMTV(C3H).

### Persistently infected human cells produce infectious MMTV(C3H) particles

To ascertain whether human cells persistently infected with MMTV(C3H) (15 weeks p.i.) or MMTV(GR) (6 weeks p.i.) produced infectious particles, the filtered supernatants obtained from these cells, after stimulation with DEX, were applied onto naïve HEK293, Hs578T, HeLa and CrFK cells. The DNA from these cells was subjected to the MMTV-LTR– gag region and quantitative (targeting the 5’ end of the env-encoding region) PCR. Importantly, an increase in proviral DNA levels was also detected for cells infected with MMTV(C3H) (Fig. 3c, f). This increase was, however, observed only when cells were cultivated with DEX (Fig. 3c–f). Virus replication in human cells was further demonstrated by increased viral RNA (vRNA) levels at later cultivation time points in the culture supernatants of cells cultured in the DEX-containing medium (Fig. 3i, j).

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MMTV(C3H) infects human mammary epithelial cells

**DISCUSSION**

The data presented here demonstrate that MMTV(C3H) is not a host-range variant of MMTV incapable of infecting human cells. Using both WT MMTV(C3H) and MMTV-based vectors carrying MMTV(C3H)Env, we established infection of human mammary epithelial cells, HS578T, previously shown to be susceptible to infection with MMTV(GR) (Indik et al., 2005a; Indik et al., 2007). In addition to establishing infection of human cells, MMTV(C3H) was, like MMTV(GR), also able to replicate in human HS578T cells. Of note, differences in the kinetics of productive infection between MMTV(C3H) and MMTV(GR) were observed. Whereas de novo MMTV(GR) was detected in supernatant fluids as soon as 14 days p.i., the appearance of de novo MMTV(C3H) was delayed to 35 days p.i. The delayed replication kinetics of MMTV(C3H) cannot be explained by a lower M.O.I. for MMTV(C3H), as RNA viral loads and infectious titres were determined in a single-round infection experiment (using quantitative PCR to quantify proviral DNA loads) prior to the time-course experiments. However, the quantitative PCR employed cannot distinguish between exogenous and endogenous MMTV sequences that may also be packaged into virions. Thus, it is conceivable that endogenous Mtv-1, Mtv-6, Mtv-8, Mtv-11 and Mtv-14 RNAs, which have been reported to be expressed in mammary glands of C3H/HeN mice (Kozak et al., 1987), may be co-packaged with the exogenous MMTV(C3H) RNA. Recombination occurring during reverse transcription may result in the formation of a crippled recombinant virus incapable of replication. Sequencing of several MMTV(C3H) proviruses did not reveal the presence of such recombinants in infected cells. Alternatively, the observed differences in replication kinetics may be due to the genetic make-up of the two betaretrovirus strains. Of note, differences in the occurrence of MMTV-induced tumours have been described previously for these two viral strains. In GR mice, MMTV infection leads to mammary tumour formation with a high incidence (>90%) and at an early age (Bentvelzen et al., 1970). In contrast, mammary tumours induced in MMTV-infected C3H/HeN mice have a longer latency period and a moderate incidence (Golovkina et al., 1993). Obviously, although other factors such as the host genetic background, in addition to the genetic make-up of the MMTVs, account for the differences in the in vivo infection progression and incidence of mammary tumours, the faster replication kinetics may also be, in part, an intrinsic property of the exogenous betaretrovirus present in the GR mice (Held et al., 1993; Hook et al., 2000; Janeway, 1991; Marrack et al., 1991).

MMTV, like parvoviruses and New World clade B arenaviruses, uses the TR1 orthologue of its host species to mediate cell entry (Abraham et al., 2009; Flanagan et al., 2008; Parker et al., 2001; Radoszitzky et al., 2008; Ross et al., 2002). Both canine parvoviruses and New World clade B arenaviruses can easily adapt to use the TR1 of new species, resulting in the cross-species transmission of these two unrelated virus families (Charrel & de Lamballerie, 2003; Choe et al., 2011; Hueffer et al., 2003). In the case of MMTV, previous studies suggest that MMTV is incapable of utilizing the TR1 of other species to establish infection.

**Fig. 3.** Quantification of proviral DNA and viral RNA in cell lysates of MMTV-infected human HS578T cells during a time-course experiment. (a–d) HS578T cells infected with either MMTV(GR) (a, c) or MMTV(C3H) (b, d) were cultured in the presence or absence of 10^{-6} M DEX. gDNA was extracted from infected cells at the indicated time points and semi-quantitative PCR performed. −, non-transduced HS578T cells; +, producer cells. Equal DNA loading was controlled using GAPDH-specific primers (bottom panels). (e–h) Real-time TaqMan PCR was performed for an accurate quantification of proviral loads in HS578T cells infected with MMTV(GR) (e) or MMTV(C3H) (f). Equal loading of the PCRs were quantified in a real-time TaqMan PCR specific for the APOB gene (g, h). (i, j) Viral RNA in cell-culture fluids of HS578T cells infected with MMTV(GR) (i) or MMTV(C3H) (j), grown in the presence or absence of 10^{-6} M DEX, was quantified by real-time RT-PCR.
(a) Hs578T+MMTV(Gr) (DEX+)
Day: 0 7 14 21 28 35 42 45 - +

(b) Hs578T+MMTV(C3H) (DEX+)
Day: 0 7 14 21 28 35 42 45 - +

(c) Hs578T+MMTV(Gr) (DEX+ AZT+)
Day: 0 7 14 21 28 35 42 45 - +

(d) Hs578T+MMTV(C3H) (DEX+ AZT+)
Day: 0 7 14 21 28 35 42 45 - +

(e) MMTV(Gr)
DNA copy number

(f) MMTV(C3H)
DNA copy number

(g) DEX +
DEX + AZT +

(h) DEX +
DEX + AZT +
The presence of endogenous betaretroviral sequences in mammalian hosts of wide geographical and evolutionary diversity (Demogines et al., 2013; Hayward et al., 2013), and the observation that the parts of the rodent Tfr1 that interact with the MMTV Env are under positive selection pressure (Demogines et al., 2013), however, suggests that cross-species transmission of betaretroviruses has commonly occurred. It is thus plausible that MMTV may adapt to use Tfr1 from non-murine species or may use other receptors or co-receptors to establish infection of heterologous cells. The concept of an alternative receptor is further supported by the observation that feline Tfr1, derived from the most susceptible cell line, CrFK, does not make cells susceptible to MMTV infection (Wang et al., 2006). Therefore, feline cells must express another molecule, different from Tfr1, that enables the virus to enter the cytoplasm very efficiently. Work to determine whether hTfr1 plays a role in MMTV-mediated infection of human cells is currently ongoing. Of note, non-pathogenic New World arenavirus strains are able to enter human and rat cells relatively efficiently, despite their inability to bind human or rat Tfr1, suggesting that non-pathogenic and possibly other clade B arenaviruses may also use more than one receptor for cell entry (Abraham et al., 2009; Flanagan et al., 2008). An alternative receptor may substitute for hTfr1; alternatively, it may function downstream of Tfr1 in manner analogous to the human immunodeficiency virus type 1 co-receptors, CCR5 and CXCR4.

There have been reports that continued passage of MMTV in human mammary carcinoma cell lines (BT474 and

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**Fig. 4.** Quantification of proviral DNA in cell lysates of MMTV-infected human Hs578T cells during AZT treatment. (a–d) Hs578T cells infected with MMTV(GR) (a, c) or MMTV(C3H) (b, d) were cultured for 7 weeks in medium containing 10^{-6} M DEX supplemented with AZT (37 μM) or not. gDNA was extracted from infected cells at the indicated time points and semi-quantitative PCR performed. −, Non-transduced Hs578T cells; +, producer cells. Equal DNA loading was controlled using GAPDH-specific primers (bottom panels). (e–h) Real-time TaqMan PCR was performed for an accurate quantification of proviral loads in Hs578T cells infected with MMTV(GR) (e) or MMTV(C3H) (f). Equal loading of the PCRs were quantified in a real-time TaqMan PCR specific for the APOB gene (g, h).

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**Fig. 5.** Immunofluorescence imaging of MMTV-infected human Hs578T cells. The expression of CA proteins in Hs578T cells infected with either MMTV(GR) (a–d) or MMTV(C3H) (e–j), during the time-course experiment, was visualized by immunofluorescence staining using a monospecific anti-CA serum. All cells were grown in medium supplemented with 10^{-6} M DEX 24 h prior to immunostaining.
MCF-7) results in an adapted virus that can infect human cells (Howard & Schlom, 1978; Lasfargues et al., 1979). Similarly, the serial passage of arenaviruses in cell lines or animals results in virus adaptation that gives the virus the capacity to infect cells, animals and tissues of different origins (reviewed by Zapata & Salvato, 2013). Finally, the passage of feline panleukopenia virus in racoons resulted in adapted virus that was capable of infecting dogs, in addition to cats, ultimately leading to the emergence of canine parvovirus type 2a (Allison et al., 2012). It would thus be interesting to see if the continued passage of MMTV(C3H) in human Hs578T cells allows the virus to adapt through, for example, alterations in the viral glycoprotein that optimize the interaction with its cellular receptor(s) (TfR1 or otherwise), allowing more efficient infection of human cells.

In conclusion, we have consolidated and expanded our previous findings by showing that MMTV(C3H), like MMTV(GR), has the potential to infect and replicate in cultured human breast cells. This observation, together with the presence of endogenous betaretroviral sequences in mammalian genomes and the finding that the parts of the rodent TfR1 that interact with the MMTV Env are under positive selection pressure, suggests that cross-species transmission of betaretroviruses may be possible.

METHODS

Cell culture. CrFK, HeLa, Hek293T, Hs578T, CHO, GR and Mm5MT (ATCC CRL-1637) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. All cultured cells were maintained at 37 °C in humidified air containing 5% CO2.

Plasmid construction. The construction of the packaging construct (pCMgpRRE17), the EGFP-labelled MMTV vector plasmid (pRRpCeGFPWPRE25) and the Rev expression construct (pLP2) has been outlined previously (Konstantoulas & Indik, 2014). Likewise, the ecotropic MLVEnv-encoding plasmid (pHCMV-EcoEnv; Addgene 15802) (Sena-Esteves et al., 2004), the amphotropic MLVEnv-encoding plasmid (pAlf) (Cosset et al., 1995) and the MMTV(GR)Env-encoding plasmid [pEnv(GR)] (Indik et al., 2005b; Müllner et al., 2008) have all been described previously. pEnv(GR) shows 99.85% nucleotide identity to WT MMTV(GR)env sequences identified in virus preparations from GR (an MMTV-induced murine mammary adenocarcinoma cell line derived from the GR mouse strain; Ringold et al., 1975) cell-culture fluids (Fig. S3). The MMTV(C3H)Env-encoding plasmid [pEnv(C3H)] was generated by high-fidelity PCR amplification of the complete env-encoding segment with primers 6684_F

Fig. 6. Infection of target cells with virus collected from persistently infected human Hs578T cells. (a, b) The cell-culture supernatant from Hs578T cells persistently infected with either MMTV(GR) (a) or MMTV(C3H) (b) was used to infected naïve target cells, following a 24 h stimulation with 10−6 M DEX. gDNA was isolated from infected cells at 1 week p.i. and analysed by PCR for the presence of MMTV sequences. (c, d) For neutralization, virus was pre-incubated (4 °C, 30 min) with either anti-MMTV neutralizing antibody or non-specific serum (diluted 1:500). For heat inactivation, virus was incubated at 60 °C for 10 min. Where indicated, AZT (37 μM) was added to the culture medium of infected Hs578T cells. –ve, Non-transduced Hs578T cells; +ve, producer cells. Equal DNA loading was controlled in the PCR assay using GAPDH-specific primers (bottom panels). The experiments were performed three times with similar results; a representative experiment is shown.
(5'-ATGCCGAAAACACCATCTG-3') and 8649-R (5'-GGAGTTCCGGCATCTTGAGTTCC-3') (position coordinates according to GenBank accession no. M15122). vRNA isolated from filtered supernatants of Mm5MT cells (an MMTV-induced murine mammary carcinoma cell line derived from the C3H/HeN mouse strain; Owens & Hackett, 1972) was used as template (see below). The resulting PCR fragments were digested with AcIII and Bgl and cloned into the corresponding sites of the pEnv(GR) vector using T4 DNA ligase (Fermentas). The env gene in pEnv(C3H) shows 99.95% nucleotide identity to WT MMTV(C3H)env sequences identified in virus preparations from Mm5MT cells and is 98.4% identical to env in pEnv(GR) (Figs S4 and S5).

Pseudovirus production and transduction. EGFP-containing MMTV recombinant virions carrying MMTV Env, ecotropic MLV Env or amphotropic MLV Env were prepared in HEK293T cells and used for transduction of target cells. Cells (7.5 x 10^5 per well in a six-well plate) were co-transfected with pCMgpRRE17, pRRpCeGFPPWPRE25, pLP2 and either pEnv(GR), pEnv(C3H), pHCMV-EcoEnv or pAIF. Transfections were performed using the calcium phosphate method. At 36 h post-transfection, the virus-containing supernatants were harvested and filtered (0.45 µm filter; Sarstedt). For transduction, target cells were seeded at a density of 5 x 10^4 cells per well in a six-well plate, 1 day before transduction. The filtered virus supernatant was added to cells together with polybrene (8 µg ml^-1) and replaced with fresh medium after 2 h.

Cell-free infection. Filtered (0.45 µm filter; Sarstedt) supernatants from DEX (10^{-7} M; Sigma)-stimulated GR cells [producing infectious MMTV(GR) particles] and Mm5MT cells [producing infectious MMTV(C3H) particles] were used to infect target cells seeded at a concentration of 5 x 10^4 cells per well in a six-well plate, 1 day before infection. The filtered virus supernatant was added to cells together with polybrene (8 µg ml^-1) and replaced with fresh medium after 2 h. For single-round infection experiments, infected cells were cultured for 1 week. For time-course experiments, infected HeLa578T cells were further cultured in cell-culture medium supplemented with DEX (10^{-6} M) for 7–16 weeks. Six weeks after the initial infection, persistently infected human HeLa578T cells were stimulated with 10^{-6} M DEX and, 24 h after induction, the filtered supernatants used to infect naive, uninfected target cells. These second-round infected cells were cultured for 1 week.

Neutralization of viral infectivity, heat inactivation and AZT treatment. Neutralization of viral infectivity, heat inactivation and AZT treatment assays were performed as described previously (Indik et al., 2007).

Antibody staining. The expression of MMTV structural proteins in infected HeLa578T cells was determined by indirect immunofluorescence staining as described previously (Indik et al., 2007).

Semi-quantitative PCR. gDNA was isolated from cells using a Qiagen DNeasy Blood and Tissue kit (Qiagen). The presence of MMTV proviral DNA in infected cells was detected by PCR using MMTV(GR)-specific (9877_R, 5'-TCAGCACTCTTTATATTATGG-3') or MMTV(C3H)-specific (9877_R, 5'-TCAGCACTCTTTATATTATGG-3') primer and Superscript II reverse transcriptase (Invitrogen). gDNA and cDNA were subsequently used for a long-template PCR (Expand Long Template PCR system; Roche), using primers 7222_F (5'-GGCCGTAACCCTACCAAGAGGCTTCCGATC-3') and 9761_R (5'-ATAAGATTCCGGCCGGCTTCAGAAGGCCGCTCAGT-3'), modified to introduce restriction sites for Accl61 and NolI (underlined). The resulting PCR fragments were cloned into the corresponding sites of the pcDNA3 vector (Invitrogen) using T4 DNA ligase (Fermentas) and sequenced using overlapping primers available from the authors upon request. All position coordinates are according to GenBank accession no. M15122.

Quantitative RT-PCR. vRNA was isolated from filtered supernatants using a QIAamp vRNA kit (Qiagen). vRNA (500 ng) was used in a one-step RT-PCR using a Power SYBR Green RNA-to-Ct 1-Step kit (Applied Biosystems) together with primers targeting the MMTV env-encoding region: MMTV_5L (5'-CCAGATCGCCTTAAGAGAGA-3') and MMTV_3L (5'-TACAGGTAGGACGAC-3') to quantify vRNA in supernatants of cells infected with WT virus or the egfp gene: EGFP_F (5'-GACCTGCTTACCCGCTCA-3') and EGFP_R (5'-AAAGAAGTGGTCGCCCTCTG-3') to quantify vRNA in supernatants of cells transduced with egfp-containing MMTV-based vectors. Cycling conditions consisted of 48 °C for 30 min, followed by 45 cycles of 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min. A dissociation curve consisting of 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s was performed after the RT-PCR to detect non-specific amplification. RNA standards were prepared by in vitro transcription of either pcCMVenv (Indik et al., 2005b) or pcCMV-GFP (Cell Biolabs) plasmid, linearized by Xbal digestion, using T7 RNA polymerase (Invitrogen). Prior to use in the one-step RT-PCR, the vRNA was treated with DNase I (TURBO DNA-free kit; Ambion).

Integration site detection. gDNA was isolated from HeLa578T cells 2 weeks after infection with virus harvested from persistently infected HeLa578T cells [MMTV(GR)hp1 or MMTV(C3H)hp1] and filtered producer cell (GR or MMSMT) supernatants, respectively, using TRI Reagent (Sigma). The integration of MMTV provirus in infected human breast cells was determined by LM-PCR as described previously (Indik et al., 2007).

MMTV virus and provirus amplification and sequencing. gDNA and vRNA were isolated from HeLa578T cells 2 weeks after infection with virus harvested from persistently infected HeLa578T cells [MMTV(GR)hp1 or MMTV(C3H)hp1] and filtered producer cell (GR or MMSMT) supernatants, respectively, using TRI Reagent. For cDNA synthesis, equivalent amounts of RNA treated with DNase I (TURBO DNA-free kit) were reverse transcribed using an MMTV(GR)-specific (9877_R, 5'-TCAGCACTCTTTATATTATGG-3') or MMTV(C3H)-specific (9877_R, 5'-TCAGCACTCTTTATATTATGG-3') primer and Superscript II reverse transcriptase (Invitrogen). gDNA and cDNA were subsequently used for a long-template PCR (Expand Long Template PCR system; Roche), using primers 7222_F (5'-GGCCGTAACCCTACCAAGAGGCTTCCGATC-3') and 9761_R (5'-ATAAGATTCCGGCCGGCTTCAGAAGGCCGCTCAGT-3'), modified to introduce restriction sites for Accl61 and NolI (underlined). The resulting PCR fragments were cloned into the corresponding sites of the pcDNA3 vector (Invitrogen) using T4 DNA ligase (Fermentas) and sequenced using overlapping primers available from the authors upon request. All position coordinates are according to GenBank accession no. M15122.

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evolution of canine parvovirus resulted from virus-specific binding to
entry pathways, and glycoproteins from human pathogenic strains are
can use transferrin receptor 1 (TfR1)-dependent and -independent
insertion elements and transitions in cloned mouse mammary tumour virus DNA: further delineation of the poison