Pro-opiomelanocortin gene delivery suppresses the growth of established Lewis lung carcinoma through a melanocortin-1 receptor-independent pathway

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Abstract

Background  Pro-opiomelanocortin (POMC) is the precursor of several neuropeptides, such as corticotropin, melanocyte-stimulating hormone and the endogenous opioid (β-endorphin). Our previous studies have indicated that POMC gene delivery inhibited the progression and metastasis of B16-F10 melanoma via the α-melanocyte-stimulating hormone/melanocortin-1 receptor (MC1R) pathway.

Methods  In the present study, the therapeutic efficacy of POMC gene therapy was evaluated in mice bearing established Lewis lung carcinoma (LLC) models both in vitro and in vivo. We also investigated the MC1R-independent mechanism underlying POMC gene therapy.

Results  We found that POMC gene delivery significantly inhibited the growth and colony formation in MC1R-deficient LLC cells. In addition, POMC gene transfer effectively suppressed the growth of established LLC in mice. The inhibitory mechanisms underlying POMC gene delivery were attributed to be inhibition of proliferation and the induction of apoptosis. Moreover, POMC gene delivery attenuated tumor β-catenin signaling by reducing protein levels of β-catenin and its downstream proto-oncogenes, including cyclin D1 and c-myc. Lastly, POMC gene delivery induced a significant suppression of tumor vasculature.

Conclusions  These results support the existence of an MC1R-independent pathway for POMC gene therapy, which further expands the therapeutic spectrum of POMC therapy for multiple types of cancer. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords  POMC; MC1R; β-catenin; gene therapy; lung cancer

Introduction

Pro-opiomelanocortin (POMC), which is a multifunctional, poly cistronic gene located on human chromosome 2p23, was found in 1979 by the cloning of bovine POMC cDNA [1]. POMC is a 31-kDa prohormone that is processed to various neuropeptides including adrenocorticotropic hormone (ACTH), melanotrophins [α-, β- and γ-melanocyte-stimulating hormone (MSH)], lipotropins and β-endorphin [2]. The POMC-derived peptides have been detected in the hypothalamus, pituitary and periphery, including the immune system, spleen, lung, melanocytes and the gastrointestinal tract [3]. The melanocortin pathway has been implicated in the regulation of a diverse set of physiological functions, including obesity, inflammation, sexual function, pigmentation, cardiovascular and steroidogenesis [4].
The melanocortins exert their effects by activating melanocortin receptors (MCRs), the smallest family of G-protein-coupled seven transmembrane receptors [5]. Five melanocortin receptors (MC-1R to MC-5R) have been identified in mammals [6]. All MCRs are coupled to adenylyl cyclase and their effects are mediated primarily by activating a cyclic AMP (cAMP)-dependent signaling pathway. Agonists α-MSH and ACTH and an antagonist agouti are some of the signaling proteins in human melanocytes that have high binding affinities to MC-1R [7]. The significance of skin pigmentation lies in the photoprotective effect of melanin against sun-induced carcinogenesis [8]. Moreover, a study by Gerstenblith et al. [9] showed that the gene for MC-1R was highly polymorphic in human populations, and allelic variation at this locus accounted, to a large extent, for the variation in pigmentation phenotypes and skin phototypes in humans. Some of these variants have been associated with specific hair and skin colour phenotypes, the presence of freckling, and melanoma and nonmelanoma skin cancer risk [9].

In a previous study, POMC gene delivery suppressed the tumorigenic processes, including anchorage-independent growth, migration and adhesion, in B16-F10 melanoma cells [10]. Recently, we showed that systemic POMC gene therapy induced re-differentiation of melanoma and attenuated tumor progression through MC-1R/cAMP pathway [11]. In the present study, we first evaluated whether POMC gene therapy suppressed tumorigenesis in lung cancer cells without functional MC-1R signaling. Subsequently, we investigated the MC-1R-independent mechanism underlying POMC gene therapy.

Materials and methods

Cell cultures

Mouse Lewis lung carcinoma (LLC) cells and mouse B16-F10 melanoma were purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) medium containing 10% fetal bovine serum (FBS; PAA, Pasching, Austria), 2 mM glutamine, 100 mg/ml streptomycin (Invitrogen) and 100 U/ml penicillin at 37°C in 5% CO2 atmosphere. Human microvascular endothelial cells (HMEC) were obtained from Lonza Inc. (Basel, Switzerland). Human umbilical vein endothelial cells (HUVEC) were obtained from ATCC. Cells were cultured in EGM-2 BulletKit (Cambrex Corp., East Rutherford, NJ, USA) containing 10% FBS at 37°C in 5% CO2 atmosphere. α-MSH, and SHU9119 were purchased from Sigma-Aldrich (St Louis, MO, USA).

Preparation of adenoviral vectors

For Ad-POMC and Ad-Laz construction, both POMC and Escherichia coli β-galactosidase (Laz) cDNA were subcloned into pShuttle-CMV to yield the transfer vector. This was then recombined with entire adenovirus genome in BJ5183 E. coli cells and transfected into 293 cells to generate recombinant virus via homologous recombination by a calcium phosphate protocol (AdEasy XL adenoviral Vector System; Stratagene, La Jolla, CA, USA). The virus was then amplified in 293 cells, purified by two rounds of cesium chloride gradient ultracentrifugation, and dialysed against buffer containing 10 mM Tris (pH 7.5), 1 mM MgCl2 and 10% glycerol at 4°C. The titer of virus solution was determined by measuring optical density at wavelength of 260 nm and plaque-forming assay in 293 cells before storage at −80°C. The enhanced green fluorescent protein (EGFP) cDNA was obtained from the pEGFP-1 (Clontech, Palo Alto, CA, USA) and prepared as described previously [12].

Western blot analysis

The protein extract was isolated from tissue or cells using buffer containing 150 mM NaCl, 50 mM HEPES (pH 7), 1% Triton X-100, 10% glycerol, 1.5 mM MgCl2, 1 mM ethylen glycol tetraacetic acid and protease inhibitors (Roche Applied Science, Indianapolis, IN, USA). After separation in 12.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis, protein was transferred onto polyvinylidene fluoride membrane using blotting apparatus. The membrane was blocked with 5% milk in Tris-buffered saline (TBS)-TWEEN 20 for 1 h and then incubated with MC-1–5R (dilution 1: 500 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), β-catenin (dilution 1:500; Cell Signaling Technology, Inc., Beverly, MA, USA), bcl-2 (dilution 1:500; Cell Signaling Technology, Inc.), c-myc (dilution 1:500; Cell Signaling Technology, Inc.) and cyclin D1 (dilution 1:500; Cell Signaling Technology, Inc.) antibodies for overnight at 4°C. After incubation with secondary antibody conjugated with horseradish peroxidase (HRP) (dilution 1:5000 in 5% milk) for 30 min, the signals on membrane were detected using ECL-plus luminal solution (Pharmacia, Piscataway, NJ, USA) and exposed to X-ray film for an autoradiogram. Digital images were quantified using Image ProPlus software (Media Cybernetics, Inc., Bethesda, MD, USA) and no modification was made to the analyzed images. Band optical density was determined relative to background levels taken from immediately above or below the band of interest within the same lane. Each experiment was carried out independently, a minimum of three times; two separate film exposures were examined for each experiment. Values obtained were normalized to an internal standard for comparison between experiments.

Real-time and semi-quantitative polymerase chain reaction (PCR)

RNA was isolated from B16-F10 and LLC cells using RNAzol (TEL-TEST, Inc., Friendswoods, TX, USA). For reverse transcription, 5 μg of total RNA was used for reverse
transcription with Superscript III (Invitrogen) using olio-dT and random primers.

For quantitative real-time PCR, reactions were performed in an ABI Prism 7700 system (Applied Biosystems, Foster City, CA, USA) using the TaqMan Universal PCR master mix and the predesigned gene-specific probe and primer sets for mouse MC-1R (Mm00434851_s1). Data were normalized to GAPDH (4326317E) and expressed as fold changes over that in the control group (B16-F10).

For semi-quantitative PCR, amplification and detection were performed by: one cycle of 95°C for 10 min, 35 cycles of 95°C for 5 s, and 60°C for 30 s; followed by one cycle of 95°C for 5 s, 57°C for 30 s, and 72°C for 30 s in a Thermal cycler (Bio-Rad-DNA Engine; Bio-Rad, Hercules, CA, USA). The primer sequences for mouse MC-1R were:

forward 5′-CATCATTGCTATAGACCGCTACAT-3′, which amplified a 295-bp fragment.
reverse 5′-AAACCCACACTGTGCCTATC-3′, which amplified a 158-bp fragment.

The β-actin mRNA level was determined using forward 5′-TCACCCACACTGTGCCTATC-3′ and reverse 5′-CAGCGGAACCGCTACATTGC-3′, which amplified a 295-bp fragment.

**Cell proliferation assay**

The effect of POMC gene transfer on the viability of various cells was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [13]. Briefly, LLC cells were cultured in a 96-well plate at a density of 4 × 10^4 cells/ml. After infection with adenovirus vectors, cells were incubated for 48 h. Cells were supplemented with fresh medium containing MTT (0.456 mg/ml) and incubated for 2 h at 37°C. The formazan in viable cells was dissolved with 100 μl of dimethyl sulfoxide and determined by reading optical densities in a microplate reader (Dynex Technologies Inc., Chantilly, VA, USA)

**Cell cycle analysis**

LLC cells were infected with adenovirus vectors at a multiplicity of infection (MOI) of 1000. After 12 h, cells were supplemented with fresh medium and continued to incubate at 37°C for an additional 48 h. The harvested cells were washed twice with PBS before fixation with ice-cold 70% ethanol and storage overnight at −20°C. Cell aliquots were again washed twice with PBS before incubation with RNase A (10 μg/ml) and propidium iodide (50 μg/ml) for 60 min at 37°C. DNA content of 10,000 events was analyzed using a Cell Lab Quanta SC and CellQuest software (Beckman Coulter Inc., Brea, CA, USA).

**Colony formation assay**

LLC cells were infected with adenovirus vectors at a MOI of 1000 or 2000. After 48 h, cells were disaggregated with trypsin/ethylenediaminetetraacetic acid and resuspended in complete medium. Aliquots (5 ml) containing 1 × 10^5 cells were seeded onto six-well plates. Cells were cultured for a further 6 days before removal of medium, washing with PBS and fixation with 70% ethanol. Colonies were then stained with 5% Giemsa and counted, after which plating efficiencies were calculated.

**Primary LLC model and gene delivery**

To induce primary LLC, LLC cells are subcutaneously injected into C57BL/6 mice (5 × 10^5 cells in 0.1 ml of PBS; n = 8) to monitor tumor growth. For liver-based gene delivery, tumor-bearing C57BL/6 mice were injected with adenovirus vectors: (i) Ad-POMC (1 × 10^9 plaque-forming units (pfu)) and (2) Ad-GFP (1 × 10^9 pfu) via the tail vein, when tumors grew larger than 100 mm^2 (approximately 7–14 days). Subsequently, the tumor volumes are measured with a dial-caliper and determined using the formula: width × length × 0.52. The mice were sacrificed on day 30 and tumors were harvested for consequence analysis.

**Histological and morphometric analysis**

For histological analysis, the dissected tumor tissues were fixed in 4% paraformaldehyde for 24 h in accordance with standard sectioning methods and staining in hematoxylin and eosin (H&E). Vessel morphometric analysis was performed as described previously [14]. The vessel density was assessed with the computer-assisted stereological toolbox system (Olympus, Tokyo, Japan). Each tissue block was evenly divided into five slices, from which a representative microtome section was obtained. Using an eyepiece with a grid, the blood vessel density percentage of area) was estimated by the number of points that fell on blood vessels divided by the total number of points counted. The vessel density values of the five sections from each single tissue were then averaged.

**Terminal deoxynucleotidyl transferase-mediated DUTP nick end labeling (TUNEL) assay**

Cell apoptosis was detected in situ in resected tissues by enzymatic labeling of DNA strand breaks using a In Situ Cell Death Detection Kit (Fluorescein; Roche Applied Science) in accordance with the manufacturer’s instructions. Images were acquired using a fluorescence microscope (DP70, Olympus).

**Immunohistochemical analysis**

Paraffin-embedded, 5 μm-thick sections were stained with H&E and CD31 (ab28364, Abcam) antibodies to examine the overall tissue. The tumor vessel density was calculated using the following formula: vessel density = (number of CD31-positive vessels/total number of points counted) × 100%.
and treated in proteinase K (Dako, Glostrup, Denmark) for 8 min. The sections were then washed in TBS (pH 7.5) and treated with 3% H$_2$O$_2$ in methanol for 10 min to block endogenous peroxidase. After another wash in TBS, the sections were incubated for 20 min in blocking solution (10% goat serum), followed by an overnight incubation in primary antibody GFP (1: 100 dilutions; Santa Cruz Inc; Santa Cruz, CA), CD31 (dilution 1:100; BD Biosciences Pharmingen, San Diego, CA, USA), Von Willebrand factor (vWF) (dilutions 1:100 dilution; BD Biosciences Pharmingen), Ki-67 (dilutions 1:100 dilution; Dako) and MC-1R (dilutions 1: 100 dilution; Santa Cruz Biotechnology Inc.) for 1 h at room temperature. The primary antibody was detected using secondary antibodies: biotinylated goat anti-rat (dilution 1:200; Dako) for 30 min at room temperature. The biotinylated complexes were detected using streptavidin-HRP (dilution 1:400; Dako) and visualized with 3,3′-diaminobenzidine (Dako). The negative controls included substitution of a primary antibody with a corresponding serum isotype (see Supporting information, Doc S1).

### Results

#### Defective MC-1R expression and signaling in mouse LLC cells

On the basis of the protein expression level shown in the western blot and immunofluorescent staining, the expression of melanocortin receptors was identified in LLC and B16-F10 melanoma cells (Figure 1a,b). Immunoblot results showed that both LLC and B16-F10 expressed MC-4R and -5R. However, MC-1R was detected in B16-F10 but not in LLC cells. The quantitative real-time-PCR analysis further confirmed that MC-1R transcript was not detectable in LLC cells (Figure 1c). Because intracellular cAMP is a key messenger in MC-1R downstream signaling, we found that treatment of B16-F10 cells with SHU9119, a MC-1R agonist, elicited a significant increase in intracellular cAMP content. However, when the same treatment was given to LLC, there was no change in cAMP content (p < 0.01; Figure 1d). These results suggested that MC-1R expression and signaling was defective in LLC cells.

#### POMC gene delivery inhibited the malignant behaviors in LLC cells.

Because MC-1R signalling plays an important role in POMC gene therapy for B16-F10 melanoma, it was...
speculated whether POMC gene delivery exerted an anti-neoplastic effect to MC-1R-lacking LLC cells. By using adenovirus encoding β-galactosidase (Ad-Laz), the optimal condition for adenoviral vector to infect LLC cells was determined at a MOI of 1000 or 2000 (see Supporting information, Figure S1), at which most LLC cells expressed β-galactosidase without overt cytotoxicity. To evaluate the efficacy of POMC expression, the levels of POMC-derived peptides in the cultured media of LLC cells were determined by an ACTH radioimmunoassay. It was found that Ad-POMC-infected LLC cells released significantly higher levels of ACTH compared to the cells of control groups ($p < 0.01$; Figure 2a).

Subsequently, we investigated the effects of POMC gene delivery on proliferation, cell cycle and colony formation in LLC with the MC-1R independent pathway. After POMC gene delivery, it was shown that POMC overexpression inhibited the proliferation of LLC cells by 20–40% compared to the control cells (Figure 2b). We further analyzed the cell cycle progression of LLC cells after POMC gene delivery by flow cytometry and found that POMC gene delivery reduced the G1/S phase, increased cells at the apoptotic pre-G0 phase, and reduced cells at the mitotic S phase in LLC cells (Figure 2c). Moreover, in the colony formation assay, POMC gene delivery significantly reduced the ability of colony formation (an approximately 40% reduction) compared to control cells (Figure 2d). These results suggested POMC gene delivery attenuated the malignant behaviors even in MC-1R-null LLC cells.

**Systemic POMC gene delivery attenuates tumor growth in mice bearing established LLC**

To evaluate the therapeutic potential of systemic POMC delivery in mice bearing established LLC, we first established viral tropism and the duration of transgene expression after intravenous injection of adenovirus vectors. We investigated GFP expression in mice after injection of adenovirus encoding GFP (Ad-GFP). After injection for 2 and 4 weeks, GFP expression was localized mainly in the liver and spleen, but not in the tumor tissues, and remained detectable for at least 4 weeks (see Supporting information, Figure 2).

![Figure 2](image-url)

Figure 2. Effect of POMC gene delivery on the feature of malignancy in LLC cells. (a) Effects of POMC gene delivery on the levels of ACTH in culture medium of LLC cells. After infection with adenoviral vectors for 48h, culture medium was collected from LLC cells and the levels of ACTH were examined by radioimmunoassay. (b) Effects of POMC gene delivery on proliferation in LLC cells. (c) Effects of POMC gene delivery on cell cycle analysis in LLC cells. (d) Effects of POMC gene delivery on colony formation in LLC cells. All data are expressed as the mean ± SEM of three experiments. Asterisks indicate statistical significance: **$p < 0.01$, *$p < 0.05$ value from corresponding Ad-GFP.
Subsequent to establishing viral tropism after intravenous injection, mice bearing established LLC were administered with adenoviral vectors and then monitored for tumor progression. Mice treated with Ad-POMC showed significantly retarded tumor growth compared to those in control animals (the tumor sizes for Ad-POMC- and Ad-GFP-treated mice were $1161 \pm 313 \text{ mm}^3$ and $2207 \pm 674 \text{ mm}^3$ at day 30, respectively; $p < 0.01$; Figure 3a).

Systemic POMC gene delivery attenuates tumor progression and inhibits $\beta$-catenin pathway in tumor tissue of LLC

Histological analysis showed that the tumor nodules in Ad-POMC-treated mice were smaller, although with a larger area of central necrosis compared to those in Ad-GFP-treated ones (Figure 3b). Quantitative histological...
analysis, using TUNEL (Figure 3c) and Ki-67 (Figure 3e) immunostaining, revealed significantly increased apoptosis and decreased proliferation in tumor cells from AdPOMC-treated tumor tissues. We further validated that the observation of the increased apoptosis in Ad-POMC-treated tumors was associated with a significant reduction in bcl-2 protein levels (Figure 3d).

β-catenin signaling plays an important role in lung carcinogenesis [15,16] and a reduced expression of downstream molecular targets such as c-myc and cyclin D1 has been found in the Ad-POMC-treated melanoma (GS Liu, HE Tsai, and MH Tai; unpublished data). Therefore, we subsequently investigated the involvement of the β-catenin pathway in tumor tissue after systematic POMC gene delivery. It was found β-catenin protein levels were significantly decreased in Ad-POMC-treated tumor tissues (Figures 4a and 4b). Moreover, Ad-POMC-treated tumor also exhibited significantly decreased expression of β-catenin downstream molecules such as c-myc and cyclin D1 compared to AdGFP-treated tumors. A similar profile of down-regulation of β-catenin/cyclin D1/c-myc was observed in Ad-POMC-treated LLC cells (see Supporting information, Figure S3). Our data suggested that systemic POMC gene delivery attenuates tumor growth by down-regulating the β-catenin pathway in LLC.

**Systemic POMC gene delivery inhibits blood vessel formation in LLC tumor tissue**

Our recent studies showed that systemic POMC gene delivery reduced tumor angiogenesis in melanoma [11]. However, it is not clear whether such an anti-angiogenic effect occurs in other tumor models such as LLC. Therefore, we examined the influence of systemic POMC gene delivery on tumor neovascularization in LLC models. Quantitative analysis of tumor neovascularization using CD31 immunostaining showed a significant decrease in blood vessel number and a reduction in vessel size in Ad-POMC-treated tumors compared to tumors of the Ad-GFP group (p < 0.05; Figure 5a). This observation of decreased angiogenesis in Ad-POMC-treated tumors was further validated with quantitative analysis using vWF immunostaining (p < 0.05; Figure 5b).

We also investigated whether the POMC-induced blockade of tumor angiogenesis was associated with endothelial apoptosis. It was observed that POMC gene delivery elicited apoptosis mainly in the tumor tissue, although not in tumor vessels (Figure 6a). To confirm such findings, we performed POMC gene delivery in
TUNEL staining

b

HUVEC

HMEC

Time (hrs)

0 48

Cell number ($x 10^4$)

0 20 40

Ad-GFP Ad-POMC

Ad-GFP Ad-POMC

NS

NS

c

HUVEC

HMEC

1.5

0.9

0.6

0.3

0.0

Ad-GFP Ad-POMC

Ad-GFP Ad-POMC

Ad-GFP Ad-POMC

Ad-GFP Ad-POMC

NS

NS

d

HUVEC

HMEC

Ad-GFP

Ad-POMC

Branch point/field

0 15 30 45

Ad-GFP Ad-POMC

Ad-GFP Ad-POMC

**

**
e

HUVEC

HMEC

Ad-GFP

Ad-POMC

Gap area (%)

0 15 30 45 60

Ad-GFP Ad-POMC

Ad-GFP Ad-POMC

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primary endothelial cells (HUVEC and HMEC) and then evaluated their influence on proliferation, apoptosis and angiogenic processes. It was observed that POMC gene delivery significantly reduced tube formation and migration (p < 0.01; Figures 6d and 6c), although it had no effect on cell proliferation or cell apoptosis (Figures 6b and 6c) in endothelial cells. This is consistent with the findings of our previous study using the EA.hy926 endothelial cell line [17]. Although MC-1R appears to be expressed in endothelial cells (see Supporting information, Figures S4a and S4b), the involvement of MC1R in POMC-mediated angiogenesis inhibition requires further investigation.

Discussion

In the present study, we first identified that MC-1R expression and signalling were both defective in LLC cells. Unexpectedly, POMC gene therapy was found to suppress the tumorigenicity of LLC both in vitro and in vivo. The anti-neoplastic effects of POMC gene therapy appear to be involved of multiple mechanisms. POMC gene therapy induced apoptosis and attenuated the proliferation and colony formation of LLC. Inhibition of the β-catenin pathway and downstream molecules such as c-myc and cyclin D1 may also contribute to the anti-tumor effect of POMC gene therapy, although further studies are warranted. Furthermore, our data suggest that POMC therapy suppressed tumor neovascularization to reduce tumor growth in the LLC animal model.

Melanocortins, particularly α-MSH, were first identified as physiological regulators of pigmentation in many vertebrate species. Five receptors have been cloned in mammals: MC-1R, MC-2R, MC-3R, MC-4R and MC-5R. The genetic variability of MC-1R, irrespective of skin type, was found to be associated with melanoma [18–20]. Recent studies have shown that MC1R agonists stimulate melanogenesis and also confer photoprotection to human melanocytes, thus preventing skin cancer formation [21]. In our previous study, we demonstrated that prophyllactic POMC gene delivery increased the release of POMC neuropeptides and attenuated the tumorigenic processes through the α-MSH/MC-1R pathway in melanoma cells [10]. The findings obtained in the present study suggest that the anti-neoplastic function of POMC gene delivery is not restricted to melanoma. Because LLC cells do not express MC-1R, and are noty responsive to MC-1R agonists, tumor inhibition by POMC gene delivery is clearly mediated through an MC-1R-independent mechanism.

β-catenin is a multifunctional protein that plays critical roles in cell adhesion as well as Wnt-activated tumorigenesis [22]. Aberrant activation of the Wnt signaling pathway is associated with a variety of human cancers and has been demonstrated to be a therapeutic target for cancer therapy [21,22]. Some studies showed a reduction of β-catenin degradation increased β-catenin levels and this led to the activation of target genes such as the proto-oncogenes c-myc [23] and cyclin D1 [24]. Recently, our microarray study showed that POMC overexpression significantly inhibited the expression of β-catenin downstream molecules such c-myc and cyclin D1 in melanoma (Liu et al. unpublished data). In the present study, we found that POMC gene delivery reduced β-catenin levels and inhibited its downstream targets such as cyclin D1, which was associated with cell cycle arrest at the G1-S checkpoint and c-myc expression. Thus, POMC gene delivery exerted a direct suppressive effect on LLC proliferation (cell cycle arrest) and such activity may involve the regulation of the β-catenin pathway and downstream cyclin D1. Future studies are warranted aiming to delineate the detailed underlying POMC-induced inhibition of β-catenin pathway.

Angiogenesis, the process of new blood vessel formation, is fundamental to the growth and dissemination of solid tumors [25]. Tumors are dependent on angiogenesis for growth and metastasis, which has led to the development of anti-angiogenic strategies for cancer treatment [26]. Previously, we showed that systematic POMC gene delivery using an adenoviral vector reduced tumor angiogenesis in melanoma models [11]. In the present study, we found that POMC gene delivery also retarded angiogenesis in LLC tumor. This observation substantiates our previous findings, and provided further evidence that the inhibition of angiogenesis by POMC gene delivery occurs through a direct effect on endothelial function. Because angiogenesis is essential for the progression of all types of cancer, the anti-angiogenic function of POMC gene delivery provides a rationale for why POMC gene therapy is effective against MC-1R-deficient LLC tumors.

In conclusion, we have demonstrated that POMC gene delivery inhibited both melanoma formation and lung cancer growth. We have shown that this effect was exerted through a MC-1R-independent pathway. Importantly, we characterized two common anti-neoplastic mechanisms underlying POMC gene therapy: angiogenesis inhibition and β-catenin inactivation. Therefore, POMC gene therapy may also have a therapeutic potential for multiple (and possibly all) types of cancer in addition to melanoma and lung cancer.

Figure 6. The effect of POMC gene delivery on proliferation, apoptosis and angiogenic activity in endothelial cells. (a) The extent of apoptosis was determined by TUNEL staining (brown, nuclear staining) in Ad-POMC-treated tumor tissue. Scale bar: left panel = 200 μm; right panel = 100 μm. The effect of POMC gene delivery on proliferation (b) and caspase 3/7 activity (c) in HUVEC and HMEC. Effects of POMC gene delivery (infected for 48h) on tube formation (d) and migration (e) responses in HUVEC and HMEC. Each point represents the mean ± SEM number of cells under high-power fields from three experiments. Asterisks indicate statistical significance: **: p < 0.01. NS, no significance.
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