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Construction of replication-competent oncolytic retroviral vectors expressing R peptide-truncated 10A1 envelope glycoprotein

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ABSTRACT

Replication-deficient retroviral (RDR) vectors have been generally used for gene therapy, but clinically beneficial transduction efficiency is difficult to achieve with these vectors. In recent times, attention has been focused on the use of murine leukemia virus (MLV)-based replication-competent retroviral (RCR) vectors. RCR vectors have been shown to achieve efficient tumor reduction in a wide variety of cancer models. Most RCR vectors have been developed from amphotropic 4070 A MLV *env*, which is broadly applied in basic research. In this study, we generated RCR vectors based on Moloney MLV by replacing the native *env* gene in a full-length viral genome with the 10A1 *env* gene. 10A1 MLV can infect a wide variety of cells. Unlike amphotropic MLV, the 10A1 MLV can use amphotropic MLV receptor Pit2 or gibbon ape leukemia virus (GaLV) receptor Pit1. The resulting construct MoMLV-10A1-EGFP was able to replicate in 293 T, NIH3T3, and *Mus dunni* cells. To evaluate the potential of MoMLV-10A1 vector as a therapeutic agent, we incorporated the yeast cytosine deaminase (CD) suicide gene into vectors. The resulting vector MoMLV-10A1-CD could inhibit the growth of human 293T cells upon 5-fluorocytosine (5-FC) administration. In addition, to lyse tumor cells by syncytium, MoMLV-10A1-R(-)-EGFP was generated by replacing wild-type 10A1 *env* with the 16-amino acid R peptide-truncated 10A1 *env* gene. Syncytium formation was observed in the TE671 human tumor cells, 293 T and PG13 cells upon transfection of the MoMLV-10A1-R(-)-EGFP vector. This result suggests that replication of this vector could be oncolytic in itself. We also found that syncytium could contribute to enhance cell-to-cell transmission of the retroviral vectors. Our results thus show that the MoMLV-10A1 vectors can be potentially useful for cancer gene therapy.

1. Introduction

Murine leukemia virus (MLV), the most commonly used retroviral vector in cancer research, is a small RNA virus with a DNA intermediate and contains three genes (*gag*, *pol*, and *env*). However, some problems associated with the retroviral vector are the use of relatively low titers (10^3–10^6 transduction units/mL) and the inability to transfer genes into nondividing cells. Recently, MLV-based replication-competent retroviral (RCR) vectors have been developed as attractive tools for cancer therapy (Kubo et al., 2018; Lu et al., 2012; Solly et al., 2003; Wang et al., 2003).

Retroviral envelope glycoproteins are critical for the virus entry into the host cell because they can recognize and bind to cell surface receptors, and are required for the fusion of the viral envelope and host membranes. Target cell tropism is mediated by the viral envelope. MLVs have been classified into ecotropic, polytropic (mink cell focus-forming), xenotropic, amphotropic, and 10A1 based on their host range/interference properties (Sommerfelt and Weiss, 1990). Infection by the ecotropic murine leukemia virus is restricted to mouse and rat cells that express the cationic amino acid transporter (CAT1). 10A1 MLV—a recombinant gammaretrovirus—is isolated from a mouse infected with amphotropic 4070 A MLV. Amphotropic 4070 A MLV uses cellular receptor Pit2(SLC20A2) and can infect most mammalian cells (but not hamster), while 10A1 MLV uses both Pit2(SLC20A2) and Pit1(SLC20A1) (Rasheed et al., 1976). Pit2 and Pit1 are both mammalian type III inorganic phosphate transporters and demonstrate 60% amino acid identity (Han et al., 1997; Miller and Chen, 1996). The 10A1 virus can infect most mammalian cells (including human cells). Recently, an amphotropic (4070 A *env*) replication-competent retroviral vector encoding a suicide gene shows efficient tumor cell killing (Hiraoka et al., 2007; Huang et al., 2015; Lu et al., 2010; Ostertag et al., 2012; Perez et al., 2012; Tai et al., 2005; Twitty et al., 2016).

In this study, we have developed novel chimeric replication-competent MoMLV-10A1 vectors. The IRES-EGFP transgene cassette was

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inserted between the *env* gene and the 5′ end of the 3′-long terminal repeat (LTR) U3 region of the pMoMLV-10A1 vector (pMoMLV-10A1-EGFP). To determine whether this vector is capable of efficient replication in culture, 293 T, NIH3T3, and *M. dunni* cells were transfected with this vector. In addition, IRES-EGFP of pMoMLV-10A1-EGFP was replaced with IRES-yeast cytosine deaminase (CD) gene, generating pMoMLV-10A1-CD. To examine whether this vector is susceptible to *in vitro* cytotoxic killing upon administration of 5-FC, the MoMLV-10A1-CD vector was transfected into 293T cells.

Fusogenic membrane glycoproteins (FMGs) are viral envelope proteins that kill cancer cells by fusing them into large multinucleated syncytia (Bateman et al., 2000; Diaz et al., 2000; Fielding et al., 2000). Syncytial killing is highly immunostimulatory. In addition, the bystander effect of FMG killing is at least a log higher than that of suicide gene/prodrug systems such as herpes simplex virus thymidine kinase-1 (HSVtk)/GCV or cytosine deaminase (CD)/5-(FC). Previous studies have reported that an R peptide (16-amino acid cytoplasmic tail of transmembrane protein)-truncated form of the GaLV envelope can cause considerable cell-cell fusion of human cells that express the Pit-1 receptor. Among the different viral FMGs, GaLV FMG is highly cytotoxic for tumor cells. GaLV FMG has a 10-fold stronger bystander killing effect than HSV-TK. However, production of high-titer viral vector encoding GaLV FMG is difficult due to the rapid fusion of vector-producing cells. To overcome this problem, the use of inducible promoter and other vector systems (lentiviral vector, Sindbis vector, oncolytic adenovirus, and oncolytic HSV) have been reported (Ebert et al., 2004; Fu et al., 2003; Guedan et al., 2012; Lin et al., 2010; Zhang et al., 2004).

Truncation of the R peptide affects envelope fusion, which has been previously observed in MoMLV. The envelope protein of the ecotropic MoMLV is synthesized as a gp85 precursor, which is then proteolytically cleaved into an extracellular surface unit (SU) and a transmembrane protein (TM). The C-terminal 16 amino acid are removed from the TM protein by the viral protease during virion maturation. Unlike wild-type Env protein bearing the R peptide, the R peptide-truncated envelope induces syncytia in susceptible cells (Chung et al., 1999; Kubo et al., 2007; Ragheb and Anderson, 1994; Rein et al., 1994).

In this study, we developed a novel chimeric retroviral vector system and evaluated the therapeutic efficacy of MoMLV-10A1-CD. In particular, MoMLV-10A1-R(-)-IRES-EGFP encoding R-truncated 10A1 *env* was used to examine whether this fusogenic oncolytic retroviral vector could be a useful tool for cancer gene therapy.

2. Materials and methods

2.1. Cell lines

The 293T human embryonic kidney (ATCC CRL-11268), TE671 human rhabdomyosarcoma cells (ATCC CRL-8805), NIH3T3, *Mus dunni* (ATCC CRL-2017), and PG13/LNc8 (ATCC CRL-10685) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.2. Construction of replication-competent retroviral vector

To create a MoMLV-10A1 novel chimeric vector, a series of exchanges were carried out as follows: First, a pMoMLV full-length clone was digested with restriction enzyme *Eag*I and an *Eag*I-*Eag*I fragment was ligated into *Eag*I-digested pCLXSN (Novus Biologicals, Littleton, USA). Second, a *Sal*I-*Nhe*I fragment of pMoMLV was ligated into pCLXSN vector containing *Eag*I-*Eag*I fragment to produce pCLXSN-MoMLV. Third, pCLXSN-MoMLV-10A1 was created by ligating the 3.9-kb *Sal*I-to-*Cla*I fragment from pCL-10A1 into the corresponding region of pCLXSN-MoMLV. Finally, the 1.5-kb *Nhe*I-*Nhe*I fragment containing IRES-EGFP was cloned into pCLXSN-MoMLV-10A1 to produce pMoMLV-10A1-EGFP. For virus production, 293 T cells were transiently transfected with retroviral vector using the Calphos Mammalian Transfection Kit (TaKaRa, Shiga, Japan). Virus-containing supernatants were harvested 48 h after transfection and used to infect 293T, NIH3T3, and *M. dunni* cells. Cells were infected with the virus in the presence of polybrene (8 μg/mL) for 3 h and 2 mL of fresh medium was then added to each well.

2.3. Construction of retroviral vector encoding cytosine deaminase

To develop pMoMLV-10A1-EGFP as a therapeutic vector for cancer gene therapy, the plasmid pMoMLV-10A1-CD was generated by replacing EGFP with a synthetic yeast CD gene. The IRES-EGFP was first removed from pIRES2-EGFP (Clontech, Mountain View, USA) using a double digestion with *Xho*I and *Not*I. The IRES-EGFP fragment was transferred into *Xho*I/*Not*I-digested pLPCX (Clontech, Mountain View, USA) to create pLPCX-IRES-EGFP. pLPCX-IRES-CD was generated by replacement of the EGFP sequence in pLPCX-IRES-EGFP with the *Nco*I/*Not*I fragment containing synthetic yeast CD gene. Finally, the *Nhe*I/*Nhe*I fragment containing IRED-CD was ligated into *Nhe*I-digested pMoMLV-10A1-EGFP to generate pMoMLV-10A1-CD. The expression of yCD was analyzed by western blotting. Two primer pairs were designed to amplify yCD by targeting a 488-nt-long coding sequence; 5′-GGTA CCATGGTGACAGGCGGCATG-3′ (*Kpn*I restriction site is underlined), and 5′-CTCGAGTTCTCCCCAATGTCCTCGAA-3′ (*Xho*I restriction site is underlined). The PCR product of the yCD was ligated into the pGEM-T Easy Vector System (Promega, Madison, USA). The yCD-ligated vector was digested with the restriction enzymes *Kpn*I and *Xho*I. C-terminal HA-tagged yCD 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 yCD was named pcDNA3-yCD-HA. Expression of yCD was evaluated by western blotting using anti-HA antibody after transient transfection of 293T cells.

2.4. MTT assay

To test the cytotoxic effect of 5-FC in 293T cells, 293T/yCD cells were determined using the MTT assay. Stable 293 T cell lines expressing yCD were generated by transfection with replication-competent retroviral vector pMoMLV-10A1-CD. The cells were seeded in a 96-well plate (1000 cells/well) and 5-FC was added at various concentrations. Cell growth kinetics was assessed using an MTT assay kit (Intron, Seoul, Korea) according to the manufacturer's protocol.

2.5. Construction of r(-) env-expressing MoMLV-10A1 vector

The R(-) Env full-length molecular clone of MoMLV vector construct, pMLV-EGFP-R(-), has been previously described (Kim et al., 2016). Briefly, *Eco*RI-*Bgl*II fragments of pCEETR-EGFP were cloned into the pIRES2-EGFP vector (pIRES2-EGFP-R(-) Env). The *Nhe*I-*Nsi*I fragment from pIRES2-EGFP-R(-) Env was inserted directly into the same sites of pMLV-EGFP (pMLV-EGFP-R(-)). To construct the R(-) Env-expressing molecular clone of MoMLV-10A1, the *Sal*I/*Cla*I fragment containing 10A1 *env* was transferred into *Sal*I/*Cla*I-digested pMLV-EGFP-R(-) to generate pMoMLV-10A1-R(-). Finally, the *Nhe*I/*Nhe*I fragment containing IRES-EGFP was transferred into *Nhe*I-digested pMoMLV-10A1-R(-) to generate pMoMLV-10A1-R(-)-EGFP. As control vector, pMoMLV-Ampho-R(-)-EGFP was constructed by substituting *Eco*RI-*Cla*I fragment from pVPack-Ampho (Agilent, Santa Clara, USA) into the corresponding region of pMoMLV-10A1-R(-)-EGFP. To investigate whether MoMLV-10A1-R(-)-EGFP can cause syncytia, TE671, 293 T and PG13 cells were transfected with this vector. The syncytium formation by R(-) Env was observed using fluorescence microscopy.

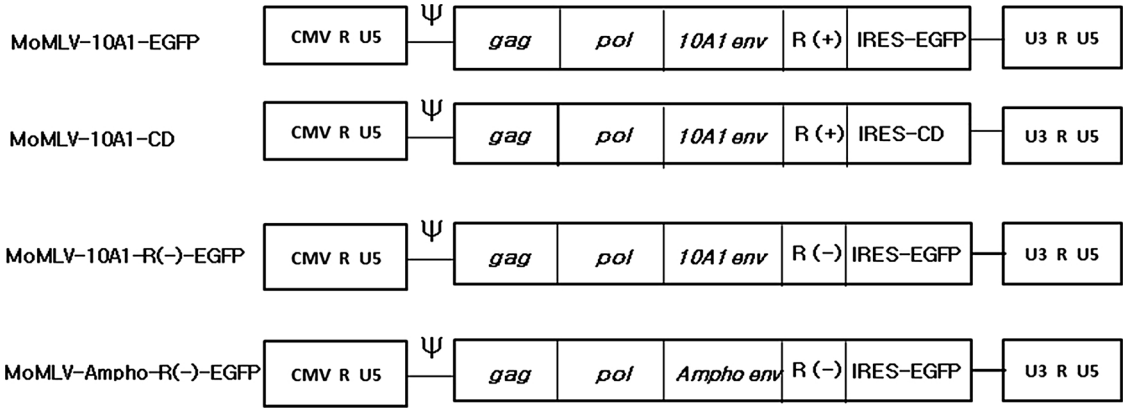


Fig. 1. Construction of replicating retroviral vectors. MoMLV-10A1-EGFP comprises Moloney MLV that contains the 10A1 *env* gene and an IRES-EGFP cassette between the *env* gene and 3′-UTR. Yeast CD gene replaced the *EGFP* gene in MoMLV-10A1-EGFP, generating MoMLV-10A1-CD. MoMLV-10A1-R(-)-EGFP is a replication-competent vector that contains the 16-amino acid R-peptide-truncated 10A1 *env*. MoMLV-Ampho-R(-)-EGFP is a replication-competent vector that contains the 16-amino acid R-peptide-truncated 4070 A *env*.

3. Results

3.1. Construction and production of MoMLV-10A1 vector

As 10A1 has an expanded host range, we developed replication-competent retroviral vector containing 10A1 *env* gene. To improve retroviral RNA transcription in 293T cell, pCLXSN retroviral vectors were used to construct replication-competent vectors. pCLXSN vectors contain a CMV promoter fused to the MoMLV LTR at the TATA box in the U3 region of 5′-LTR. An *Eag*I-*Nhe*I fragment from pMoMLV was inserted into pCLXSN to generate pCLXSN-MoMLV. The ecotropic envelope from pCLXSN-MoMLV was replaced with 10A1 *env*, generating pMoMLV-10A1. Finally, an IRES-EGFP was inserted at the boundary between the *env* gene and the 3′-UTR (Fig. 1). To determine whether the replication-competent vector could express GFP, 293T cells were transiently transfected for virus production. Viral supernatants were used to infect 293T, NIH3T3, and *M. dunni* cells. After another three passages, fluorescence microscopic analysis revealed efficient propagation of the replication-competent retroviral vector in 293 T, NIH3T3, and *M. dunni* cells (Fig. 2).

3.2. Cytotoxic efficacy of the MoMLV-10A1 vectors mediated suicide gene therapy

The replication-competent amphotropic MLV vector (ACE-GFP) contains the ecotropic Moloney MLV provirus in which the envelope was replaced with 4070A envelope and IRES-EGFP, as previously described by other groups (Hiraoka et al., 2007; Lu et al., 2010; Tai et al., 2005). In addition, they constructed replication-competent retroviral vector (ACE-CD)—expressing the yeast cytosine deaminase suicide gene—by replacing the IRES-GFP cassette in ACE-GFP. ACE-CD exhibited enhanced cytotoxicity upon administration of the prodrug 5-FC.

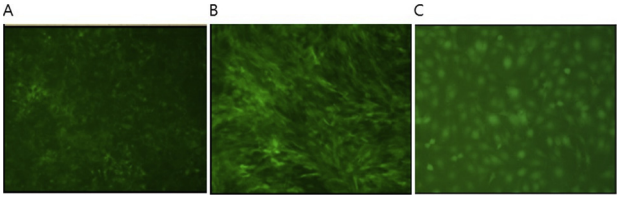


Fig. 2. Transduction of the replication-competent vector. 293T cells were transiently transfected with replication-competent MoMLV-10A1-EGFP vector. After 48 h, the undiluted supernatant containing vectors was transferred to 293T(A), NIH3T3 (B), and *M. dunni* cells (C). At 7 days postinfection, more than 90% of the cells expressed EGFP as detected using fluorescence microscopy.

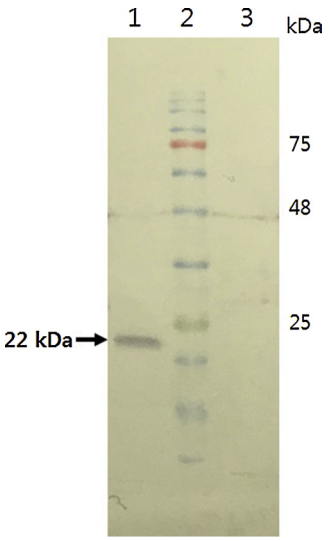


Fig. 3. Expression of yeast CD in 293T cells. 293T cells were transiently transfected with yeast CD-3 × HA expression vector (lane 1). Protein expression was detected using western blotting upon probing with anti-HA antibody. Lane 1293 T cells transfected with yeast CD-3 × HA expression vector. Lane 2, solgent™ triple color protein. Lane 3293 T cell lysates were used as negative control.

In this study, we also chose a codon-optimized and heat-stabilized mutant yeast CD as a therapeutic suicide gene. Expression of yCD was confirmed in transiently pcDNA3-yCD-HA-transfected 293 T cells by western blotting. As shown in Fig. 3, SDS-PAGE revealed a molecular weight of 22 kDa for HA-tagged yCD as expected. To verify the enzyme activity of yCD, yCD-expressing 293T cell lines were prepared by stable transfection with pMoMLV-10A1-CD. 293T−CD cells were exposed to the 5-FC at different concentrations and the number of viable cells was monitored using the MTT assay every 2 days. In 293T cells, decreased cell viability was observed in MoMLV-10A1-CD-transduced cultures in a 5-FC dose-dependent manner (Fig. 4).

3.3. Construction of MoMLV-10A1 vector encoding fusogenic membrane glycoproteins

GaLV FMG, VSV-G, and the measles virus proteins F and H are commonly used as potential cancer gene therapy agents. It was of interest to know whether 10A1-R(-) Env is capable of inducing cell-cell fusion. We, therefore, subcloned the *Sal*I/*Cla*I fragment containing

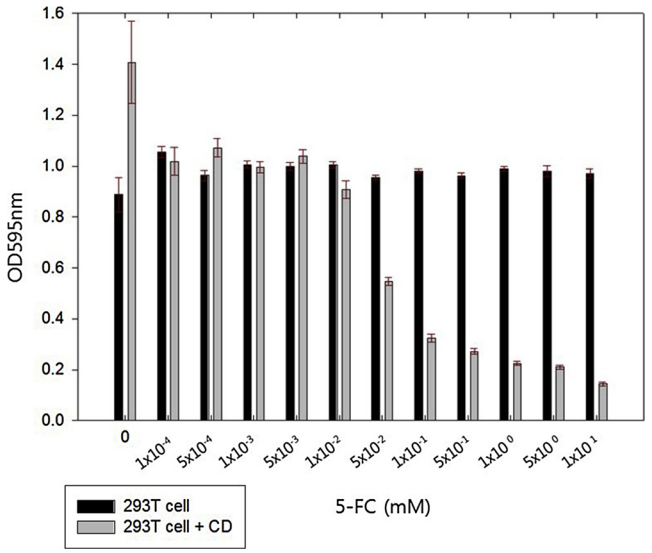


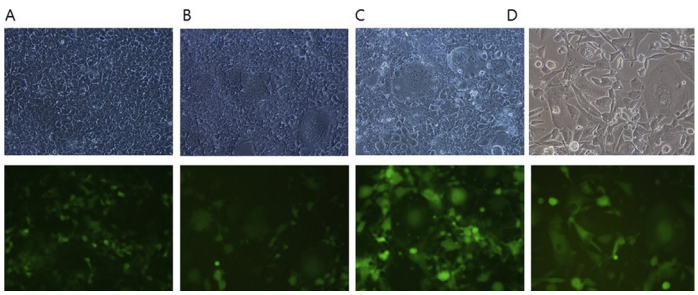
Fig. 4. Cytotoxic effect of MoMLV-10A1-CD/5-FC in 293T cells. MoMLV-10A1-CD transduced and untransduced 293T cells were exposed to various concentrations of 5-FC. Cell viability was determined using the MTT assay every 2 days.

10A1 *env* into R(-) Env expression vector pMLV-EGFP-R(-), generating MoMLV-10A1-R(-)-EGFP. As syncytium-inducing retroviral vector control, pMoMLV-Ampho-R(-)-EGFP was used. Fig. 5(B) shows that control vector causes syncytium formation in transiently transfected 293T cells. When MoMLV-10A1-R(-)-EGFP vector was transfected into TE671, 293T and PG13 cells, extensive syncytium formation was observed (Figs. 5 and 6). Three days after transfection, syncytia formation was visible either using phase contrast microscopy or fluorescence microscopy. When transfected PG13 cells were passaged after transfection, the number of nuclei per syncytia was increased (Fig. 6C).

4. Discussion

Recently, two different replication-competent retroviral vectors derived from amphotropic MLV and gibbon ape leukemia virus (GaLV) were developed that use different receptors to infect target cells (Kubo et al., 2018). However, the replicative spread of these two vectors in solid tumors was potentially affected by cellular receptor expression level. Therefore, the use of multiple replication-competent retroviral vectors based on cellular receptor expression level may enhance antitumoral therapeutic efficacy. In this study, we have developed new replication-competent retroviral vectors derived from 10A1 MLV, which have divergent host ranges. 10A1 MLV can use either human Pit1(SLC20A1) or human Pit2(SLC20A2) for human cell entry. As shown in Fig. 2, almost the entire target cells exhibited EGFP fluorescence after another three passages in 293T, NIH3T3, and *M. dunni* cells. These results suggest that MoMLV-10A1 recombinant vectors replicate efficiently in human and murine cells *in vitro*.

Previous study reported that artificially constructed chimeric



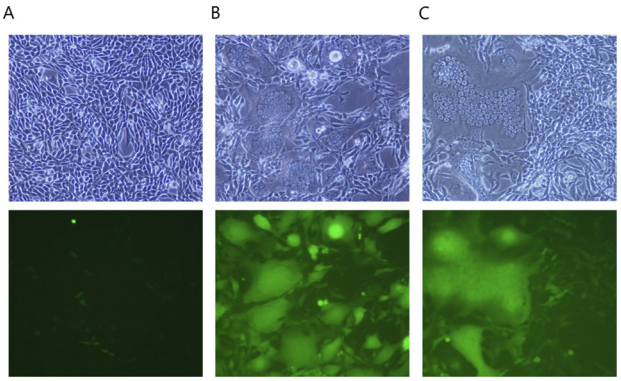


Fig. 6. Syncytia-mediated retroviral cell-to-cell spread. PG13 cells were transfected with MoMLV-10A1-EGFP (A) and MoMLV-10A1-R(-)-EGFP (B). Two days post-transfection, PG13 cells were observed using phase contrast microscopy (top panel) or fluorescence microscopy. (C) PG13 cells transfected with MoMLV-10A1-R(-)-EGFP (B) were cultured for another 6 days.

MoMLV-GaLV was partially replication-impaired (Logg et al., 2007). However, they found that prolonged passage of cells exposed to this MoMLV-GaLV chimeric viruses allowed the selection of rapidly replicating mutants. To determine whether additional mutations might arise after sequential passages, we directly sequenced PCR products from 5′LTR and splice acceptor site of MoMLV-10A1 provirus. As expected, no alterations were observed in these regions (data not shown). This is because MoMLV-Ampho and MoMLV-10A1 chimeras are efficiently replicating retroviral vectors, unlike MoMLV-GaLV (Ott et al., 1992).

To investigate the efficacy of replication-competent retroviral vector-mediated prodrug activated cancer gene therapy, we generated MoMLV-10A1-CD. Human 293T cells pretransduced with MoMLV-10A1-CD were cultured in 96-well plates with various concentrations of 5-FC to quantitatively analyze drug cytotoxicity. MoMLV-10A1-CD showed efficient killing of MoMLV-10A1-CD-transduced 293T cells. These data indicate the potential utility of MoMLV-10A1-CD in a wide variety of cancer cells.

A previous study has shown that R peptide-truncated MoMLV envelope can be incorporated into the virion, which can exhibit nearly normal titers, whereas those with R peptide-truncated amphotropic MLV envelope exhibit 10-fold lower titers using the pseudotype assay. They also found that infection of NIH3T3 cells with culture fluids from CHO cells stably transfected with R(-) MLV full-length clone or R(-) MoMLV-4070A full-length clone could induce the formation of large syncytia (Ragheb and Anderson, 1994; Rein et al., 1994). Based on these results, we developed replication-competent retrovirus-encoding FMGs, which kill cells by fusing them into syncytia. A full-length molecular clone expressing R peptide-truncated 10A1 *env* was constructed (Fig. 1). As shown in Fig. 5, TE671 and 293T cells transfected with MoMLV-10A1-R(-)-EGFP vector caused extensive syncytia. To compare with conventional amphotropic vector, MoMLV-Ampho-R(-)-EGFP was used for syncytium formation. Both retroviral vectors could efficiently spread throughout entire cell populations and induced syncytium

Fig. 5. Syncytia formation mediated by 10A1-R(-) *env* gene. 293T cells were transfected with MoMLV-10A1-EGFP (A), MoMLV-Ampho-R(-)-EGFP(B), and MoMLV-10A1-R(-)-EGFP (C). TE671 cells were transfected with MLV-10A1-R(-)-EGFP (D). 293 T and TE671 cells were observed using phase contrast microscopy (top panel) or fluorescence microscopy.

formation. MoMLV-10A1-R(-)-EGFP was replicated comparable rate to MoMLV-Ampho-R(-)-EGFP. In addition, a cell-free MoMLV-10A1-R(-)-EGFP virus obtained from transfected 293T cells was used to infect 293T cells and these produced syncytia (data not shown). In agreement with previous studies, we have observed that the level of production of MoMLV-10A1-R(-)-EGFP virus in the presence of syncytia is lower than that of wild-type virus in transfected 293T cells. This result suggests that rapid cell fusion may inhibit retroviral vector replication.

To determine the contribution of the *gag*, *pol*, and *env* products to syncytium formation, PG13 cells were transfected with MoMLV-10A1-R(-)-EGFP. PG13, which is derived from TK-NIH3T3 cells, constitutively expresses a GaLV envelope in addition to the *gag, pol,* and retroviral vector genome (LN c8). As shown in Fig. 6C, the number of nuclei per syncytia was increased after another three passages. This result suggests that the *gag* and *pol* from PG13 may augment syncytium formation and then syncytia may enhance cell-to-cell transmission of retroviral vectors. When we compared previous fluorescence microscopic images of non-replicable vector-transfected NIH3T3 cells (Kim et al., 2016) with the present replicable vector-transfected PG13 cells, we could not clearly detect the syncytia. We suggest that this may be due to enhanced virus release from syncytial lysis. As retroviral infections are known to spread by cell-to-cell transmission (Jin et al., 2009; Jones and Risser, 1993; Mothes et al., 2010), we propose that syncytial formation might enhance viral dispersion.

In conclusion, the novel chimeric vector MoMLV-10A1 developed in this study can efficiently replicate in human 293T, NIH3T3, and *M. dunni* cells. Genetically engineered MoMLV-10A1-encoding suicide gene CD or R peptide truncated 10A1 *env* were also generated as more specific and effective agents for cancer gene therapy. These newly generated replication competent retroviral vectors will be useful tools for cancer gene therapy.

Conflict of interest

The authors declare no conflict of interest.

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