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Mouse tetherin enhances moloney murine leukemia virus-induced syncytium formation

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Summary. – Tetherin (also referred to as BST-2 or CD317) is an antiviral cellular restriction factor that inhibits the release of many enveloped viruses. It is a 30–36 kDa type II transmembrane protein, expression of which is induced by type I interferon. Mouse tetherin inhibits nascent cell-free particle release. However, it is unclear whether mouse tetherin restricts cell-to-cell spread of moloney murine leukemia virus (Mo-MLV) or whether is the mouse tetherin involved in syncytium formation. To examine cell-to-cell spread and syncytium formation of Mo-MLV in the presence or absence of mouse tetherin, R peptide (the cytoplasmic tail of the transmembrane protein (TM); 16 amino acids) truncated Env expressing vector was constructed. It contained enhanced green fluorescent protein (EGFP) in the proline rich region (PRR) of Env. This R(-)Env full-length molecular clone could rule out virus-cell transmission due to the slightly reduced R(-)Env protein incorporation into the viral particles. When NIH3T3 cells stably expressing mouse tetherin were transfected with R(-)Env full-length molecular clone, syncytium formation was significantly enhanced in the tetherin-expressing cells. These data suggest that tetherin-mediated retention of R-defective virions on the cell surface could enhance syncytium formation. In addition, we found that the R(-)Env full-length molecular clone containing EGFP in the PRR of Env to be a useful tool allowing fast and convenient detection of syncytia by fluorescence microscopy.

Keywords: cell-to-cell transmission; R(-)Env full-length clone; syncytium; tetherin

Introduction

For the retrovirus infection, the virus needs to deliver its genome into the cell either by fusion of the viral membrane with the plasma membrane, or by fusion with the endosome membrane after endocytosis (Jones and Risser, 1993). In addition to allowance of viral entry, the fusion mediated by viral surface proteins can cause cell-to-cell fusion, resulting in the formation of multinucleate giant cells (syncytia) (Chung *et al*., 1999; Park *et al*., 1994). Syncytium formation is thought to arise by at least two routes. A single virion can simultaneously fuse with two cells (fusion from without), or an infected cell expressing viral envelope (Env) glycoprotein on its cell surface can fuse with an adjacent cell (fusion from within) (Jung and Kozak, 2003). The envelope protein of the ecotropic moloney murine leukemia virus (Mo-MLV) is synthesized as a gp85 precursor which is then proteolytically cleaved into an extracellular surface unit (SU) and the transmembrane protein (TM). The cytoplasmic tail (16 amino acids; R peptide) of the TM protein is further cleaved by the viral protease during virion maturation. Unlike wild-type Env protein bearing the R peptide, the R-peptide-truncated Env induces syncytia in susceptible cells (Kubo *et al*., 2007; Ragheb and Anderson, 1994; Rein *et al*., 1994).

Tetherin was first identified as a cellular restriction factor that blocks the release of human immunodeficiency virus (HIV) (Liberatore and Bieniasz, 2011; Neil *et al*., 2008; Perez-Caballero *et al*., 2009). Several viruses have evolved viral-encoded antagonists to overcome the restrictions imposed by tetherin. HIV-1 Vpu, HIV-2 Env, SIV-Nef, KSHV

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Abbreviations: Env = envelope; EGFP = enhanced green fluorescent protein; HIV = human immunodeficiency virus; MLV = murine leukemia virus; Mo-MLV = moloney MLV virus; PRR = proline rich region; TM = transmembrane protein; VS = virological synapses

K5, and Ebola GP are examples of such antagonists (Jia *et al*., 2009, Kaletsky *et al*., 2009, Kirchhoff, 2010, Mansouri *et al*., 2009). However, gammaretroviruses (murine leukemia virus (MLV) and porcine endogenous retrovirus) are not able to overcome restriction by tetherin (Goffinet *et al*., 2010; Mattiuzzo *et al*., 2010).

Retroviruses are enveloped viruses that carry virally encoded glycoprotein spikes on their surfaces. HIV-1 and other human retroviruses can spread via either a cell-free virus mode or through direct cell-to-cell contact via the virological synapses (VS), which facilitate escape of viral particles from neutralizing antibodies (Mothes *et al*., 2010). HIV-1 is known to spread much more efficiently under conditions of direct cell-cell contact compared to cell-free virus (Carr *et al*., 1999; Monel *et al*., 2012).

A similar phenomenon has also been observed in MLV. Productive cell-to-cell infection of MLV requires interaction between the viral envelope glycoprotein expressed in the infected cell and the viral receptor expressed on the target cell, leading to the formation of the VS (Jin *et al*., 2009). In addition to broad synaptic contacts, virus-induced filopodia are utilized by MLV. The majority of virus particle assembly is highly polarized at the cell-cell contact sites. Interaction between the cytoplasmic tail of Env and matrix direct this contact-induced polarized assembly (Jin *et al*., 2011). In case of HIV-1, tetherin is present at VS and synapse is formed normally (Casartelli *et al*., 2010; Jolly *et al*., 2010). Productive cell-to-cell spread of HIV-1 was observed in the presence of lower levels of tetherin (Jolly *et al*., 2010). In the presence of higher levels of tetherin, cell-to-cell spread of HIV-1 was inhibited (Casartelli *et al*., 2010). It is likely that the results are influenced by cell type and tetherin expression levels. However, the effect of tetherin on VS formation in MLV is only partly understood. Moreover, it is not clear whether tetherin-mediated restriction enhances syncytium formation by MLV. It remains to be determined whether mouse tetherin promotes or blocks cell-to-cell transmission.

In this study, R-truncated full length Mo-MLV molecular clone was used to examine whether mouse tetherin could promote cell-to-cell transmission and enhance syncytium formation.

Materials and Methods

Cell lines. The 293T human embryonic kidney, NIH3T3 and SC-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Germany) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cloning and stable expression of mouse tetherin. Total RNA was extracted from NIH3T3 cells using TRI-Reagent (Molecular Research Center, USA) according to the manufacturer's instructions. Two primer pairs were designed to amplify mouse tetherin by targeting a 540 nt long coding sequence: moTHN-for (nucleotides 8–27: GenBank Acc. No. NM_198095), 5'-GGTACCA TGGCGCCCTCTTTCTA-3' (*Kpn*I restriction site is underlined); and moTHN-rev (nucleotides 512–531), 5'-CTCGAGTTAA AGAGCAGGAACAG-3' (*Xho*I restriction site is underlined). cDNA synthesis was performed in 20 μl volumes using a Random primer RT PreMix kit (Intron, Korea) according to the manufacturer's instructions. The PCR products of the mouse tetherin were ligated into the pGEM-T Easy vector system (Promega). The tetherin-ligated vectors were digested with the restriction enzymes *Kpn*I and *Xho*I. C-terminal HA-tagged mouse tetherin was produced by introducing the PCR product into the pcDNA3-mCAT-HA vector using the *Kpn*I and *Xho*I sites present in the primers. The expression plasmid for mouse tetherin was named pcDNA3-moTHN-HA. The resulting clone only expresses mouse tetherin. Stable mouse tetherin-expressing NIH3T3 cell lines were selected using medium containing G418 (1 mg/ml, Sigma-Aldrich, Germany), after which tetherin-expressing clones were identified by Western blot using anti-HA antibody.

Western blot assay. Cell lysates from the pcDNA3-moTHN-HA-transfected 293T cells and stable tetherin-expressing NIH3T3 cells were prepared by lysing the cells in 300 μl of Thermo Scientific M-PER mammalian protein extraction reagent lysis buffer (Thermo Scientific). All samples were denatured at 100°C for 10 min and then separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane and then probed with anti-HA antibodies (1:200; Bethyl, USA) for 1 hr or overnight, followed by incubation with a goat anti-rabbit conjugated horseradish peroxidase (1:5,000; Komabio, Korea) for 1 hr. The blots were visualized using 3,3-diaminobenzidine.

Construction of R(-)Env-expressing molecular clone of Mo-MLV. To examine cell-to-cell spread of Mo-MLV in the presence or absence of mouse tetherin and to rule out virus-to-cell infection, we have used R(-)Env expressing full-length molecular clone. A nucleotide sequence coding for EGFP was introduced into the PRR of the Env coding region of the infectious proviral plasmid pMoMLV, resulting in the final construct pMoMLV-EGFP. The 5'LTR U3 region of pMoMLV-EGFP was replaced with the cytomegalovirus promoter to increase initial transcription, generating pCLXSN-MLV-EGFP. R(-)Env-expressing molecular clone pCLXSN-MLV-EGFP-R(-) was constructed by replacing the R(+)Env of pCLXSN-MLV-EGFP with R(-)Env from pCEETR-EGFP. pCEETR-EGFP is Mo-MLV *Env* expression vector which contains EGFP within PRR of Env. Briefly, *Eco*RI-*Bgl*II fragments of pCEETR-EGFP were cloned into the pIRES2-EGFP vector. The *Nhe*I-*Nsi*I fragment from pIRES2-EGFP-R(-)Env was inserted directly into the same sites of pCLXSN-MLV-EGFP (pCLXSN-MLV-EGFP-R(-)). To determine whether mutation in the receptor binding region affects syncytium formation by R(-)Env, pCLXSN-S82F-MLV-EGFP-R(-) was constructed by substitution of serine with phenylalanine (S82F) at the receptor binding domain (Jung and Kozak, 2003). The syncytium formation was observed using fluorescence microscopy.

Virus production and pseudotype assay. To determine whether R(-)Env carrying EGFP can be incorporated into a viral particle, cell-free virus was produced. For transient transfection with split-genome packaging constructs, pVPack-Eco, pVPack-GP (Agilent Technologies, USA), pCLMFG-lacZ (Novus, USA) and the R(-)Env expression plasmid pCEETR-EGFP were used. Viral supernatants were collected from each transfection culture and used to infect NIH3T3 cells that had been plated in 6-well culture plates at a density of 1.5×10^5 per well. The cells were infected with 1 ml of virus in the presence of 8 μg/ml polybrene for 3 hr, after which 2 ml of fresh medium was added to each well. Two days after infection, the cells were fixed with 0.5% glutaraldehyde and stained to reveal the presence of ß-gal activity. Infectious titers were expressed as the blue CFU count per milliliter of virus supernatant.

Quantification of virion release by real-time PCR. To determine whether mouse tetherin in stably transfected NIH3T3 cell lines has anti-retroviral activity, the amount of virus in the supernatant after transfection with an infectious molecular clone was analyzed by infecting SC-1 target cells. Twenty-four hours after infection, the genomic DNA was extracted using the G-spin total DNA extraction kit (Intron). Real-time PCR was conducted using a SYBR Premix Ex Taq II kit (Takara). A Thermal cycler dice real time system (Takara, USA) was used for the thermal cycling and to record fluorescence changes. The primers EGFP-for 5'-GACGTAACGGCCACAGTT-3', EGFP-rev 5'-GGTCTTGTAGTTG-CCGTCGT-3', mouse actin-for 5'-GGGCTGTATTCCCCTCCATCG-3', and mouse actin-rev 5'-GCAGCTCATTGTAGAAGGTGTGGTG-3' were used. Standard curves were generated from 10-fold serial dilutions of infectious clones containing the EGFP gene. EGFP copy numbers were normalized to those of mouse actin.

Syncytium formation. To measure syncytia formation in NIH3T3 cells and mouse tetherin-expressing NIH3T3 cells, NIH3T3 cells were transfected with the molecular clones pCLXSN-MLV-EGFP, pCLXSN-MLV-EGFP-R(-), and pCLXSN-S82F-MLV-EGFP-R(-). Syncytium formation was then examined by fluorescence microscopy.

Results

Cloning of mouse tetherin and construction of stable NIH3T3 cell lines expressing mouse tetherin

Tetherin is constitutively expressed on a few cell types (mature B cells, plasma cells and plasmacytoid dendritic cells), but is absent from many other cells in mice. Although tetherin is not constitutively expressed on NIH3T3 cells, molecular cloning of mouse tetherin was possible by performing RT-PCR on RNA extracted from interferon-treated NIH3T3 cells. The amino acid sequences of cloned mouse tetherin show 39.8% identity with human tetherin. Three cysteine residues and two N-glycosylation sites are conserved in mouse tetherin. As shown in Fig. 1a, the expression of tetherin in 293T cells was confirmed by Western blot analysis using an antibody against the hemagglutinin (HA) tag. Tetherin signals were diffuse due to glycosylation. To examine the effect of tetherin expression on syncytium formation, stable NIH3T3 cell lines expressing mouse tetherin were

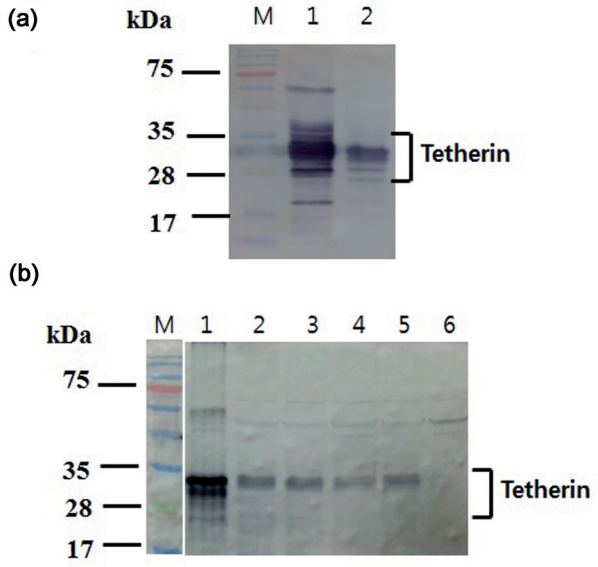

Fig. 1

Expression of mouse tetherin in 293T cells and construction of stable NIH3T3 cell lines expressing mouse tetherin

(a) 293T cells were transiently transfected with mouse tetherin-3×HA expression vector (lane 2). The protein was detected by Western blot probed with anti-HA antibody. Lane 1, 293T cells transfected with human tetherin-3×HA expression vector as positive control. Lane M, solgentTM triple color protein. (b) A comparison of the tetherin expression of transiently transfected 293T cells (lane1) and stable NIH3T3 cell lines (lane 2–5). NIH3T3 cell lysates were used as negative control (lane 6).

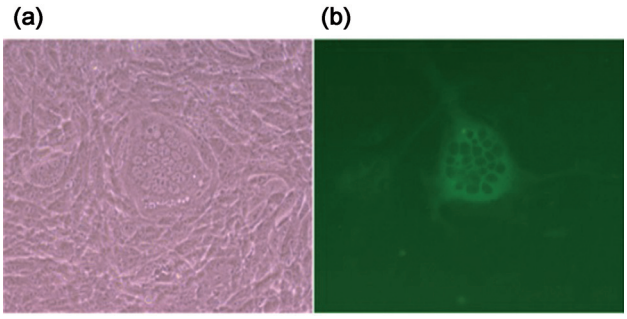

Fig. 2

Light microscopy image (a) and fluorescence microscopy image (b) of syncytium in NIH3T3 cells

NIH3T3 cells were transfected with EGFP-R(-)Env protein expression plasmids of Mo-MLV. The image shows a syncytium displaying EGFP fluorescence (b).

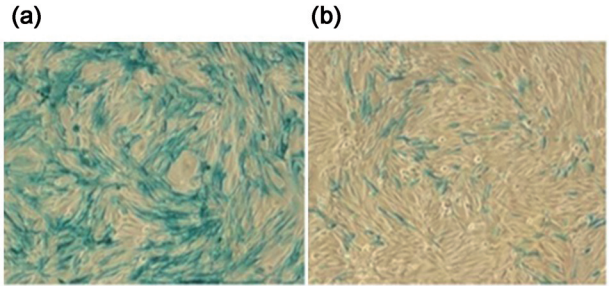

Fig. 3

Properties of EGFP-R(-)Env proteins
293T cells were co-transfected with pVPack-Eco (a), pVPack-GP, pCLMFG-lacZ and pCEETR-EGFP (b). Viral supernatants were harvested 48 hr post-transfection and used to infect NIH3T3 cells. After 48 hr, the number of cells expressing β-galactosidase was evaluated by X-gal staining.

established. The stable expression of tetherin was compared to expression of mouse tetherin in transiently transfected 293T cells. The rate of synthesis in transiently transfected cells was much greater than in the stable cell line, whereas NIH3T3 cell lysate, as the negative control, did not show tetherin expression (Fig. 1b).

Syncytium formation of R(-)Env and transduction titers of pseudotype virus

It has been reported that the R(-)Env protein is fusogenic in NIH3T3 cells (Kubo *et al*., 2007; Ragheb and Anderson, 1994; Rein *et al*., 1994). To confirm the previous results and show syncytia by displaying EGFP fluorescence, EGFP was inserted into the R(-)Env expression plasmid pCEETR (pCEETR-EGFP). As expected, the R(-)Env proteins induced syncytia formation in the mouse NIH3T3 cells (Fig. 2). To investigate the effect of the R(-)Env encoding EGFP on transduction efficiency, 293T cells were co-transfected with pVPack-GP, pCEETR-EGFP and pCLMFG-lacZ for the production of pseudotype virus. Pseudotype virus showed lower transduction efficiency than R(+)Env (Fig. 3). These results indicated that R(-)Env encoding EGFP was not efficiently incorporated into viral particles. To minimize virus-to-cell transmission, R(-)Env was used for the construction of full-length molecular clone.

Construction of R(-)Env-expressing molecular clone of Mo-MLV

When transfection was carried out with pCLXSN-MLV-EGFP, the percentage of EGFP-positive cells rapidly increased over time, approaching 100% by day 6 (Fig. 4a). In contrast, the R(-)Env expressing molecular clone induced syncytia formation in NIH3T3 cells 2 days after transfection (Fig. 4b). However, fluorescence was not detected on the 6th day post transfection.

Effect of stable expression of mouse tetherin on Mo-MLV replication

To examine whether tetherin has inhibitory activity against infectious Mo-MLV molecular clone, pCLXSN-MLV-EGFP,

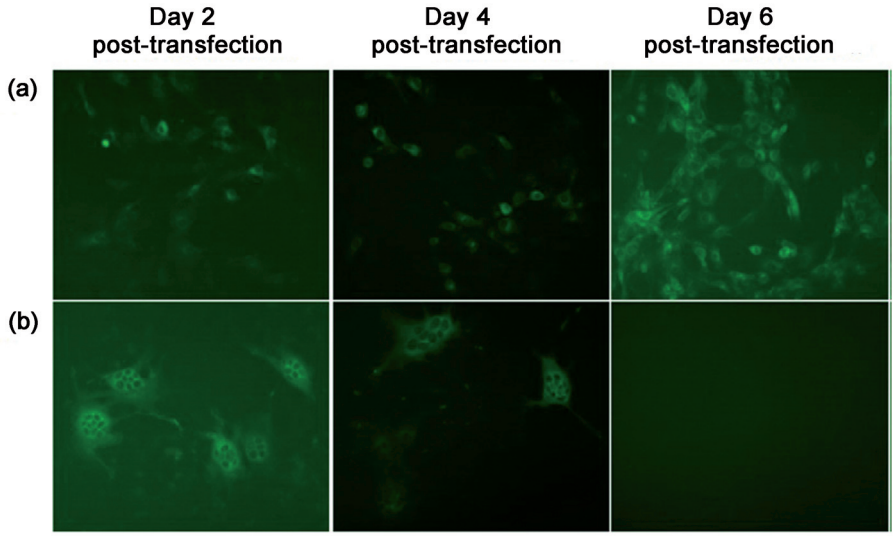

Fig. 4

Generation of replication-competent EGFP-R(-)Env protein bearing viruses
NIH3T3 cells were transfected with pCLXSN-MLV-EGFP (a) and R(-)Env expression molecular clone pCLXSN-MLV-EGFP-R(-) (b). When NIH3T3 cells were transfected with pCLXSN-MLV-EGFP-R(-), syncytia were observed 48 hr after transfection.

was transfected into the stable NIH3T3 cell line expressing mouse tetherin. The supernatant collected from pCLXSN-MLV-EGFP-transfected stable NIH3T3 cells was titrated on SC-1 cells. Total DNA was isolated from infected SC-1 cells, and the amount of Mo-MLV was demonstrated by real-time PCR. Standard curves were generated from the 10-fold serial dilution of pCLXSN-MLV-EGFP containing the gene for EGFP protein. Remarkably, the mouse tetherin expressed in stable NIH3T3 cells was able to inhibit the replication of Mo-MLV (Fig. 5).

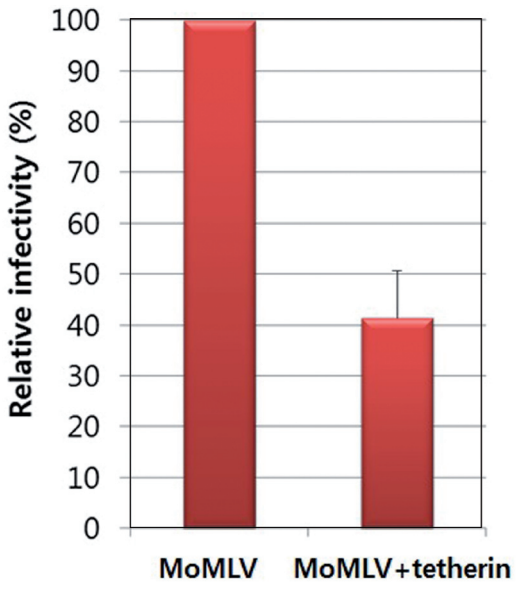

Fig. 5

Antiviral function of mouse tetherin from stable NIH3T3 cells
Wild type Mo-MLV was prepared from NIH3T3 cells and tetherin-expressing NIH3T3 cells by transient transfection of infectious Mo-MLV clone. Viral supernatants were harvested 48 hr post-transfection and used to infect SC-1 cells. Error bars represent the standard deviations of three independent experiments.

Syncytium formation induced by cell-to-cell transmission

The effect of tetherin on directional cell-to-cell transmission of Mo-MLV is not clear. Herein, NIH3T3 cells stably expressing mouse tetherin were used to examine whether mouse tetherin could enhance syncytium formation. As shown in Fig. 6, in comparison to control cells, syncytium formation following transfection with pCLXSN-MLV-EGFP-R(-) was enhanced in the NIH3T3 cells stably expressing mouse tetherin. To test whether amino acid change in the receptor binding domain of the *env* gene (S82 in Mo-MLV) affects syncytium formation, pCLXSN-S82F-MLV-EGFP-R(-) was constructed by the substitution of serine with phenylalanine (S82F) in the receptor binding domains. When pCLXSN-S82F-MLV-EGFP-R(-) was transfected into NIH3T3 cells stably expressing mouse tetherin, syncytium formation was also observed (Fig. 6). These results suggest that a weak interaction between envelope protein and receptor does not affect enhancement of syncytium formation.

Discussion

Tetherin-mediated inhibition of cell-free virus infection has been well reported (Dietrich *et al.*, 2011; Hu *et al.*,

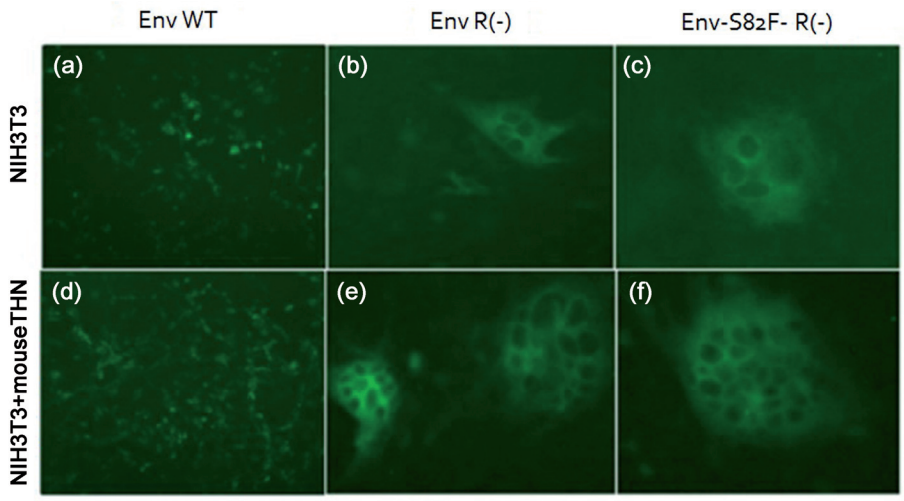

Fig. 6

Effects of stable expression of mouse tetherin on syncytium formation
Syncytium formation in the absence or presence of mouse tetherin was analyzed at day 3 post-transfection. NIH3T3 cells were transfected with pCLXSN-MLV-EGFP (a), pCLXSN-MLV-EGFP-R(-) (b), and pCLXSN-MLV-S82F-EGFP-R(-) (c). The stable mouse tetherin-expressing NIH3T3 cells were transfected with pCLXSN-MLV-EGFP (d), pCLXSN-MLV-EGFP-R(-) (e), and pCLXSN-MLV-S82F-EGFP-R(-) (f).

2012; Mattiuzzo *et al.*, 2010; Neil *et al.*, 2008). However, it is not clear whether tetherin also restricts cell-to-cell spread or whether it affects syncytium formation. The cell-to-cell transmission of HIV-1 takes place at the VS where tetherin is present. MLV-infected fibroblasts could also establish similar cell-to-cell contacts between infected and uninfected target cells. The establishment of cell-to-cell contacts is followed by the polarized assembly of viruses at the cell-to-cell interface. In the case of MLV, Env cytoplasmic tail directs polarized Gag assembly to the cell-to-cell contact (Jin *et al.*, 2011). Previous results suggest that endogenous tetherin can limit replication of HIV-1 and MLV (Goffinet *et al.*, 2010). However, two conflicting results have shown the effects of tetherin on cell-to-cell transmission. Some groups observed that tetherin inhibited the cell-to-cell transmission of Vpu-defective HIV-1 (Casartelli *et al.*, 2010; Kuhl *et al.*, 2010). In contrast, other groups found that tetherin could promote cell-to-cell virus transmission (Jolly *et al.*, 2010). Variations in tetherin expression may account for these conflicting results in cell-to-cell transmission. It was reported that syncytium formation following feline immunodeficiency virus infection was enhanced in tetherin-expressing cells (Dietrich *et al.*, 2011).

To investigate the effect of mouse tetherin on syncytium formation, a cell line stably expressing mouse tetherin was constructed. The stable expression of mouse tetherin obtained was compared to tetherin expression in 293T cells transiently transfected with the tetherin expression vector by Western blot. As expected, mouse tetherin was expressed in the stable NIH3T3 cell line at lower levels than in 293T cells. The highest-expressing tetherin cell line was used for examining the mechanism of cell-to-cell spread and enhancement of syncytium formation. To generate a tool allowing for the fast and convenient detection of syncytia by fluorescence microscopy, EGFP was inserted at a position within the PRR of R(-)Env, as previously reported (Erlwein *et al.*, 2003). EGFP-positive syncytia were detected after transient transfection of R(-)Env, indicating the presence of functional Env protein on the membrane even though the *egfp* gene was inserted within PRR. This result confirmed that R(-)Env could induce syncytia formation in NIH3T3 cells. The EGFP-R(-)Env expression plasmid showed lower transduction efficiency than R(+)Env. This result suggests that R(-)Env could not be efficiently incorporated into virions and cell-free virus production would be inhibited in stable tetherin-expressing cells. Therefore, R(-)Env was used for the construction of a full-length molecular clone to rule out induction of syncytia formation by cell-free virus. To examine the cell-cell spread of Mo-MLV between NIH3T3 cells in the presence or absence of tetherin, full-length pCLXSN-MLV-EGFP or pCLXSN-MLV-EGFP with R(-)Env (pCLXSN-MLV-EGFP-R(-)) was constructed. In agreement with previous reports, the pCLXSN-MLV-EGFP virus showed efficient replication and EGFP transmission (Erlwein *et al.*, 2003). Although syncytium formation in pCLXSN-MLV-EGFP-R(-)-transfected cells was detectable for the first few passages, no spread of EGFP fluorescence was observed. These observations suggest that pCLXSN-MLV-EGFP-R(-) virus cannot replicate over several rounds in NIH3T3 cells. Syncytium formation by pCLXSN-MLV-EGFP-R(-) might trigger an apoptotic response. This pCLXSN-MLV-EGFP-R(-) would be useful for cell-to-cell virus transmission studies to rule out cell-free virus dissemination.

To test whether functional levels of tetherin are expressed in stable NIH3T3 cells, viruses were produced from the transfected cells and infectivity was quantified by real-time PCR. The results showed that stable ectopic expression of mouse tetherin could restrict the replication of Mo-MLV. Using the mouse tetherin-expressing NIH3T3 cells transfected with pCLXSN-MLV-EGFP-R(-), we have observed increase in syncytium size. These results suggest that tetherin-mediated retention of Mo-MLV at the cell surface could promote cell-to-cell transmission via the VS, and that the presence of tetherin at the VS may also enhance syncytium formation. We have found that productive cell-to-cell spread occurs under conditions where stably transfected cells express lower levels of tetherin.

In conclusion, we here report the generation of R(-) Env replication-competent Mo-MLV reporter viruses that express EGFP. Transfection of proviral DNA into NIH3T3 showed that reporter viruses were infectious, allowing for the fast and convenient detection of syncytia by fluorescence microscopy. In addition, the use of the newly developed pCLXSN-MLV-EGFP-R(-) allowed us to study the mechanism of Mo-MLV cell-to-cell transmission and examine the effect of mouse tetherin on syncytium formation. We found that Mo-MLV cell-to-cell transmission requires the high local concentration of virus particles, and stable expression of tetherin could enhance the formation of syncytia.

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