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Title
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Permalink
https://escholarship.org/uc/item/7gn8z1w0

Journal
Cancer Gene Therapy, 20(12)

ISSN
0929-1903

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Publication Date
2013-12-01

DOI
10.1038/cgt.2013.67

Peer reviewed

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Author manuscript
Cancer Gene Ther. Author manuscript; available in PMC 2021 June 08.

Published in final edited form as:
Cancer Gene Ther. 2013 December ; 20(12): 671–677. doi:10.1038/cgt.2013.67.

Highly efficient tumor transduction and antitumor efficacy in experimental human malignant mesothelioma using replicating gibbon ape leukemia virus

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Abstract

Retroviral replicating vectors (RRVs) have been shown to achieve efficient tumor transduction and enhanced therapeutic benefit in a wide variety of cancer models. Here we evaluated two different RRVs derived from amphotropic murine leukemia virus (AMLV) and gibbon ape leukemia virus (GALV), in human malignant mesothelioma cells. In vitro, both RRVs expressing the green fluorescent protein gene efficiently replicated in most mesothelioma cell lines tested, but not in normal mesothelial cells. Notably, in ACC-MESO-1 mesothelioma cells that were not permissive for AMLV-RRV, the GALV-RRV could spread efficiently in culture and in mice with subcutaneous xenografts by in vivo fluorescence imaging. Next, GALV-RRV expressing the cytosine deaminase prodrug activator gene showed efficient killing of ACC-MESO-1 cells in a prodrug 5-fluorocytosine dose-dependent manner, compared with AMLV-RRV. GALV-RRV-mediated prodrug activator gene therapy achieved significant inhibition of subcutaneous ACC-MESO-1 tumor growth in nude mice. Quantitative reverse transcription PCR demonstrated that ACC-MESO-1 cells express higher PiT-1 (GALV receptor) and lower PiT-2 (AMLV receptor) compared with normal mesothelial cells and other mesothelioma cells, presumably accounting for the distinctive finding that GALV-RRV replicates much more robustly than AMLV-RRV in these cells. These data indicate the potential utility of GALV-RRV-mediated prodrug activator gene therapy in the treatment of mesothelioma.

Keywords

gibbon ape leukemia virus vector; replicating retrovirus vector; malignant mesothelioma; molecular imaging

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INTRODUCTION

Malignant mesothelioma is an aggressive cancer that arises from the mesothelial cells of serous membranes lining the pleural, peritoneal and pericardial cavities. This malignancy is an attractive target for gene therapy based on the following: (1) it is an aggressive neoplasm with poor prognosis (median survival of 1–2 years after initial diagnosis),[1–3] (2) the tumor is usually localized in a cavity, and vectors can be easily administered simply via a drainage tube, which also makes it easy to monitor the status of the lesion treated[3,4] and (3) morbidity and mortality are primarily related to regional tumor extension.[5] Several gene therapy strategies for mesothelioma are being tested in clinical trials; the first trial involved prodrug activator gene therapy mediated by a replication-defective adenoviral vector expressing the herpes simplex virus thymidine kinase gene (HSV-TK), resulting in very limited efficacy.[5,6] Dose-related intratumoral HSV-TK gene transfer was demonstrated in most patients who received intrathoracic administration with the vector, but HSV-TK protein expression proved relatively superficial at tumor surfaces up to 20–50 cell layers deep by immunohistochemical assessment,[6] indicating that even high-titer adenoviral vectors only achieve limited transduction into tumor cells and this is confined to areas surrounding the needle track. It may therefore be technically difficult to achieve efficacious levels of gene delivery by conventional replication-defective vectors, because virus diffusion in the tumor is limited and pleural mesothelioma is usually widely spread over a large area.

The use of replication-competent viruses that are capable of tumor-selective replication represents an emerging technology that shows considerable promise as novel cancer therapeutics.[7,8] Various species of virus have been examined as potential oncolytic agents for treatment of mesothelioma, including adenovirus,[9,10] vaccinia virus,[11] herpes simplex virus[12] and vesicular stomatitis virus.13 Recently, we have demonstrated that amphotropic murine leukemia virus (AMLV)-based retroviral replicating vectors (RRVs) show tumor selectivity because of an inherent and stringent specificity for mitotically active cells.[14] In contrast to other viruses used in cancer virotherapy, AMLV-based RRVs are non-cytolytic by nature, but can be engineered to carry prodrug activator genes, which mediate synchronized cell killing of infected tumor cells upon prodrug administration. Using RRVs expressing the yeast cytosine deaminase (CD) prodrug activator gene, we have demonstrated highly efficient killing of a wide variety of cancer cells both in vitro and in vivo upon administration of its prodrug, 5-fluorocytosine (5FC).[15–19] Based on these promising preclinical results, two clinical trials for RRV-mediated prodrug activator gene therapy in patients with recurrent malignant brain tumor have recently been started in the United States (http://www. clinicaltrials.gov NCT01470794, NCT01156584). In experimental human malignant mesothelioma, after a single intratumoral injection of RRV delivering the CD prodrug activator gene into xenografted human mesothelioma, RRV-mediated prodrug activator gene therapy also achieved significant inhibition of subcutaneous tumor growth, and significantly prolonged survival in the disseminated peritoneal model of malignant mesothelioma.[19]

In addition, we have recently developed another RRV, which is derived from the gibbon ape leukemia virus (GALV).[20] Although GALV and AMLV both belong to the gammaretrovirus genus, they have divergent host ranges and are not in the same interference class,[21,22] as

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these viruses employ different receptors to infect target cells.[20,22,23] GALV and AMLV use distinct proteins as cellular receptors. These proteins, designated as PiT-1 (SLC20A1) and PiT-2 (SLC20A2), respectively, are both mammalian type III inorganic phosphate transporters; orthologs of these proteins are present in all phyla and function as ubiquitously expressed facilitators of phosphate uptake.[20–23]

In the present study, we evaluated the specificity and efficiency of gene delivery and therapeutic efficacy of GALV-RRVs, comparing our previously validated AMLV-RRVs, in experimental human malignant mesothelioma. Our results demonstrate the potential utility of GALV-RRV-mediated prodrug activator gene therapy in the treatment of malignant mesothelioma, especially in cases in which the tumor is not permissive for AMLV-RRV.

MATERIALS AND METHODS

Cell lines

Normal human adult mesothelial cells (NMCs) were purchased from Zen-Bio, Inc. (Research Triangle Park, NC, USA) and maintained in Mesothelial Cell Growth Medium (Zen-Bio, Inc.). Non-malignant human pleural mesothelial cells transformed with SV40 T antigen (Met5A) and three human mesothelioma cell lines, MSTO-211H(MSTO), NCI-H2052 (H2052) and NCI-H2452 (H2452), were obtained from American Type Culture Collection (Manassas, VA, USA). ACC-MESO-1 (MESO1) was purchased from the RIKEN BioResearch Center (Tsukuba, Ibaraki, Japan). These cells were grown in Roswell Park Memorial Institute 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA). Transformed human embryonic kidney 293 and 293 T cell lines were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque) supplemented with 10% fetal bovine serum. Human dermal fibroblasts and their specific media were purchased from Cell Systems (Kirkland, WA, USA). All the cells were grown in 5% CO2 at 37 °C.

Viral vector plasmid and virus production

The AMLV-RRV vector plasmids, pAMLV-green fluorescent protein (GFP) and pAMLV-CD, have been described previously as AZE-emd and AZE-CD, respectively;[24,25] each contains a full-length replication-competent amphotropic AMLV provirus with an internal ribosome entry site (IRES)-transgene cassette (Figures 1a and 3a). The GALV-RRV vector plasmid, pGALV-GFP, described previously as pGS4-GFP,[20] is a replication-competent GALV vector containing an IRES-GFP (Figure 1a). pGALV-CD was generated by replacement of the IRES-GFP sequence in pGALV-GFP with the IRES-CD cassette (Figure 3a). For virus production, 293 T cells were transiently transfected with the vector plasmid using LipofectAMINE 2000 (Life Technologies Japan, Tokyo, Japan), replenished with serum-free medium, and 48 h later the supernatant medium was harvested, filtered and stored frozen at −80 °C.[19] These vectors were prepared side by side and confirmed to have comparable titers by quantitative reverse transcription and the polymerase chain reaction (qPCR) (data not shown). The virus preparation of a self-inactivating replication-defective lentivirus vector expressing GFP, sinSKcmv-EGFP (LV-GFP),[26] was produced by transient co-transfection of 293 T cells as described previously.[15,19,26,27] The titers of these vectors

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were determined by analysis of GFP expression using a FACScalibur flow cytometer (Becton Dickinson Japan, Tokyo, Japan) and expressed as transducing units (TUs) per ml.

Replication kinetics of RRVs in vitro

For analysis of replication kinetics in vitro, virus vector stock was used for infection of various human cell lines at 20% confluency at a multiplicity of infection (MOI) of 0.01. At serial time points, the cells were trypsinized, onefourth of the cells were replated and the remainder analyzed for GFP expression by flow cytometry as above.

Replication kinetics in subcutaneous tumor models

BALB/c-nu/nu (nude) mice (Charles River Japan Co., Yokohama, Japan) were bred and maintained under specific pathogen-free conditions, and all studies conducted under protocols approved by the Hyogo College of Medicine Animal Research Committee. Human malignant mesothelioma MSTO or MESO1 xenografts were established in 6- to 8-week-old female nude mice by subcutaneous inoculation of 1×10^6 tumor cells into the right dorsal flank. When tumors reached a diameter of B5 mm, three groups of mice (n=8 per group) were injected intratumorally with 1×10^4 TU of LV-GFP, AMLV-GFP or GALV-GFP on day 0. Over sequential days, mice were anesthetized with sodium pentobarbital, and spectral fluorescence imaging was performed using the Maestro in vivo fluorescence imaging system (Cambridge Research and Instrumentation, Woburn, MA, USA), as previously described.[19,28,29] For detection of GFP fluorescence (maximum excitation 488 nm; maximum emission 507 nm), whole body images (0.05- to 0.5-s exposure) were captured at a 500–600nm range in 10nm steps with a band-pass filter from 445 to 490nm and a long-pass filter over 515 nm. All images were analyzed by Maestro 2.2 software in order to create spectral unmixed images of fluorescein and autofluorescence. To evaluate the increase of fluorescence intensities in tumors, the total signal intensities (scaled counts/sec) were divided by tumor volumes (mm^3).[19]

Cytotoxicity assay in vitro

For quantitative analysis of the drug cytotoxicity, triplicate wells containing human NMC, Met5A, H2052, H2452, MSTO or MESO1 cells (1×10^4 cells per well), which had been pretransduced with AMLV-CD or GALV-CD at an MOI of 0.01 and maintained for 15 days, were cultured in 96-well tissue-culture plates with various concentrations of 5FC. On day 3, the viable cell numbers of triplicate cultures were measured by the Alamar Blue method according to the manufacturer’s instructions (Alamar Biosciences, Inc., Sacramento, CA, USA). Briefly, 40 ml of Alamar Blue was aseptically added to the cultures, which were then returned to the incubator for 3 h; fluorescence was measured by an ARVO X4 multilabel plate reader with a 544nm excitation wavelength and a 590nm emission wavelength (Perkin Elmer Japan, Tokyo, Japan). The percentage of viable cells was determined by calculation of the fluorescence of viable cells as measured against wells containing no 5FC.

Prodrug activator gene therapy in subcutaneous tumor models

MESO1 cells were grown subcutaneously in nude mice to a diameter of 5–6mm as above, and three groups of mice (n=10 per group) were then injected intratumorally with 50 ml of

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phosphate-buffered saline, AMLV-CD or GALV-CD on day 0, followed by intraperitoneal administration of either 5FC (500mgkg^{-1} day^{-1}) every other day from day 14 to day 28. The mice were observed closely and the tumors were measured twice a week. The tumor volume was calculated as $a \times b^2 \times 0.5$, where a and b were the largest and smallest diameters, respectively.

PiT-1 and PiT-2 mRNA expression profile analysis

The expression of PiT-1 and PiT-2 mRNA in cell lines was analyzed by qPCR. Total RNA was extracted from semiconfluent cell cultures on 10 cm dishes using the ISOGEN RNA extraction solution (Nippon Gene, Tokyo, Japan), whereupon it was treated with DNase to remove genomic DNA contamination. The RNA was analyzed by qPCR for expression of PiT-1, PiT-2 and glyceraldehyde-3-phosphate dehydrogenase RNA with the Taqman One-step RT-PCR master mix reagent kit (Applied Biosystems Japan Ltd., Tokyo, Japan) as described by the manufacturer. The primers and TaqMan probe for PiT-1 (Hs00965587_m1), PiT-2 (Hs00198840_m1) and glyceraldehyde 3-phosphate dehydrogenase (Hs99999905_m1) were purchased from Applied Biosystems Japan Ltd. (Taqman gene expression assays). Briefly, 20 ng of total RNA was added to the reaction mixture (TaqMan one-step RT-PCR master mix reagents), containing 18 pmol of each of the primers and 5 pmol of the probe, and amplified for 1 cycle of 30 min at 48 °C and 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C.

Statistical analysis

The results are presented as the mean ± s.d. Statistical significance of differences was calculated using Student's *t*-test, and a P value of <0.01 was considered significant in all analyses.

RESULTS

GALV vectors replicate efficiently in human malignant mesothelioma cell lines in vitro

To evaluate replication kinetics in human malignant mesothelioma cells, we first used the AMLV-based RRV vector (AMLV-GFP) and GALV-based RRV vector (GALV-GFP); both RRVs contain an IRES-GFP cassette inserted precisely at the env-3' untranslated region boundary (Figure 1a). GFP expression was monitored by flow cytometry at serial time points after inoculation of cells with AMLVGFP or GALV-GFP at an MOI of 0.01 (Figure 1b). In H2052, H2452 and MSTO cells, the two RRVs showed similar replicative kinetics; the percentage of GFP-expressing cells quickly increased in a logarithmic manner and reached 480% within 16 days and 497% within 20 days after virus inoculation, and then remained stable thereafter. In contrast, in MESO1 cells inoculated with GALV-GFP, GFP-expressing cells quickly increased and reached 495% within 12 days after virus inoculation, whereas GFP-expressing cells after AMLV-GFP inoculation remained less than 20% at day 40. Thus, the GALV vector exhibited a far greater proclivity for transduction of MESO1 cells. In addition, in NMCs and non-malignant transformed pleural mesothelial cells (Met5A), both RRVs exhibited severely attenuated infectivities (Figure 1b), confirming that RRV spread is highly selective for malignant mesothelioma cell lines and the nonmalignant cells are non-permissive for retroviral replication.

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GALV vectors replicate efficiently in human malignant mesothelioma cell lines in vivo

To examine the spread of RRV vectors in vivo, nude mice with subcutaneous MSTO or MESO1 tumors received a single intratumoral injection of GALV-GFP, AMLV-GFP or LV-GFP replication-defective lentiviral vector on day 0, and GFP expression was monitored at serial time points thereafter by in vivo fluorescence imaging (Figure 2). In both MSTO- and MESO-1-inoculated group of mice injected with the replication-defective lentiviral vector, LV-GFP, intratumoral GFP expression was observed on week 1, and proportionally increased with an increase in tumor size (Figures 2a and c). In MSTO tumor-bearing animals injected with either AMLV-GFP or GALV-GFP, intratumoral GFP expression increased robustly and reached throughout the entire tumors by week 3, without detectable signal in extratumoral normal tissue (Figure 2a). The fluorescence intensities were normalized to the tumor volumes and found to be highly increased in both AMLVGFP- and GALV-GFP-injected tumors compared with LV-GFPinjected tumors (Figure 2b), indicating efficient RRV spread in these tumors. On week 4, tumors were harvested, immediately digested with collagenase into cell suspensions, and the percentage of GFP-positive cells was 7.9±4.1% with LV-GFP, 92.7±6.1% with AMLV-GFP and 94.4±5.8% with GALV-GFP by flow cytometry.

In MESO1-bearing animals injected with AMLV-GFP, intratumoral GFP expression was not observed by week 4 (Figures 2c and d), consistent with the results that MESO1 was not permissive for AMLV-RRV (Figure 1b). However, GALV-GFP spread robustly and reached throughout entire tumor by week 3, (Figure 2c). The fluorescence intensities also increased highly in the GALV-GFPinjected tumors compared with AMLV-GFP- and LV-GFP-injected tumors (Figure 2d), indicating efficient GALV-RRV spread in these tumors. On week 4, tumors were harvested, immediately digested with collagenase into cell suspensions, and the percentage of GFP-positive cells was 4.7±2.7% with LV-GFP, 1.1±0.2% with AMLV-GFP and 97.3±4.9% with GALV-GFP.

These results were consistent with the results *in vitro* RRV replication kinetics in MSTO and MESO1, and indicate that the GALV-RRV vector was also capable of efficient replication and progressive spread through MESO1 mesothelioma tumors *in vivo*.

Prodrug activator gene-mediated cell killing effect of RRVs in human malignant mesothelioma cells

To investigate the efficacy of RRV-mediated prodrug activator gene therapy in human malignant mesothelioma cells, we used AMLV-CD and GALV-CD, which express the *CD* prodrug activator gene (Figure 3a). The human mesothelioma cells were infected with AMLV-CD or GALV-CD at an MOI of 0.01 on day 0 and exposed to the 5FC prodrug at various concentrations from day 15 for 3 days. On day 18, cell viability was examined by Alamar blue assay. In H2052, H2452 and MSTO cells, decreased cell viability was observed in both AMLV-CD and GALV-CD-transduced cultures in a 5FC dose-dependent manner (Figure 3b). At the highest 5FC dose level (10 mM), the viability of all RRV-transduced malignant mesothelioma cells was significantly decreased to <10%, whereas RRV-transduced non-malignant Met5A cells showed significantly higher levels of survival (~80%). In contrast, in MESO1 cells, decreased cell viability was observed significantly higher in GALV-CD-transduced cultures than AMLV-CD (Figure 3b). At the 10 mM 5FC

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dose level, the viability of GALV-CD-transduced MESO1 cells was significantly decreased to (11.9±4.4%), compared with AMLV-CD (83.3±12.7%).

These results were consistent with *in vitro* RRV-GFP replication kinetics in these cells (Figure 1b), and indicate that the GALV-CD vector could achieve selective spread and cytotoxicity in malignant mesothelioma cells, as well as enhanced transduction efficiency by using a different physical binding mechanism.

GALV-mediated CD/5FC prodrug activator gene therapy shows potent *in vivo* antitumor effects in subcutaneous human mesothelioma xenograft models

To examine the antitumoral therapeutic efficacy of RRV-mediated CD/5FC prodrug activator gene therapy, nude mice bearing established MESO1 tumors were treated with a single intratumoral injection (1×10^4 TU total dose) of either AMLV-CD or GALV-CD, or of phosphate-buffered saline vehicle control on day 0, followed by intraperitoneal administration of 5FC. As shown in Figure 4, subcutaneous tumors treated with AMLV-CD showed no obvious inhibition of tumor growth after 5FC administration by day 28 post-infection (3171.0 ± 485.8 mm^3), as compared with the phos- phate-buffered saline control group (3209.2 ± 510.7 mm^3). In contrast, the growth of GALV-CD transduced tumors was significantly inhibited by 5FC treatment by day 28 post-infection (1715.5 ± 212.7 mm^3, $P<0.01$ vs AMLV-CD group). Thus, GALV- mediated prodrug activator gene therapy could achieve effective *in vivo* growth inhibition in this subcutaneous MESO1 model of human malignant mesothelioma.

mRNA expression of PiT-1 and PiT-2 in human cell lines

To elucidate the difference in replicative spread of GALV-RRV and AMLV-RRV in MESO-1 cells, we analyzed the expression levels of cellular receptors for GALV (PiT-1) and AMLV (PiT-2) in human mesothelioma cell lines by quantitative RT-PCR. In MESO1 cells, high PiT-1 but low PiT-2 expression were observed, presumably leading to distinctive findings that GALV-RRV predominates over AMLV-RRV in these cells.

In addition, PiT-1 mRNA was expressed in all of the four mesothelioma cell lines tested (H2052, H2452, MSTO and MESO1), but was low in NMC and Met5A (Figure 5). In contrast, PiT-2 mRNA was higher in NMC and Met5A than in mesothelioma cell lines. These results suggested an advantage of GALV-RRV over AMLVRRV for targeting human mesothelioma in order to ensure safety and efficacy.

DISCUSSION

For the ultimate success of cancer gene therapy, improved overall transduction efficiency throughout the entire tumor is critically required. The use of replication-competent viruses represents an emerging technology with the potential to achieve highly efficient gene transfer to tumors, as each successfully transduced tumor cell itself becomes a virus-producing cell, sustaining further transduction events even after initial administration.7,8 In contrast to various other replicating viruses now in development as naturally oncolytic agents, RRVs can replicate without immediate lysis of host cells and can spread via direct

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cell-tocell budding, and may be less likely to elicit robust immune responses that prematurely terminate virus propagation.

Replicative spread of RRVs in solid tumors is potentially affected by many factors, including antiviral innate immunity, acquired immunity and cellular receptor expression levels. In this study, we investigated cellular receptor expression and found high PiT-1 but low PiT-2 expression in MESO1 cells (Figure 5), presumably leading to the distinctive finding that GALV-RRV predominates over AMLVRRV in these cells. In this case, GALV-RRV is an alternate to AMLVRRV in MESO1 cells and could be vice versa with regard to other cancer cells. Therefore, to line up multiple different RRVs that use different receptors may be practically useful for RRV-mediated prodrug activator gene therapy against many types of solid tumors, thereby allowing a tailor-made virotherapy on the basis of the cellular receptor expression.

As a cancer therapeutic agents, RRV-mediated prodrug activator gene therapy would be the ultimate ‘intracellular’ chemotherapy. However, cancer eventually becomes resistant to any one chemotherapeutic agent. In an ideal clinical setting, therefore, combination regimens will be essential for long-term success, thereby reducing the risk of resistance as well as presumably enhancing antitumor effect by addictive or synergetic effects. Previously, coinfections with AMLV- and GALV-RRV have been shown in MDA-MB-435 human breast cancer cells in vitro, demonstrating that dual-vector prodrug activator gene therapy could achieve synergistic cytotoxic efficacy than single-vector gene therapy.[23] Thus, coinfection of cancer cells with AMLV and GALV vectors supplied with different prodrug activator genes may be employed to achieve combination intracellular chemotherapy, leading to the enhancing cytotoxic effects. When bystander effects are also taken into consideration, such high levels of tumor transduction may allow sufficient tumor cell killing to destroy dormant cancer stem cells by prodrug activator gene therapy.

As another solution to further improve the transduction efficiency of RRV vectors, we previously developed a novel method in which high-titer adenovirus was employed to deliver RRV vectors. These chimeric vector systems (adenovirus-retrovirus hybrid vectors) exhibited significantly higher initial transduction and higher levels of second-stage RRV production in situ, subsequently leading to accelerated RRV vector spread, and thereby achieving enhanced therapeutic efficacy of prodrug activator gene therapy for cancer.[30] This hybrid vector system may also have additional advantages for combined tumor targeting via fiber modification of the first-stage adenovirus[31,32] and transcriptional regulation of the second-stage RRV vectors.[33]

Gammaretroviruses contain no nuclear localization signals in their capsid and can only infect cells that are actively dividing.[14] This absolute selectivity for dividing cells will be of significant utility for RRV vector replication in solid tumors. There will be little risk of inadvertently transducing normal quiescent epithelium or stroma, whereas actively dividing tumor neovasculature or cancerassociated hyperproliferative stroma may also provide a potentially useful target. Also, retroviral replication is inhibited even in actively dividing normal cells, which have intact innate antiviral immune mechanisms, as indicated by restriction of both AMLV-RRV and GALV-RRV in the non-malignant cell lines.

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Accordingly, we have previously shown that RRVs achieve highly tumor-selective replication.[15–19,23] In this study, we showed that PiT-1 mRNA is expressed in all of the four mesothelioma cell lines tested (H2052, H2452, MSTO and MESO1), but is low in normal cells (NMC and Met5A; Figure 5). In contrast, PiT-2 mRNA was higher in NMC and Met5A than mesothelioma cell lines. This differential expression of PiT-1 and PiT-2 indicates an advantage of GALV-RRV over AMLV-RRV for targeting human mesothelioma in order to ensure safety and efficacy. For more precise tumor targeting, we have indeed been developing RRV vectors that are targeted specifically to cancer cells. Our initial strategy has been to limit vector replication through transcriptional control of the retroviral RNA genome using cell type-specific promoters, for example, probasin promoter, which directs high level expression selectively upon androgen induction in prostate cells. We have demonstrated proof-of-concept for this strategy, achieving stringent targeting of RRV vector replication and transgene expression to relatively well-differentiated prostate cancer cells.[33]

In conclusion, our results show that GALV-RRV vectors can efficiently replicate and achieve significant levels of tumor transduction in human malignant mesothelioma cells, with minimal spread in normal human mesothelial cells, and without detectable spread to normal cells in vivo. The present study represents the first report to show efficient transduction of human malignant mesothelioma cells by GALV-based RRV and the therapeutic efficacy of GALV-RRV-mediated CD/5FC prodrug activator gene therapy in the mesothelioma model, suggests a new treatment paradigm for malignant mesothelioma.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (25460484; SK); MEXT-Supported Program for the Strategic Research Foundation at Private Universities (SK); a Pilot and Feasibility Study Award through NIDDK P30 DK 41301 (CRL) and NIH R01CA121258 (NK).

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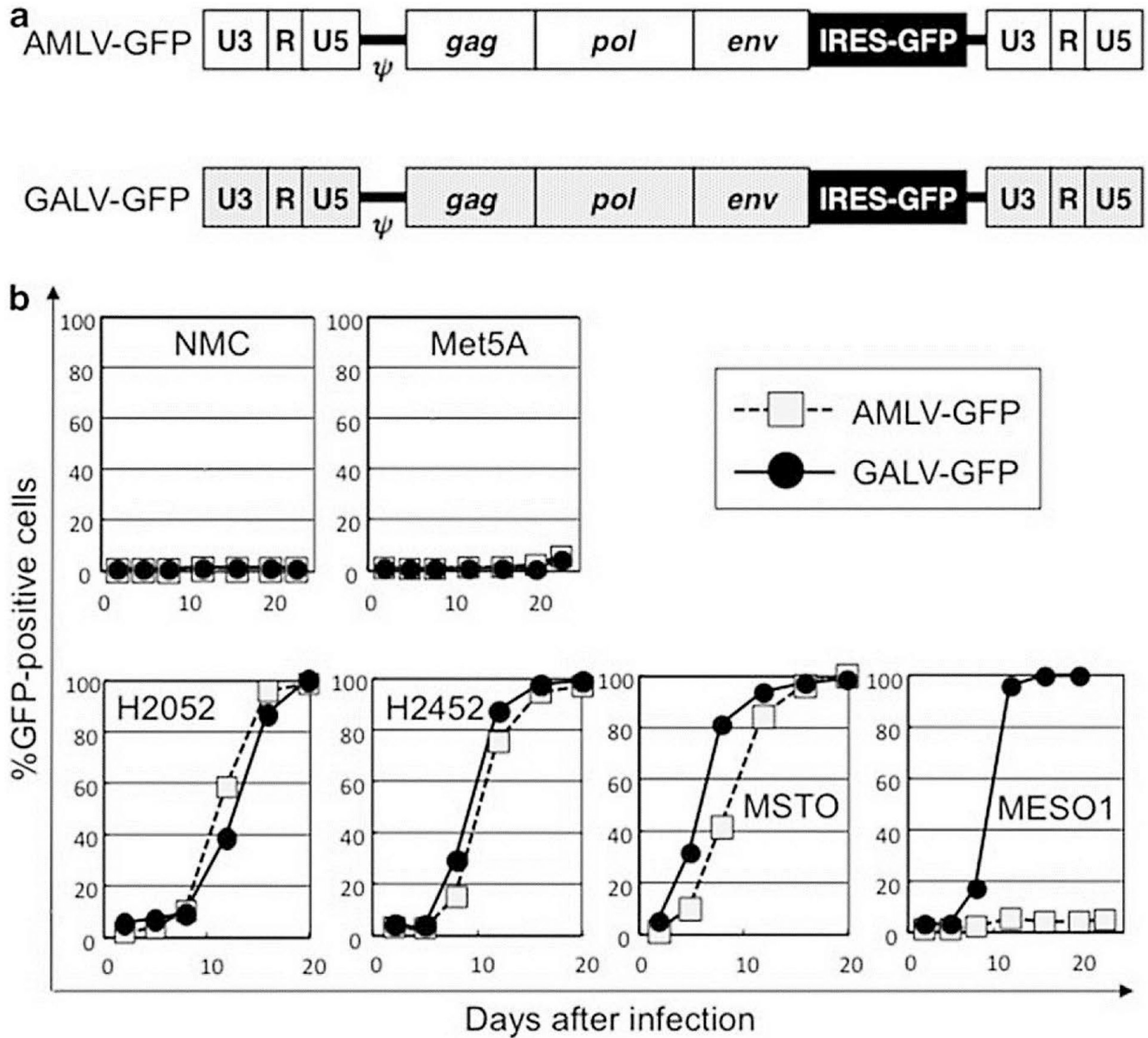

Figure 1.

Replication kinetics of amphotropic murine leukemia virus (AMLV) vs gibbon ape leukemia virus (GALV) vectors in human mesothelioma cells. (a) Schematic structure of AMLV-GFP and GALV-GFP vectors. These vectors contain a full-length replication-competent AMLV or GALV provirus, in which an internal ribosome entry site (IRES)-green fluorescent protein (GFP) cassette has been inserted between the *env* gene and 3'-untranslated region. *Ψ*, packaging signal; *gag-pol*, AMLV or GALV structural genes. (b) Replication kinetics of AMLV vs GALV vectors in human mesothelioma cells. Human non-malignant mesothelial cells (NMC and Met5A) and mesothelioma cells (H2052, H2452, MSTO and MESO1) were inoculated with AMLV-GFP or GALV-GFP vector at a multiplicity of infection of 0.01. On the days of passage, cells were analyzed for GFP expression by flow cytometry. Data are representative of three independent experiments, all yielding similar results.

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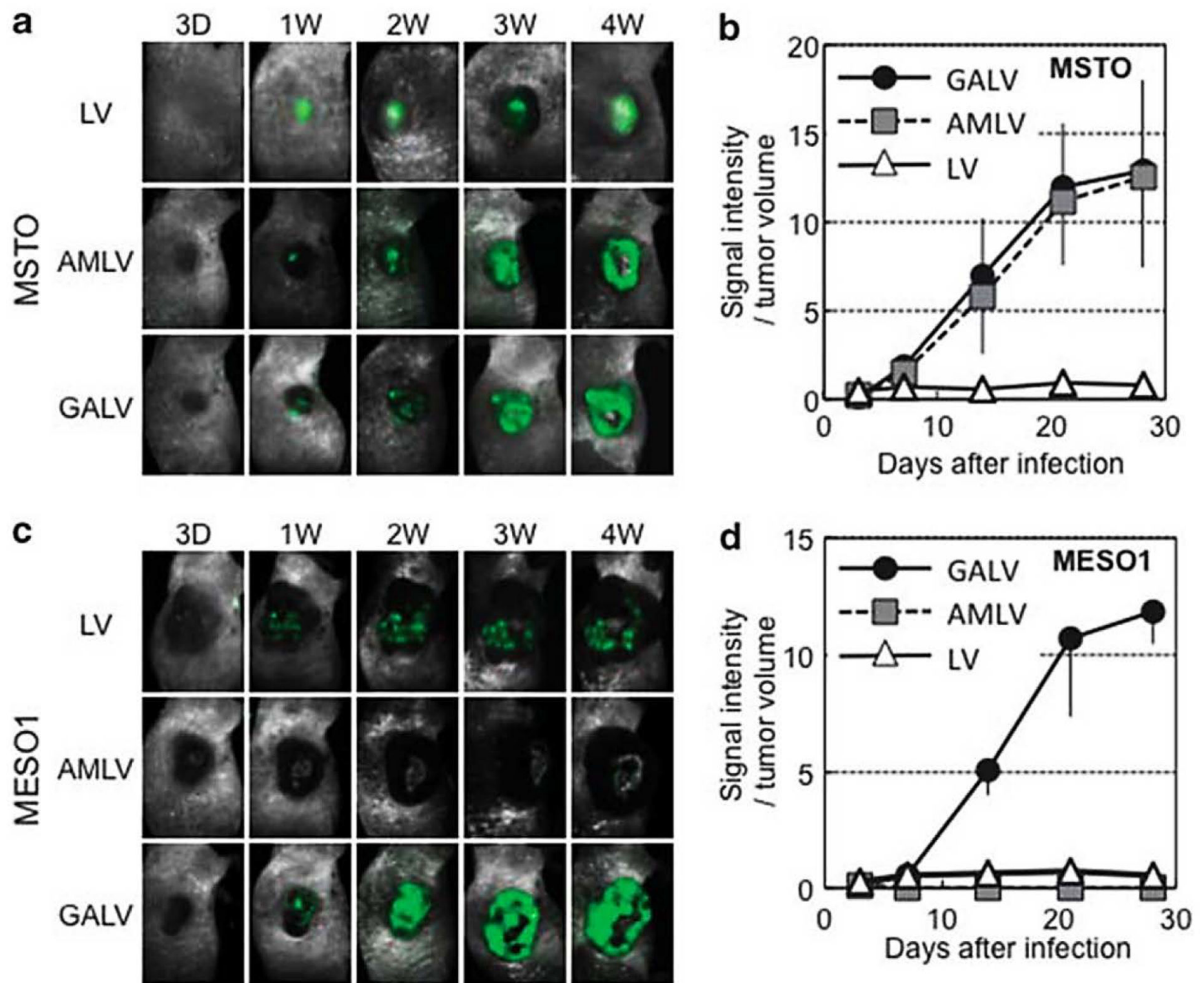

Figure 2.

In vivo spread of amphotropic murine leukemia virus (AMLV) vs gibbon ape leukemia virus (GALV) vectors in malignant mesothelioma xenograft tumors. (a) *In vivo* fluorescence imaging. Human malignant mesothelioma MSTO tumors were grown subcutaneously in nude mice to 5–6mm in diameter, and injected intratumorally with LV-GFP (replication-deficient lentiviral vector), AMLV-GFP or GALV-GFP on day 0 (n=8 per group). At different time points indicated in the figure, whole body images (0.05- to 0.5-s exposure) were taken and analyzed by *in vivo* fluorescence imaging system. Representative images are shown from each group. (b) Comparison of the fluorescence intensities in MSTO tumors injected with LV-GFP, AMLV-GFP or GALV-GFP. The fluorescence intensities were normalized to the tumor volumes. Data shown are averages – (AMLV-GFP) or + (LV-GFP and GALV-GFP) s.d. from experiments (n=8 per group). (c) *In vivo* fluorescence imaging. Subcutaneous MESO1 tumors established in nude mice were injected intratumorally with LV-GFP, AMLV-GFP or GALV-GFP on day 0 (n=8 per group). Representative images are shown from each group. (d) Comparison of the fluorescence intensities in MESO1 tumors injected with LV-GFP, AMLV-GFP or GALV-GFP. The fluorescence intensities were

normalized to the tumor volumes. Data shown are averages – (GALV-GFP) or + (LV-GFP and AMLV-GFP) s.d. from experiments (n=8 per group). D, day; W, week.

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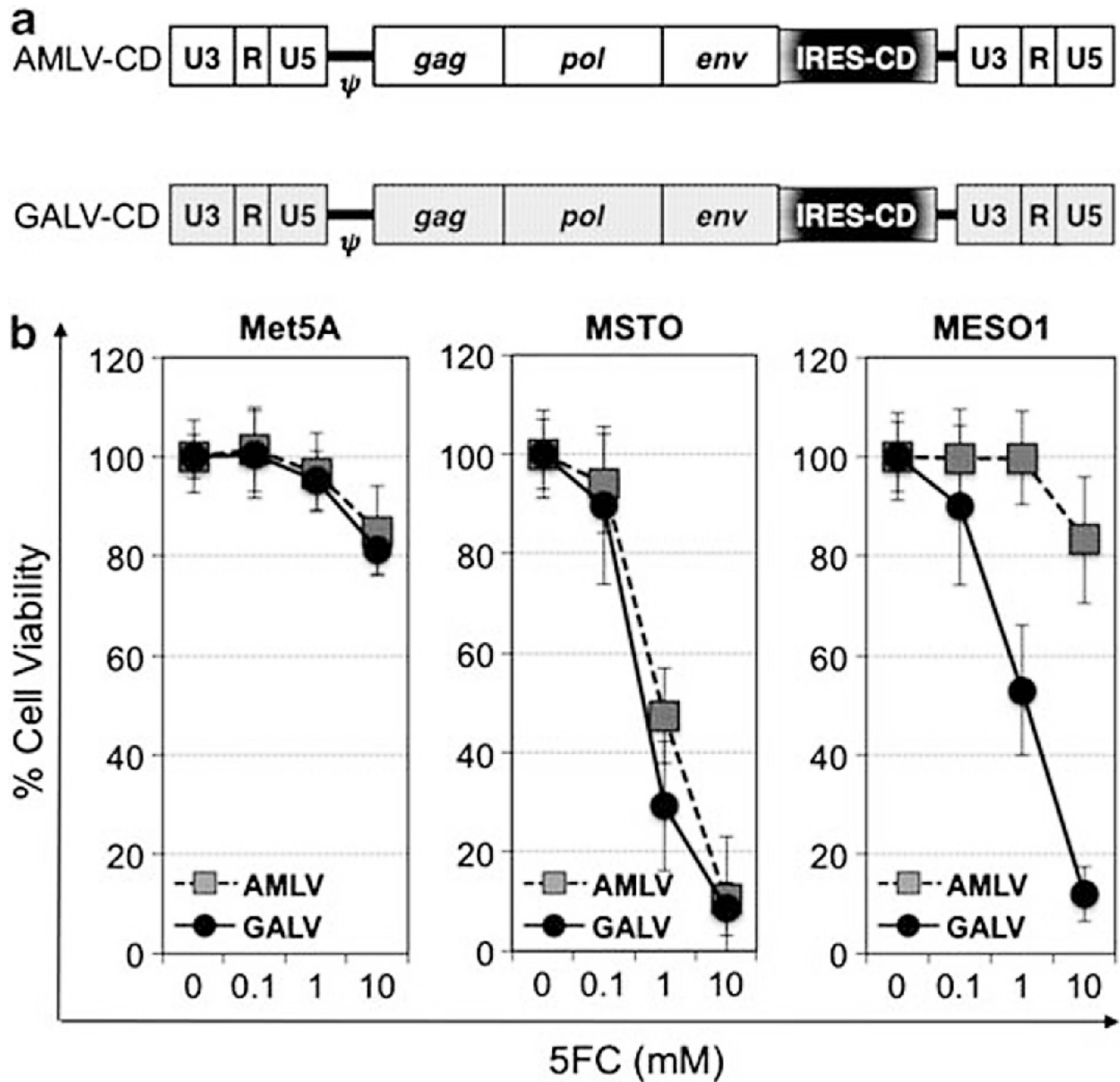

Figure 3.

Prodrug activator gene-mediated cell killing effect after amphotropic murine leukemia virus (AMLV) vs gibbon ape leukemia virus (GALV) infection in vitro. (a) Schematic structure of AMLV-CD and GALV-CD vector. These vectors were created by replacement of the internal ribosome entry site (IRES)-green fluorescent protein (GFP) cassette of AMLV-GFP and GALV-GFP with IRES-CD, respectively. *CD*, yeast cytosine deaminase prodrug activator gene. (b) Cell viability of Met5A, MSTO and MESO1 cells on day 18 after infection at a multiplicity of infection of 0.01 with AMLV-CD or GALV-CD. Data shown are averages ± s.d. from experiments performed in triplicate. 5FC, 5-fluorocytosine.

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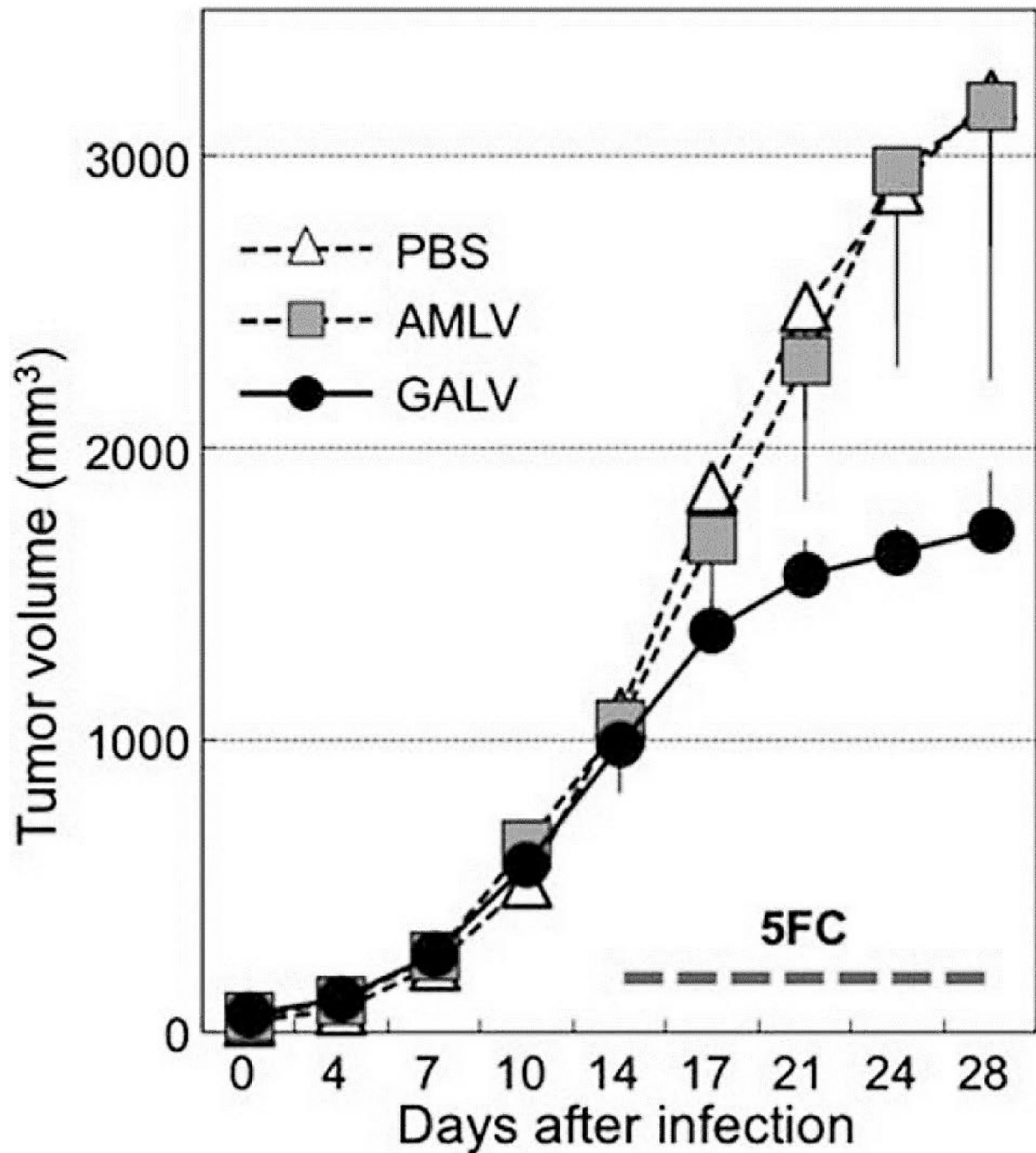

Figure 4.

In vivo antitumor effect of amphotropic murine leukemia virus (AMLV)- vs gibbon ape leukemia virus (GALV)-mediated prodrug activator gene therapy in subcutaneous xenograft model of human malignant mesothelioma. MESO1 tumors were grown subcutaneously in nude mice to 5–6mm in diameter, and injected intratumorally with 1×10^4 TU (50 ml) of either AMLV-CD or GALV-CD, or phosphate-buffered saline (PBS) vehicle control on day 0, followed by intraperitoneal administration of 5-fluorocytosine (5FC; 500mgkg^{-1} day^{-1}) every other day from day 14 to day 28 (n=10 per group). Tumor volumes were measured twice a week, and data shown are averages – (PBS and AMLV-GFP) or + (GALV-GFP) s.d. from experiments.

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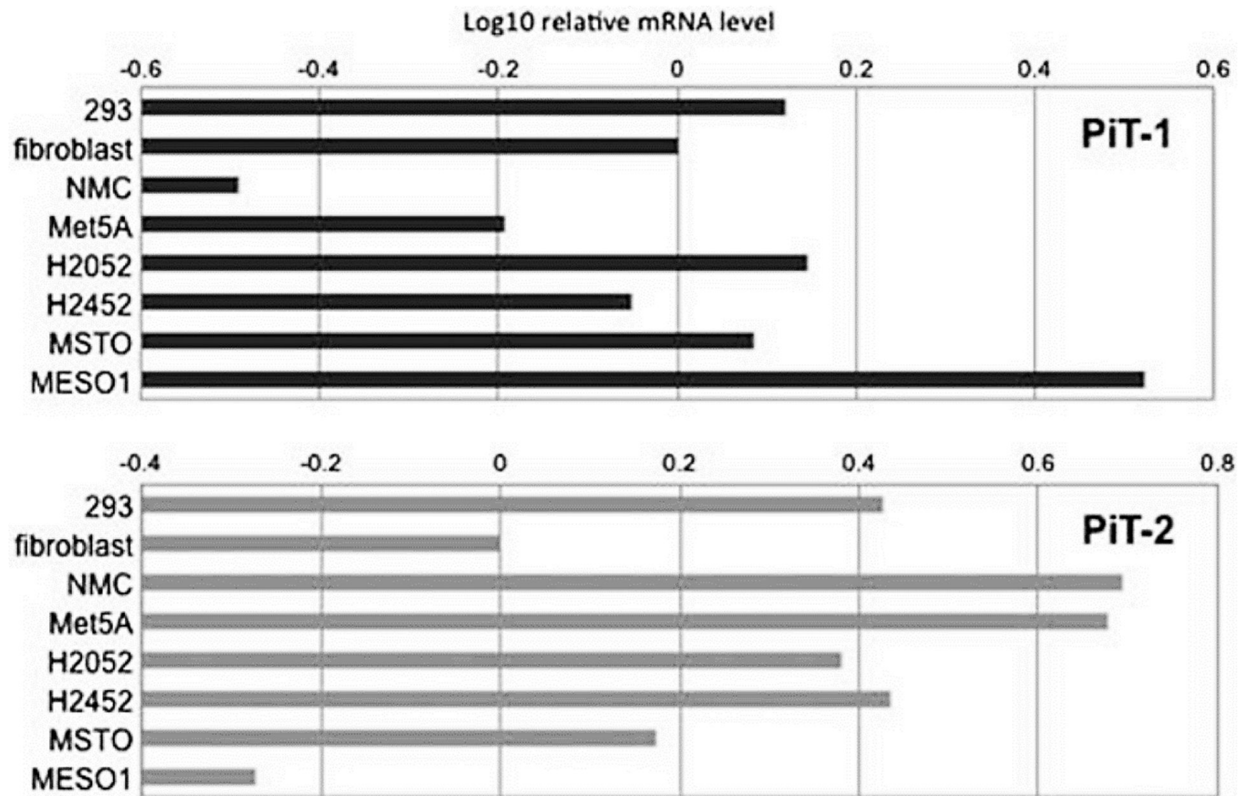

Figure 5.

Relative messenger RNA levels of PiT-1 and PiT-2 in cell lines by quantitative reverse transcription PCR. Total RNA was extracted from various human cells, including non-malignant human cell lines (293, fibroblast, NMC, Met5A) and malignant mesothelioma cell lines (H2052, H2452, MSTO and MESO1). The RNA was reverse-transcribed and amplified by PCR with specific primers for PiT-2, PiT-1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH was used as an endogenous RNA control to normalize for differences in the amount of total RNA.