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Title page

Gene Transfer of Pro-opiomelanocortin Prohormone Suppressed the Growth and Metastasis of Melanoma: Involvement of α-MSH-mediated Inhibition of $NF_{\kappa}B$/COX-2 Pathway

Guei-Sheung Liu, Li-Fen Liu, Che-Jen Lin, Jui-Cheng Tseng, Ming-Ju Chuang, Hing-Chung Lam, Jenn-Kuen Lee, Lin-Cheng Yang, Julie Hwa Yu Chan, Shen-Long Howng, Ming-Hong Tai

Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan. Liu, G.-S., Howng, S.-L., Tai, M.-H.; Department of Medical Education & Research, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan. Liu, G.-S., Lin, C.-J., Lam, H.-C., Chan, J. Y.-H., Tai, M.-H.; Department of Biological Science and Technology, I-Shou University, Kaohsiung, Taiwan. Liu, L.-F.; Division of Allergy, Immunology, and Rheumatology, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan. Tseng, J.-C.; Division of Endocrinology and Metabolism, Department of Medicine, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan. Chuang, M.-J., Lam, H.-C., Lee, J.-K.; Department of Anesthesiology, E-DA Hospital, I-Shou University, Kaohsiung, Taiwan. Yang, L.-C.; Department of Biological Sciences, National Sun Yat-Sen University, Kaohsiung 804, Taiwan. Tai, M.-H.

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Running title page

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POMC Gene Transfer for Melanoma Suppression

Correspondence to:

Ming-Hong Tai, Ph. D.

Department of Medical Education and Research, Kaohsiung Veterans General Hospital

386 Ta-Chung 1st Road, Kaohsiung 813, Taiwan.

Tel: 886-7-3422121 Ext. 1510. FAX: 886-7-3468056. E-mail: mhtai@isca.vghks.gov.tw

or

Sheng-Long Howng, MD.

Department of Neurosurgery, Kaohsiung Medical University, 100 Shih-Chuan 1st Road

Kaohsiung 80708, Taiwan

Tel: 886-7-3121101 x 5104, Fax: 886-7-3215039, E-mail: shloho@ms.kmuh.org.tw

Abbreviations:

ACTH, adrenocorticotrophic hormone; α-MSH, α-melanocyte-stimulating hormone

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Abstract

Pro-opiomelanocortin (POMC) is a prohormone of various neuropeptides, including adrenocorticotrophic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH) and β-endorphin (β-EP). POMC neuropeptides are potent inflammation inhibitors and immunosuppressants, and may exert opposite influences during tumorigenesis. However, the role of POMC expression in carcinogenesis remains elusive. We evaluated the anti-neoplastic potential of POMC gene delivery in syngenic B16-F10 melanoma model. Adenovirus-mediated POMC gene delivery in B16-F10 cells increased the release of POMC neuropeptidesin cultured media, which differentially regulated the secretion of pro- and anti-inflammatory cytokines in lymphocytes. POMC gene transfer significantly reduced the anchorage-independent growth of melanoma cells. Moreover, prior- or post-treatment with POMC gene delivery effectively retarded the melanoma growth in mice. Intravenous injection of POMC-transduced B16-F10 cells resulted in reduced foci formation in lung by 60-70% of control. The reduced metastasis of POMC-transduced B16-F10 cells could be attributed to their attenuated migratory and adhesive capabilities. POMC gene delivery reduced the cycolooxygenase-2 (COX-2) expression and prostaglandin E_2 (PGE_2) synthesis in melanoma cells and tumor tissues. Besides, application of NS-398, a selective COX-2 inhibitor, mimicked the anti-neoplastic functions of POMC gene transfer in melanoma. The POMC-mediated COX-2 downregulation was correlated with its inhibition of nuclear factor kappa B ($NF_\kappa B$) activities. Exogenous supply of α-MSH inhibited $NF_\kappa B$ activities while application of α-MSH antagonist, GHRP-6, abolished the POMC-induced inhibition of $NF_\kappa B$ activities and melanoma growth in mice. In summary, POMC gene delivery suppresses melanoma

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via α-MSH-induced inhibition of $NF_{\kappa}B$/ COX-2 pathway, thereby constituting a novel therapy for melanoma.

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Introduction

POMC is a multifunctional, polycistronic gene located on human chromosome 2p23.3. POMC is a 31 kDa prohormone that is processed into various neuropeptides including adrenocorticotrophic hormone (ACTH), melanotrophins (α-, β- and γ-MSH), lipotropins and β-endorphin (β-EP)(Catania et al., 2004; Solomon, 1999). POMC peptides possess pleiotrophic functions including pigmentation, adrenocortical function, regulation of energy stores, the immune system, and the central and peripheral nerve systems(Catania et al., 2004). POMC expression in the hypothalamus or brainstem gives rise to melanocortinergic neurotransmitters, while expression in the pituitary gland gives rise to circulating melanocortin hormones(Raffin-Sanson et al., 2003). The specific patterns of post-translational POMC processing dictate whether an individual cell releases α-MSH, ACTH, or other melanocortin peptides, which in turn control overlapping arrays of endocrine, metabolic and neurologic endpoints.

POMC processing was thought to occur primarily in the pituitary gland and the central nerve system. Recently, POMC neuropeptides such as α-MSH and β-EP are identified in various non-neuronal cells including memory T cells, macrophages and melanocytes(Cabot et al., 1997; Raffin-Sanson et al., 2003; Schauer et al., 1994). In addition, POMC gene delivery in muscle or bladder by gene gun approach alleviates pain via local generation of β-EP (Chuang et al., 2003; Lu et al., 2002), indicating that POMC could be processed in the peripheral tissues to elicit anti-nociceptive effect.

The anti-inflammatory and immunosuppressive functions of POMC neuropeptides have been characterized (Catania et al., 2004; Luger et al., 2003). POMC neuropeptides, particularly ACTH and α-MSH, are potent inhibitors of inflammation. ACTH stimulates

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the adrenal gland to release cortisol, which exerts anti-inflammatory functions directly or via the synthesis of glucocorticoid. The anti-inflammatory nature of α-MSH has been proposed for the treatment of various inflammation-related diseases (Catania et al., 1999; Catania et al., 2004). The immune-modulating functions of POMC neuropeptides have been elucidated (Luger et al., 2003). ACTH and its downstream products, cortisol and glucocortioid, are potent immunosuppressive agents. α-MSH regulates the immune responses by impairing the functions of both antigen-presenting cells and T cells (Luger et al., 1998), and modulating the production of pro-inflammatory and anti-inflammatory cytokines (Luger et al., 2003). β-EP inhibits the immune system at multiple levels that elevated splenocytes proliferation and cytokines production were observed in β-EP-deficient mice (Refojo et al., 2002).

The influence of POMC expression during tumor progression remains elusive. POMC expression is repressed in tumor tissues due to promoter methylation (Newell-Price et al., 2001). In malignant mesothelioma cells, expression of POMC transcripts, POMC-processing enzymes and melanocortin 1 receptor (MC1R), the high-affinity receptor for α-MSH, constitutes an autocrine-inhibitory circuit to suppress the proliferation of tumor cells(Catania et al., 2004). Furthermore, α-MSH inhibits the production of prostaglandins (Nicolaou et al., 2004) and contribute to the prevention of melanoma by reduction of UV-induced DNA damage in melanocytes (Kadekaro et al., 2003). These findings supported that POMC overexpression might inhibit inflammatory responses to intervene tumor progression (Coussens and Werb, 2002; Manna and Aggarwal, 1998). However, based on the concept of immunosurveillance against cancer development, POMC expression could result in dysfunction of host immune system,

thereby allowing tumor cells to escape from immune scrutiny. Since elevated plasma α-MSH levels was found to correlate with malignancy in patients with melanoma(Ghanem et al., 1989; Liu and Johansson, 1995), the correlation between α-MSH levels and melanoma progression implicated that POMC expression might modulate the immune response and create favorable environment for tumor invasion. Therefore, it remains controversial whether POMC expression promoted or suppressed tumor progression. In the present study, we investigated the outcome of POMC gene delivery on growth and metastasis of melanoma using syngenic B16-F10 melanoma model, thereby to delineate the interplay between anti-inflammatory and immunosuppressive functions by POMC overexpression.

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Materials and methods

Cell cultures and reagents

For production and propagation of Ad5 adenovirus, E1a-transformed human embryonic kidney 293 cells were purchased from Microbix Biosystems Inc. (Toronto, Canada) and maintained at low-passage. B16-F10 melanoma cells were purchased from ATCC (Manassas, VA) and cultured in DMEM (Invitrogen; Carlsbad, CA) medium containing 10% fetal calf serum (FCS), 2 mM glutamine, 100 mg/ml streptomycin and 100 U/ml penicillin at 37 ^{o}C in 5% CO_2 incubator. ACTH, α-MSH, β-EP and GHRP-6 were purchased from BACHEM (Torrance, CA). NS-398 was from Cayman Chemical Co. (Ann Arbor, MI). Naloxone, H-89 and Concanavalin A were from Sigma (St. Louis, MO)

Generation of recombinant Ad-POMC

Human POMC cDNA (Lin et al., 2002) was subcloned into *Kpn*I site of adenovirus transfer vector Ad5-2 to yield Ad5-2-POMC, in which the transgene was driven by long-terminal-repeat promoter from the Rous sarcoma virus and flanked by polyadenylation sequences from bovine growth hormone. Recombinant adenovirus was generated by cotransfection of pAd5-2-POMC with pJM17 vector (Microbix; Toronto, Canada), a plasmid containing the entire type 5 Ad genome with E1-insertion and E3-deletion, into 293 cells as previously described(Tai et al., 2003). After homologous recombination, the virus plaques were verified by checking for cytopathic effect (CPE) and PCR. The virus was amplified by two rounds of cesium chloride ultracentrifugation and desalted by G-25 gel-filtration chromatography. The titer of virus solution was

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determined by measuring optical density at 260 nm and plaque-forming assay on 293 cells before storage at -80°C.

Radioimmunoassays (RIAs)

ACTH and β-EP concentrations were determine using their RIA kits (Nichols Institute Diagnostics, San Juan Capistrano, CA) with a linear range of measurement between 5-1000 pg/ml for ACTH or β-EP and a detection threshold of 5 pg/ml. α-MSH RIA kit (Euro-Diagnostica, Malmö, Sweden) was used for measurement of α-MSH. The detection limit of this assay was 5 pg/ml with no crossreactivity. A standard curve was constructed for each assay. Experiments are performed at least three times.

Melanin assay

The melanin production in the cultured media of B16-F10 cells was determined by measurement of the absorbance at the wavelength of 475 nm and the cultured media from untreated cells was used as blank. The melanin content in cell culture was normalized with the cell number.

Isolation and stimulation of peripheral blood mononuclear cells (PBMC)

PBMCs were prepared from heparinized whole blood using Ficoll-Hypaque (Amersham-Pharmacia, Buckinghamshire, UK) gradient centrifugation. The blood samples were diluted 1:1 with sterile PBS and carefully layered over Ficoll-Hypaque followed by centrifugation at 1800 rpm at 4 °C for 20 min. The interface containing PBMC was harvested and washed twice with cold serum-free RPMI-1640 medium

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(Invitrogen; Carlsbad, CA). After resuspension in RPMI 1640 media containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine, the viability of PBMC was analyzed by trypan blue exclusion assay. In the presence of Concanavalin A (Con A; 25 μg/ml), PBMC (1×10^6 cells per well in 6-well plate) in one milliliter cultured media were incubated with equal volume of conditioned media from infected B16-F10 cells at 37 °C for 24 h. Subsequently, cultured media of stimulated PBMC was harvested for cytokines determination.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-2, IFN-γ and IL-10 in cultured media of PBMC were measured by their specific ELISA kits (R&D Systems Inc., Minneapolis, Minnesota, USA) following protocols provided by manufacturer. The release of PGE_2 in cultured media was determined by PGE_2 EIA kit (Cayman; Ann Arbor, MI) following protocol of manufacturer.

Cell proliferation assay

B16-F10 cells were cultured in a 96-well plate at a density of 4×10^4 cells per ml. After infection with adenovirus vectors, cells were supplemented with fresh medium containing 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT; 0.456 mg/ml) and incubated for 1-2 hours at 37 □. The formazan in viable cells were dissolved with 100 μl of dimethyl sulfoxide and determined by reading optical densities in microplate reader (DYNEX Technologies, Inc.; Chantilly, Virginia) at an absorption wavelength of 570 nm.

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Soft agar assay

After infection for 12 hours, B16-F10 cells (5 x 10^3 cells) were seeded in 0.3% agarose (Difco; MD, USA) containing growth medium and added onto 6-cm plate prelayered with 0.5% bottom agarose (Difco; MD, USA) containing growth medium. The plates were incubated at 37 □ and 5% CO_2 for 1-2 weeks until colonies developed. The number of colonies (> 50 cells per colony) in each well was scored.

GFP-expressing melanoma cells and GFP fluorescence assays

pEGFP-C1 plasmid (Clontech; Palo Alto, CA) was transfected into B16-F10 cells by Lipofectamine (Invitrogen; Carlsbad; CA). The GFP-expressing clones were obtained by G418 selection and verified using western blotting and fluorescence microscope analysis. The tissue extracts were isolated using lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 7.8, 1% NP-40 and complete protease inhibitors (Roche Applied Science; Indianapolis, IN). After extraction, the GFP fluorescence intensity was determined in an fluorometer (FLUOstar, BMG Labtechnologies Inc; Germany).

Primary and metastatic melanoma models

Animal experiments were carried out in Animal Center of Kaohsiung Veterans General Hospital in accordance with institutional guidelines. Male C57BL/6 mice (6- to 8-week-old; The Animal Center of National Science Council; Taipei, Taiwan) were acclimated and caged in groups of four or less. All mice were fed with a diet of animal chow and water ad libitum. Animals were anesthetized in a methoxyflurane chamber

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prior to all procedures and sacrificed by a lethal dose of methoxyflurane.

To induce the primary melanoma, B16-F10 cells were infected with adenovirus vectors for 12 h, then subcutaneously injected into C57BL/6 mice (5×10^5 cells in 0.1 ml PBS per mice) to monitor tumor growth. To evaluate the therapeutic potential of POMC gene delivery, melanoma were grown in mice to the size of at least 100 mm^3 then administrated with adenovirus vectors (1×10^9 pfu) in 100 μl PBS by intratumor injection. NS-398 was administered into mice via intraperitoneal route daily at a dose of 10 mg/kg as previously described (Duff et al., 2003). The tumor volumes were measured with a dial-caliper and determined using the formula: $width^2 \times length \times 0.52$.

In the pulmonary metastasis model, mice were divided into three groups and administrated with: 1) untreated, 2) Ad-POMC-infected and 3) Ad-GFP-infected B16-F10 cells (5×10^5 cells in 200 μl PBS per mice) via tail vein to induce metastatic melanoma in lung. The metastasis to lung was detected at day 14 after injection.

Cell adhesion assay

The adhesion assay was performed as previously described (Zhong Wu et al., 2004). laminin (Roche Applied Science; Indianapolis, IN) or fibronectin (Roche Applied Science; Indianapolis, IN) was dissolved in PBS to final concentration of 50 μg/ml and used for coating by adding 0.1 ml solution per well in 96-well plate at 4°C overnight. All groups were assayed at the same time in quadruplicates. After overnight incubation, the wells were washed with PBS twice and incubated with serum-free DMEM at 37 °C for 30 min. Cells (2×10^4) were added to each well and incubated at 37 °C from 30 min to 4

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h for adhesion. After incubation, the adhered cells were washed with PBS twice gently and measured using MTT assay.

Scratch migration assay

The migration of melanoma cells was assessed as using a scratch migration assay as previously described(Zhu et al., 2004). Briefly, after infection with adenovirus vectors for 12 h, a gap of approximately 1 mm was created in the adherent layer of confluent B16-F10 cells (in 6-well plates) by introducing a scratch using a sterile 0.1 ml pipette tip (Gilson, Inc.; Middleton, WI). The closure extent of the cell-free gap was recorded by microscopy at different time intervals and counted over a time period of 24 h.

Quantitative real time RT-PCR (qRT-PCR)

RNA was isolated from cells using RNAzol (TEL-TEST Inc., Friendswoods, TX). For reverse transcription, 5 μg of total RNA was used for reverse transcription with Superscriptase II (Invitrogene; Carlsbad, CA) using olio-dT and random primers. One twentieth of reverse-transcription products were used as template for qRT-PCR in ABI Prism 7700 sequence detector (Applied Biosystems; Foster City, CA) using a SYBR green assay. PCR reaction was performed in 50 μl SYBR Green PCR Master Mix (Applied Biosystems; Foster City, CA) containing 10 μM forward primers and reverse primers, and approximately 30 ng cDNA. Amplification and detection were performed by: 1 cycle of 95 □ for 10 min, 40 cycles of 95 □ for 15 s, and 62 □ for 20 s, and 72 □ for 15 s. After completion, a final melting curve was performed by denaturation at 95 □ for 15 s and then was recorded by cooling to 60 □ and then heating slowly until 95 □ for

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20 min according to the dissociation protocol of ABI7700 instrument. The primer sequences for COX-2: forward primer 5' GGTGTATCCCCCCACAGTCA -3', reverse primer 5'-CCAGGCACCAGACCAAAGAC-3', which amplified a 110 bp COX-2 cDNA fragment. The β-actin mRNA level was determined using: forward primer 5'-TCACCCACACTGTGCCCATCTACGA-3' and reverse primer 5'-CAGCGGAAC CGCTCATTGCCAATGG -3', which amplified a 295-bp β-actin cDNA fragment.

Western blot analysis

B16-F10 cells were infected with adenovirus vectors at MOIs of 500 or 1000. After 12 h, cells were supplemented with fresh medium and continued to incubate at 37 ^{o}C for an additional 48 h. The protein extract was isolated using buffer containing 150 mM NaCl, 50 mM HEPES pH 7, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA and protease inhibitors (Roche Applied Science; Indianapolis, IN). After separation in 12.5% SDS-PAGE, protein are transferred onto polyvinylidene fluoride membrane using blotting apparatus. The membrane was blocked with 5 % milk in TBS-T for 1 h then incubated with $NF_{\kappa}B$ antibodies (1:500 dilutions; Santa Cruz Inc; Santa Cruz, CA) or COX-2 antibodies (1:500 dilutions; Santa Cruz Inc; Santa Cruz, CA) for 1 h at room temperature. After with secondary antibody conjugated with HRP (1:5000 dilutions in 5 % milk) for 30 minutes, the signals on membrane were detected using ECL-plus luminol solution (Pharmacia; Piscataway, NJ) and expose to X-ray film for autoradiogram.

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Immunohistochemistry

The paraffin-embedded melanoma tissue blocks were sectioned into 3 μm slices and mounted on the poly-L-lysine slides. After deparaffinization, the slides were blocked with 3% hydrogen peroxide for 10 min and subjected to antigen retrieval with microwave in 10 mM citrate buffer for 15 min. The slides were incubated with COX-2 antibodies (1:200 dilution; Cayman) at 4 ℃ overnight. After wash with PBS, the sections were incubated with horseradish peroxidase/Fab polymer conjugate (Polymer detection system, Zymed, USA) for 30 min and detected using diaminobenzidine (1:20 dilution, Zymed).

$NF_{\kappa}B$ activities assays

The $NF_{\kappa}B$ activities in melanoma cells were investigated by luciferase activities assay and electrophoretic mobility shift assay (EMSA). For luciferase assay, B16-F10 cells (in 6-well plate) at 80% confluence were co-transfected with $NF_{\kappa}B$-driven luciferase (Stratagene; La Jolla, CA) vector and the renilla luciferase reporter vector (Promega; Madison, WI) at a ratio of 1: 1/10 using the lipofetamine (Invitrogen; Carlsbad, CA). After subsequent infection with adenovirus vectors, the $NF_{\kappa}B$-driven luciferase activities in cells were determined using a Dual-Light kit (Promega; Madison, WI) in luminometer (Microlumat Plus LB96V; EG& G Berthold, Germany) and normalized with that of renilla luciferase according to manufacturer's instructions.

For EMSA, the double-stranded oligonucleotides for the consensus binding sites of $NF_{\kappa}B$ (5'-AGTTGAGGGGACTTTCCCAGGC-3') were labeled using Klenow fragment (Invitrogen; Carlsbad, CA) in the presence of 100 μCi [α-^{32}P]-dCTP (3000 Ci/mmol;

NEN, Boston, MA) and unlabeled dATP, dGTP, and dTTP at 100 µM in a final volume of 50 µl. The unincorporated nucleotides were removed using a G-25 spin column (Pharmacia; Piscataway, NJ). Five micrograms of nuclear proteins were incubated with reaction buffer (50 µg/ml poly-[dI–dC], 5% glycerol, 10 mM Tris–HCl, pH 7.8, 1 mM EDTA, 40 mM KCl, and 1 mM dithiothreitol) on ice for 20 min before addition of the radiolabeled oligonucleotide probe for additional 20 min at room temperature. The reaction products were separated in a 4% polyacrylamide gel. Gels were run at room temperature at 10 V/cm for 45 min, dried in a gel dryer under vacuum at 80 °C and analyzed by autoradiography.

Statistic analysis

Differences between the groups were statistically evaluated using the unpaired Student's t-test. The results are presented as mean ± sd. All p values were two-tailed, and a P value of less than 0.05 was considered to be statistically significant.

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Results

Adenovirus-mediated POMC overexpression in B16-F10 melanoma cells increased the production of immunomodulatory POMC neuropeptides

The E1-, E3-defective recombinant adenovirus encoding POMC (Ad-POMC) was generated for gene delivery studies. By infection with adenovirus encoding green fluorescent protein (Ad-GFP) at various multiplicity of infection (MOI), the optimal condition for adenovirus vectors to infect B16-F10 melanoma cells was determined at a MOI of 500 or 1000 (Supplementary data). Under this condition, most B16-F10 melanoma cells expressed GFP without overt cytotoxicity. To evaluate the efficacy of POMC processing, the levels of POMC neuropeptides in the cultured media of B16-F10 cells were determined by RIAs. Ad-POMC-infected B16-F10 cells released significantly higher levels of ACTH, β-EP and α–MSH compared with cells of control groups ($P < 0.001$; Fig. 1a).

Since α–MSH stimulates melanogenesis in melanocytes, we examined the morphologies and melanin secretion in POMC-transduced melanoma cells. Indeed, Ad-POMC-infected B16-F10 cells exhibited characteristic features of melanogenesis including a significantly higher melanin release and dendritic phenotype with increased dark deposits (Supplementary data). Thus, POMC gene delivery increases the production of bioactive POMC neuropeptides, such as α–MSH, in melanoma cells.

To investigate the immunomodulatory influence of POMC neuropeptides produced by melanoma cells, the cytokines release from peripheral blood mononuclear cells (PBMC) was evaluated after incubation with cultured media from POMC-transduced melanoma cells. Treatment with cultured media of POMC-transduced cells significantly

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attenuated the release of anti-inflammatory cytokines such as interleukin-2 (IL-2) and interferon γ (IFN-γ) in PBMC (Fig. 1b). On the contrary, incubation with cultured media of POMC-transduced cells significantly increased the production of anti-inflammatory interleukin-10 (IL-10) in PBMC. Together, these results validate that POMC gene transfer in melanoma cells leads to enhanced secretion of POMC neuropeptides, which differentially regulate the expression of pro- and anti-inflammatory cytokines in lymphocytes.

POMC gene transfer inhibited the growth of melanoma *in vitro* and *in vivo*

To evaluate the anti-tumor effect of POMC gene delivery, the proliferation and colonies-forming capability of B16-F10 cells were investigated after POMC gene delivery. Despite marginal inhibition of cell proliferation (Fig. 2a), POMC gene transfer prominently inhibited the anchorage-independent growth of B16-F10 cells in soft agar by more than 50% of control groups (Fig. 2b). To further explore the anti-neoplastic potential of POMC gene delivery *in vivo*, B16-F10 cells were infected with adenovirus vectors then injected subcutaneously into C57BL/6 mice to induce melanoma (Fig. 2c). The tumor size in mice implanted with POMC-transduced B16-F10 cells was significantly retarded (1625 ± 305 mm^3 at day 19) compared with mice implanted with cells of control groups (3098 ± 525 and 2866 ± 559 mm^3 for PBS- and Ad-GFP-treated B16-F10 cells, respectively, at day 19; $P < 0.01$). Moreover, in mice with established melanoma, administration of Ad-POMC, but not Ad-GFP or PBS, potently inhibited the progression of melanoma (tumor size in Ad-POMC-, PBS- or Ad-GFP-treated mice was 1420 ± 145 mm^3, 4512 ± 548 or 4286 ± 259 mm^3, respectively, at day 21; $P < 0.01$; Fig.

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2d). These results indicate that POMC gene transfer effectively suppresses the tumorigenicity of primary melanoma.

POMC gene transfer perturbed the lung metastasis of melanoma cells

To investigate the effect of POMC gene transfer on the metastatic potential of melanoma cells, GFP-expressing B16-F10 cells (GFP-B16-F10 cells) were generated for tracking of metastatic melanoma cells (Fig. 3a). Intravenous injection of GFP-B16-F10 cells resulted in formation of numerous metastatic foci in lung within 14 days. The events of lung metastasis could be quantified by measuring either the foci number or GFP fluorescence intensity in lung (Fig. 3b). After infection with adenovirus vectors, the transduced GFP-B16-F10 cells were intravenously injected into mice at day 0 and the formation of pulmonary colonies was evaluated on day 14. The number of metastatic foci in mice injected with Ad-POMC-treated GFP-B16-F10 cells (6.05 ± 4.47 foci per lung) was significantly lower than that of control groups (17.4 ± 4.38 and 24.4 ± 5.78 foci per lung for Ad-GFP- and PBS-treated group, respectively; $P < 0.01$; Fig. 3c, d). Besides, quantification of GFP fluorescence in lung tissues revealed that GFP expression lung tissues from mice injected with Ad-POMC-infected cells was significantly decreased compared with control groups (Fig. 3e). Together, these results suggest that POMC gene delivery attenuates the metastatic potential of B16-F10 cells *in vivo*.

POMC gene transfer attenuated the motility and adhesion to extracellular matrix of melanoma cells

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To investigate the mechanism underlying attenuated metastasis by POMC gene delivery, the migration of infected B16-F10 cells was investigated by a scratch wound assay (Fig. 4a). The gap area in PBS- or Ad-GFP-treated cells was completely healed within 24 h, whereas the gap in Ad-POMC-infected cells was not filled up in the same time interval. Quantitative analysis of wound area revealed a significant reduction of wound closure in Ad-POMC-infected cells compared with cells of control groups at 24 h ($P < 0.01$; Fig. 4a). These data suggest that POMC gene delivery reduces the motility of melanoma cells.

Because the attachment to extracellular matrix is pivotal to the colonization of metastatic cells in distant organs, we examined the influences of POMC gene transfer on the adhesion properties of melanoma cells to extracellular matrix proteins. Despite moderate effect in binding due to viral infection, POMC gene transfer caused significantly reduced binding affinity in B16-F10 cells to fibronectin ($P < 0.05$; Fig. 4b) or laminin ($P < 0.05$; Fig. 4c). Together, these findings indicate that POMC-mediated suppression in migratory and adhesive properties of melanoma cells may contribute to their reduced metastatic potential.

POMC gene transfer suppressed COX-2 expression in melanoma cells

COX-2 has been the therapeutic target of many anti-inflammatory agents for cancer therapy. To investigate whether COX-2 pathway participated in the anti-neoplastic and anti-metastatic functions of POMC gene delivery, the COX-2 expression and PGE_2 production were analyzed in B16-F10 cells and melanoma tissue after POMC gene delivery. We found that POMC gene delivery potently reduced the COX-2 expression at mRNA and protein levels by more than 70% of that in control groups ($P < 0.001$; Fig. 5a,

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b). Moreover, the release of COX-2 enzyme product, PGE_2, in Ad-POMC-treated B16-F10 cells were also decreased by 50-70% of that in control groups (Fig. 5c). Similarly, in tumor tissues, POMC gene delivery also led to a significant reduction in COX-2 protein levels compared with Ad-GFP-infected tumors (Fig. 6a). Besides, the PGE_2 level in POMC-transduced melanoma was also significantly lower than that of control groups (Fig. 6b). Histological analysis revealed a prominent COX-2 immunostaining in melanoma, but not in normal skin, indicating that COX-2 is overexpressed in B16-F10 melanoma (Fig. 6c). Above all, the COX-2 immunoreactivities were significantly lower in Ad-POMC-infected melanoma than Ad-GFP-treated ones (Fig. 6d). Therefore, POMC gene transfer leads to reduction in COX-2 expression and PGE_2 synthesis in B16-F10 melanoma *in vitro* and *in vivo*.

POMC gene transfer mimicked the anti-neoplastic effect of a selective COX-2 inhibitor, NS-398, in B16-F10 melanoma cells

To evaluate whether POMC gene delivery indeed acted like a COX-2 inhibitor, we made an extensive, parallel comparison on the cellular effect of POMC gene delivery with a selective COX-2 inhibitor. Although NS398 had no effect on cell proliferation even at dose as high as 100 μM (Fig. 7a), treatment with NS398 significantly attenuated the PGE_2 production, anchorage-independent growth and cell migration of B16-F10 cells in a dose-dependent manner (Fig. 7b-d). Moreover, continuous administration of NS-398 prominently reduced the melanoma growth of implanted B16-F10 cells in mice (Fig. 7e). Besides, the reduced tumor volume was associated with a decreased PGE_2 content in

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melanoma tissues (Fig. 7f). These data indicate that the cellular influences of POMC gene delivery in B16-F10 cells are remarkably similar to that of COX-2 inhibitor.

POMC gene transfer inhibited $NF_\kappa B$ activities in melanoma cells

Since $NF_\kappa B$ is an upstream regulator of COX-2 pathway, the effect of POMC gene delivery on the endogenous and stimulated $NF_\kappa B$ activities in B16-F10 cells was investigated. POMC gene delivery resulted in a significant reduction in nuclear $NF_\kappa B$ level, nuclear binding activities to $NF_\kappa B$ motif and $NF_\kappa B$-driven luciferase activities by 30-60% of control groups (left panels in Fig. 8). Even in the presence of TNF-α, which markedly elevated the $NF_\kappa B$ activities, POMC gene delivery still effectively repressed the TNF-α-stimulated $NF_\kappa B$ activities in melanoma cells (right panels in Fig. 8). These results indicate that POMC gene transfer potently inhibits the $NF_\kappa B$ activities in melanoma cells.

POMC gene transfer inhibited the $NF_\kappa B$ activities, COX-2 expression and melanoma growth via α-MSH pathway

To identify the POMC neuropeptide(s) that contributed to inhibition of $NF_\kappa B$ activity and PGE_2 production by POMC gene delivery, B16-F10 cells were treated with various POMC peptides and assayed for $NF_\kappa B$-driven luciferase activities. Treatment with α-MSH, but not ACTH or β-EP, mimicked the effect of POMC gene transfer in inhibition of $NF_\kappa B$-driven luciferase activities (Fig. 9a). Conversely, application of a selective α-MSH antagonist, GHRP-6, abolished the POMC-induced inhibition on $NF_\kappa B$

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activities (Fig. 9b) and PGE_2 release (Fig. 9d). Since protein kinase A (PKA) is a general downstream effector of melanocortin receptors including MC1R, we investigated whether H-89, a specific inhibitor of PKA, influenced the effect of POMC gene delivery in $NF_\kappa B$ activities. It was found that H-89 treatment also reverted the POMC-induced inhibition of $NF_\kappa B$ activities (Fig. 9c). Thus, α-MSH pathway plays an important role in POMC-mediated inhibition of $NF_\kappa B$/COX-2 pathway. To validate the involvement of α-MSH pathway, the effect of GHRP-6 on melanoma growth was investigated. Application of GHRP-6 alone had no effect on the growth of B16-F10 melanoma in mice (Fig. 9e). However, administration of GHRP-6 effectively reverted the POMC-mediated melanoma suppression in mice that the tumor size in mice implanted with Ad-POMC-infected cells increased from 2119 ± 950 mm^3 to 3814 ± 1104 mm^3 after GHRP-6 administration (Fig. 9e). Together, α-MSH/MC1R pathway is critical to the POMC-mediated melanoma suppression.

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Discussion

The present study demonstrates for the first time that gene delivery of POMC prohormone in melanoma cells increases the release of POMC neuropeptides, which simultaneously modulates the cytokines expression of cultured lymphocytes and attenuates the tumorigenic properties of melanoma cells, including proliferation, anchorage-independent growth, migration and adhesion. Besides, prior- or post-treatment with POMC gene vector suppresses, rather than promotes, the growth and metastasis of melanoma in immune-competent mice. Finally, α-MSH-induced inhibition of pro-inflammatory $NF_{\kappa}B$/COX-2 pathway is involved in the anti-neoplastic mechanism of POMC gene delivery. Similar α-MSH-induced inhibition of $NF_{\kappa}B$/COX-2 activities has been reported in hypothalamus of lipopolysaccharide-treated rats yet via melanocortin 4 receptor (Caruso et al., 2004). Due to its potent suppression of melanoma progression in either the chemoprevention study using engineered melanoma cells or therapeutic study with established tumors, POMC gene delivery may constitute a novel chemoprevention or treatment modality for melanoma. Nevertheless, the optimal dose and gene delivery route for POMC gene delivery require further investigation. In addition, it remains to be explored whether POMC gene-based drugs could be applied to the treatment of other types of cancer.

Other than anti-inflammation, there may be other mechanisms participating in melanoma suppression by POMC gene delivery. Due to its marginal influence on cell proliferation, the anti-tumor function of POMC gene delivery is likely due to indirect mechanisms rather than the direct cytotoxicity to melanoma cells. Because POMC gene transfer inhibits $NF_{\kappa}B$/COX-2 pathway, one probable mechanism is the blockade of

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tumor angiogenesis because either $NF_{\kappa}B$ or COX-2 pathway may regulate the expression of vascular endothelial growth factor (VEGF), an important angiogenic factor for tumor blood supply (Ditsworth and Zong, 2004; Rigas and Kashfi, 2005). Additionally, a significant correlation was observed between overexpression of $NF_{\kappa}B$-p65 and vascular development in clinical melanoma specimens (Kashani-Sabet et al., 2004). Another plausible mechanism for POMC-mediated melanoma suppression is the activation of immune surveillance since the POMC-transduced cells are capable of altering the cytokines expression by lymphocytes (Fig. 1). However, given that POMC neuropeptides are potent immunosuppressive agents, the probability of induction of systemic immunity by POMC gene delivery is relatively remote. Future studies are warranted to evaluate these mechanisms, thereby shedding light on the POMC-mediated melanoma suppression.

COX-2 and its product PGE_2 are critical to tumor growth and progression (Hla et al., 1999; Trifan and Hla, 2003). COX-2 is overexpressed in malignant melanoma, and involved in the progression and metastasis of melanoma (Denkert et al., 2001). In the present study, we provide convincing evidence for POMC gene delivery attenuates the COX-2 expression and PGE_2 synthesis in melanoma. It is still not clear how the POMC-mediated COX-2 inhibition may lead to suppression of melanoma progression and metastasis. Recent evidence points out that COX-2 overexpression renders cancer cells resistant to apoptosis, and promotes angiogenesis, invasive potential and metastasis. Besides, melanoma inhibits the tumoricidal activity of macrophage via COX-2 expression (Duff et al., 2003). Conversely, inhibition of COX-2 could restore macrophage function in melanoma and hence maximize the antitumor activity of immune

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stimulant such as IFN-γ. Thus, COX-2 inhibition can suppress melanoma by induction of apoptosis and immune responses, while reducing the neovascularization and invasive potential of tumor cells.

Melanoma exhibit constitutive activation of $NF_{\kappa}B$, which leads to endogenous expression of number of factors associated with escape from apoptosis, tumorigenesis, and metastasis(Dolcet et al., 2005). On the contrary, the inhibition of $NF_{\kappa}B$ has resulted in significant anti-tumor effects in melanoma(Amiri and Richmond, 2005). In the present study, POMC-mediated $NF_{\kappa}B$ inhibition is mimicked by α-MSH but abolished by antagonist of MC1R or PKA, demonstrating at least the involvement of α-MSH/MC1R/PKA signaling pathway. However, the exact mechanism underlying POMC-induced inhibition of $NF_{\kappa}B$ activities remains to be delineated.

Since several POMC neuropeptides are produced in POMC-expressing melanoma cells, it is important to characterize the peptide(s) pathway that contributes to melanoma suppression by POMC gene delivery. The production of α-MSH by POMC gene transfer is postulated because the anti-neoplastic efficacy of α-MSH has been extensively characterized in melanoma. Treatment with α-MSH attenuates the growth of melanoma cells (Robinson and Healy, 2002) while the presence of wild-type MC1R is essential to the inhibitory function of α-MSH (Robinson and Healy, 2002). Recently, α-MSH is identified as a potential attenuating factor during progression of neuroendocrine neoplasm (Zhou et al., 2005). In the present study, we provide evidence for α-MSH treatment mimics the POMC-induced inhibition of $NF_{\kappa}B$ activities in melanoma cells. Because MC1R is functional in B16-F10 cells, the anti-neoplastic effect of POMC gene delivery in melanoma supports the autocrine-inhibitory circuit for tumor suppression as

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in malignant mesothelioma cells (Catania et al., 2004). Besides, application with α-MSH inhibits the migration or invasiveness (Canton et al., 2003; Eves et al., 2003; Murata et al., 1999; Zhu et al., 2002; Zhu et al., 2004), and attachment to extracellular matrix protein(Robinson and Healy, 2002) in melanoma cells. Therefore, the anti-metastatic effect of POMC gene delivery could also be attributed to α-MSH generation. Taken together, α-MSH/MC1R pathway plays an essential role in the anti-tumor mechanism of POMC gene delivery in melanoma. In contrast, ACTH or β-EP is not known for its role in skin carcinogenesis. Furthermore, treatment with naloxone did not affect the POMC-induced $NF_{\kappa}B$ inhibition nor tumor suppression (data not shown), suggesting that β-EP/opioid receptors pathway is unlikely involved. Nevertheless, we cannot exclude the probable contribution of other POMC neuropeptides or the interaction between several POMC neuropeptides to the POMC-induced melanoma suppression.

Gene delivery of POMC mini-gene, such as α-MSH, has been applied to the treatment of inflammation in the central nervous system (Ichiyama et al., 1999), autoimmune encephalomyelitis(Yin et al., 2003) and liver failure (Wang et al., 2004). In the present study, the strategy of using the entire POMC precursor gene may be advantageous over the mini-gene approach in the following. First, the utilization of POMC precursor gene may be more efficient in production of POMC-derived peptides due to the preservation of the secretory and processing signals for POMC prohormone. Second, the use of natural POMC gene may be less immunogenic than the artificially engineered mini-genes during clinical application. Third, other than α-MSH, POMC gene delivery also generates other beneficiary neuropeptides such as β-EP in tumors or the peripheral tissues. The production of anti-nociceptive β-EP via POMC gene delivery may

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confer relief to the life quality of cancer patients. Previous studies indicated that transplantation of encapsulated tumor cells secreting opioid peptides such as β-EP or enkephalin provided anti-nociceptive effect (Gonzalez-Navarro et al., 1987; Saitoh et al., 1995; Wu et al., 1994). In addition, recent evidence indicates that POMC gene delivery in muscle or kidney alleviates formalin-induced pain(Lu et al., 2002) or bladder pain(Chuang et al., 2003). Hence, β-EP production in the peripheral tissues via POMC gene delivery may exert anti-nociceptive function. Since cancer pain is a devastating problem for the life quality of cancer patients, it seems plausible that POMC gene delivery may inhibit neoplasm through the anti-inflammation functions, but also alleviate cancer pain via the analgesic effect of β-EP.

Although *in situ* POMC gene delivery was effective and well tolerant in tumor-bearing mice, probable obstacles exist that might hinder the clinical application of POMC gene delivery. First, the ectopic ACTH production due to POMC gene transfer might result in disease states such as Cushing's syndrome(Labeur et al., 2004). Second, POMC gene delivery might influence various physiological parameters such as energy balance, steroid synthesis and behavior. Based on these aspects above, future studies are warranted to shed lights on the adverse effects of POMC gene delivery to the physiological processes particularly steroidgenesis and native immune functions. In summary, we demonstrate the potential of POMC gene delivery for treatment of melanoma and probably cancer pain. The feasibility and limitations of peripheral POMC gene transfer for cancer therapy remain to be elucidated.

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Footnotes

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Figure Legends

Figure 1. The immunomodulatory effect of POMC neuropeptides in B16-F10 cells after POMC gene delivery.

(a) Effect of POMC gene delivery on the levels of ACTH, α-MSH and β-EP in conditioned medium of B16-F10 cells. After infection with adenovirus vectors for 48 h, conditioned medium were harvested from B16-F10 cells and analyzed for the levels of POMC-derived peptides (ACTH, α-MSH and β-EP) by RIAs. There was significant difference between control and Ad-POMC-treated groups. **: $P < 0.001$. (b) Effect of conditioned media from POMC-transduced B16-F10 cells on inflammation-related cytokines expression in peripheral blood mononuclear cells (PBMC). After incubation of PBMC with conditioned media from various adenovirus-infected B16-F10 cells for 24 h in the absence or presence of Concanavalin A (Con A; 25 μg/ml), the secretion level of interleukin-2 (IL-2), interferon γ (IFN-γ) and interleukin-10 (IL-10) in stimulated PBMC was determined by ELISA. The results were expressed as mean ± sd of triplicate experiments There was significant difference between control and Ad-POMC-treated groups. *: $P < 0.05$, **: $p < 0.01$

Figure 2. Effect of POMC gene delivery on the tumorigenesis of B16-F10 melanoma cells

(a) Effect of POMC gene delivery on proliferation of B16-F10 melanoma cells. After infection with adenovirus vectors for 48 h, the proliferation of B16-F10 melanoma cells was determined by MTT assay and expressed as mean ± sd percentages of control from quadruplicate experiments. (b) Effect of POMC gene delivery on anchorage-independent

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growth of B16-F10 melanoma cells in soft agar. After 14 days in soft agar, the number of colonies in different groups of B16-F10 melanoma cells were scored and expressed as mean ± sd percentages of control from triplicate experiments. (c) Tumor growth in mice after implantation of POMC-transduced B16-F10 melanoma cells. B16-F10 cells were infected with adenovirus vectors at a MOI of 1000 for 12 h then implanted into C57BL/6 mice at day 0. The tumor volumes in mice were measured at different time intervals. The results were representative of three independent experiments. *: $p < 0.05$, **: $p < 0.01$. (d) Effect of POMC gene delivery on established melanoma in mice. Mice were implanted with B16-F10 melanoma cells at day 0. After tumor size in each mouse was at least 100 mm^3 at day 10, mice were randomly divided into three groups (n = 8) and treated with Ad-GFP (1 x 10^9 pfu), Ad-POMC (1 x 10^9 pfu) or PBS by intratumor injection. Arrow indicated the date of injection. The results were representative of three independent experiments. **: $p < 0.01$.

Figure 3. Effect of POMC gene transfer on metastasis of melanoma cells in mice

(a) Characterization of GFP-expressing B16-F10 melanoma cells. The morphologies of GFP-B16-F10 cells were examined under phase contrast (left panel) and fluorescence microscope (right panel). (b) The metastatic foci of GFP-B16-F10 cells in lung. After intravenous injection of GFP-B16-F10 cells for 14 days, the pulmonary foci were examined by phase contrast (left panel; 100x) and fluorescence microscope (right panel; 200x). (c) The appearance of lung foci after intravenous injection of various B16-F10 cells for 14 days. (d). Quantification of metastatic event by counting lung foci number in various groups of mice. The number of lung foci were counted and expressed as mean ±

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sd after intravenous injection of different engineered B16-F10 cells into mice for 14 days (n = 8). **: $P < 0.001$ (e) Quantification of metastatic event by measuring GFP fluorescence intensity in lung extracts from mice injected with various groups of B16-F10 cells. After extraction, the GFP fluorescence intensity was determined in fluorometer and expressed mean ± sd arbitrary units for each group (n = 8). The results were representative of three independent experiments. **: $P < 0.001$

Figure 4. Effect of POMC gene transfer on the motility and adhesion capability of B16-F10 melanoma cells.

(a) Healing of scratch wound in POMC-transduced B16-F10 cells. After infection with adenovirus vectors for 12 h, a scratch was made and the closure extent of gap was monitored by phase-contrast microscope for 24 h. Quantification of gap area in POMC-transduced B16-F10 cells at 24 h. (b, c) Effect of POMC gene transfer on the adhesion capacity of B16-F10 cells to extracellular matrix proteins. After infection with adenovirus vectors for 12 h, the binding extent of B16-F10 melanoma cells to wells coated with (b) fibronectin or (c) or laminin was measured at different time courses. **: $P < 0.001$, *: $P < 0.05$

Figure 5. Effect of POMC gene delivery on cyclooxygenase-2 (COX-2) expression and prostaglandin E_2 (PGE_2) production in B16-F10 cells.

(a) Effect of POMC gene delivery on COX-2 mRNA level. Real time of COX-2 mRNA in B16-F10 after POMC gene transfer. After infection with adenovirus vectors for 24 h, total RNA was isolated from different groups of B16-F10 cells for measurement of

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mRNA level by quantitative RT-PCR analysis using actin as internal control. (b) Effect of POMC gene delivery on COX-2 protein level. After infection with adenovirus vectors for 24 h, protein extract was isolated from different groups of B16-F10 cells for determination of COX-2 protein level by western blot analysis. (c) Effect of POMC gene delivery on the PGE_2 production. After infection with adenovirus vectors for 24 h, cultured media was collected from different groups of B16-F10 cells to evaluate PGE_2 production using PGE_2 immunoassay. *: $P < 0.05$; **: $P < 0.001$

Figure 6. Effect of POMC gene delivery on cyclooxygenase-2 (COX-2) expression and prostaglandin (PGE_2) production in melanoma tissues.

After implantation of various adenovirus-infected B16-F10 cells into C57BL/6 mice for 14 days, melanoma was dissected and analyzed for COX-2 expression. (a) Western blot analysis of COX-2 protein level in melanoma tissues. *: $P < 0.05$ (b) ELISA analysis of PGE_2 level in melanoma tissues extract. *: $P < 0.05$ (c) Immunohistochemical analysis of COX-2 expression in normal skin (left panel) and melanoma (right panel). (d) Effect of POMC gene delivery on COX-2 expression in melanoma tissues. Tumors derived from Ad-GFP- or Ad-POMC-infected B16-F10 cells were analyzed for COX-2 expression by immunohistochemistry. Magnification: x 20 and x 40.

Figure 7. Comparison of the effect of COX-2 inhibitor, NS398, and POMC gene transfer on the tumorigenecity of B16-F10 melanoma cells

(a) cell proliferation. After treatment with NS398 (1, 10 and 100 μM) or infection with adenovirus vectors at a MOI of 1000 for 24 h, the proliferation of B16-F10 melanoma

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cells was determined by MTT assay. (b) PGE_2 production. After treatment with NS398 (1, 10, and 100 μM) or infection with adenovirus vectors at a MOI of 1000 for 24 h, the culture medium of B16-F10 cells were collected for analysis of PGE_2 release by ELISA. (c) Anchorage-independent growth. After treatment with NS398 (1, 10, and 100 μM) or infection with adenovirus vectors at a MOI of 1000 for 24 h, B16-F10 cells were plated on 5% soft-agar to evaluate their colonies-forming capabilities. (d) cell migration. After treatment with NS398 (1, 10, and 100 μM) or infection with adenovirus vectors at a MOI of 1000 for 24 h, B16-F10 cells were introduced with a scratch wound then measured for the healing extent of gap after 24 h. (e) melanoma growth in mice after implantation of B16-F10 cells. After treatment with NS-398 or infection with adenovirus vectors at a MOI of 1000 for 24 h, B16-F10 cells were implanted to monitor tumor growth in C57BL/6 mice (n = 6 per group). For NS-398 treatment, the implanted mice received daily administration of NS-398 (at a dose of 10 mg/kg) via intraperitoneal route. At day 14, the tumor volumes in different groups of mice were measured and expressed as mean ± sem mm^3 and (f) PGE_2 production in melanoma tissues. The protein extracts were isolated from melanoma tissues and analyzed for PGE_2 level by ELISA. *: $P < 0.05$ **: $P < 0.01$.

Figure 8. Effect of POMC gene delivery on the $NF_\kappa B$ activity in B16-F10 cells

The basal and stimulate $NF_\kappa B$ activities in B16-F10 cells were measured in the absence (right panel) and presence (left panel) of TNF-α (10 ng/ml), respectively. (a) Western blot analysis of $NF_\kappa B$ expression in the nuclear extract of B16-F10 cells after POMC gene delivery. The protein expression of histone was measured as loading control for the

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nuclear extract. (b) The electrophoretic mobility shift assay of $NF_{\kappa}B$ binding activities in B16-F10 cells after POMC gene delivery. (c) The $NF_{\kappa}B$-driven luciferase activities in B16-F10 cells after POMC gene delivery. **: $P < 0.001$.

Figure 9. Effect of α-MSH antagonists, GHRP-6, on POMC-induced inhibition of $NF_{\kappa}B$ activities, PGE_2 production and melanoma growth.

(a) Effect of POMC peptides on the $NF_{\kappa}B$-driven luciferase in B16-F10 cells. After transfection with luciferase vectors, B16-F10 cells were treated with 1 μM of ACTH, α-MSH and β-EP, respectively, for 24 h then assayed for luciferase activities. (b) Effect of GHRP-6 on POMC-induced inhibition of $NF_{\kappa}B$-driven luciferase activities in B16-F10 cells. After transfection with luciferase vectors and infection with adenovirus vectors, B16-F10 cells were treated with PBS or GHRP-6 (1 μM) for 24 h then assayed for $NF_{\kappa}B$-driven luciferase activities. (c) Effect of H-89 on POMC-induced inhibition of $NF_{\kappa}B$-driven luciferase activities in B16-F10 cells. After transfection with luciferase vectors and infection with adenovirus vectors, B16-F10 cells were treated with PBS or H-89 (5 μM) for 24 h then assayed for $NF_{\kappa}B$-driven luciferase activities. (d) Effect of GHRP-6 on POMC-induced inhibition of PGE_2 production in B16-F10 cells. After infection with adenovirus vectors, B16-F10 cells were treated with PBS or GHRP-6 (1 μM) for 24 h. The culture media were collected for PGE_2 ELISA assay. (e) Effect of co-administration of GHRP-6 on the POMC-mediated suppression of melanoma growth in C57BL/6 mice. After infection with various adenovirus vectors, B16-F10 cells were implanted in C57BL/6 mice to monitor tumor growth (n = 12 per group). After implantation for 3 days, mouse was either injected with PBS (n = 6) or GHRP-6 (100 μg;

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n = 6). At day 21, the representative profile of melanoma growth in different groups of mice was recorded (left panel). The tumor size in different groups of mice were measured and expressed as mean ± sem mm^3 (n = 6; right panel). *: $P < 0.05$, **: $P < 0.001$.

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Figure 1

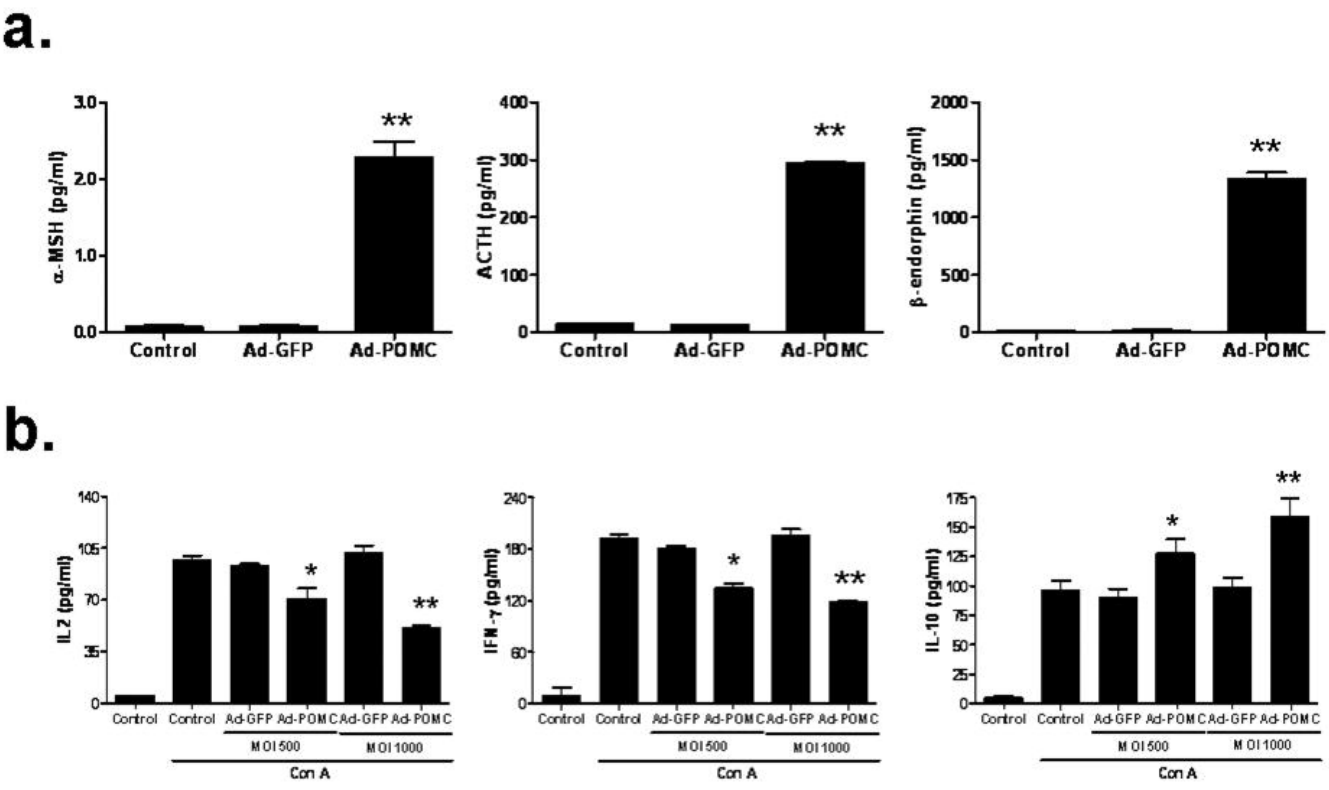

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Figure 2

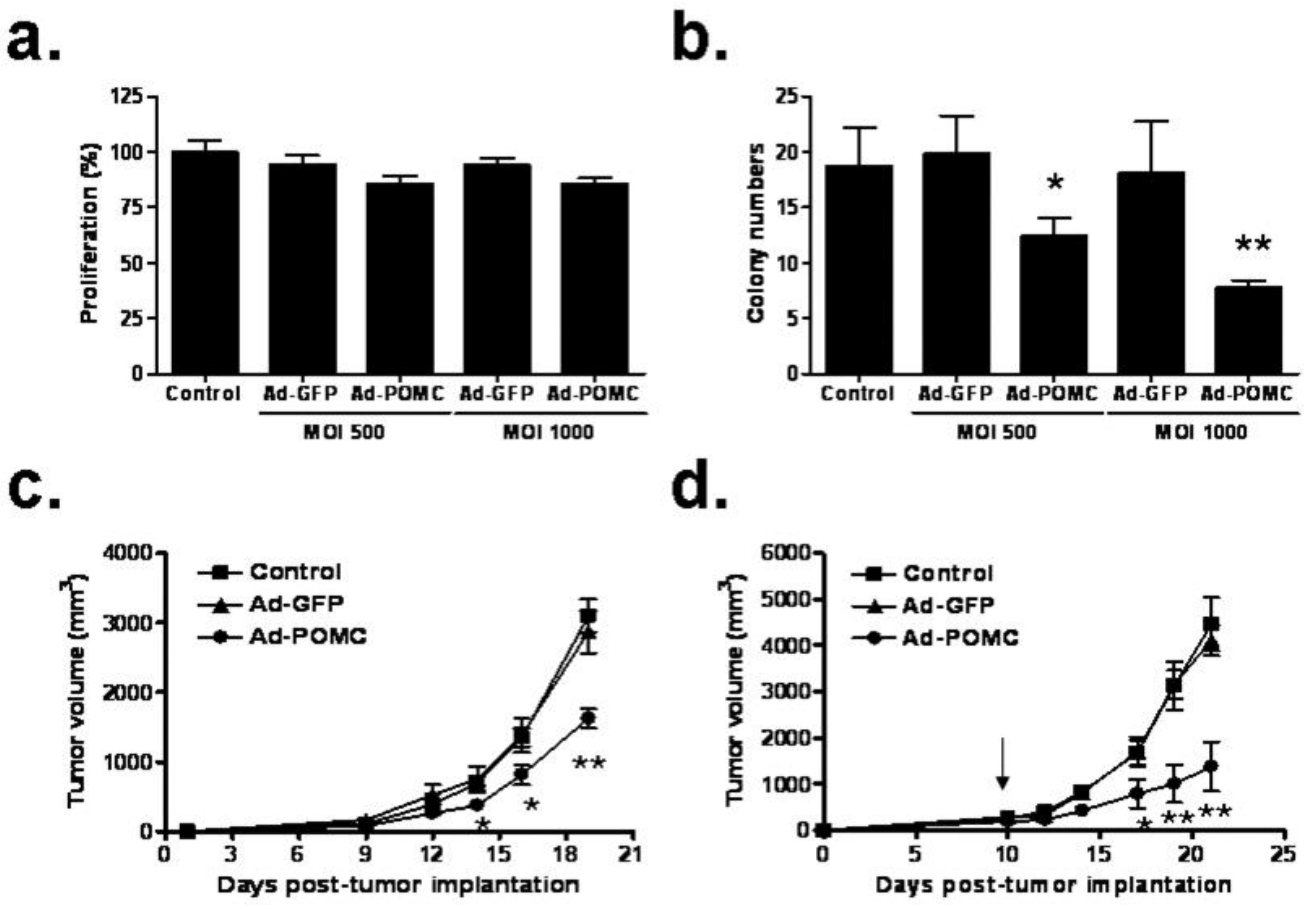

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Figure 3

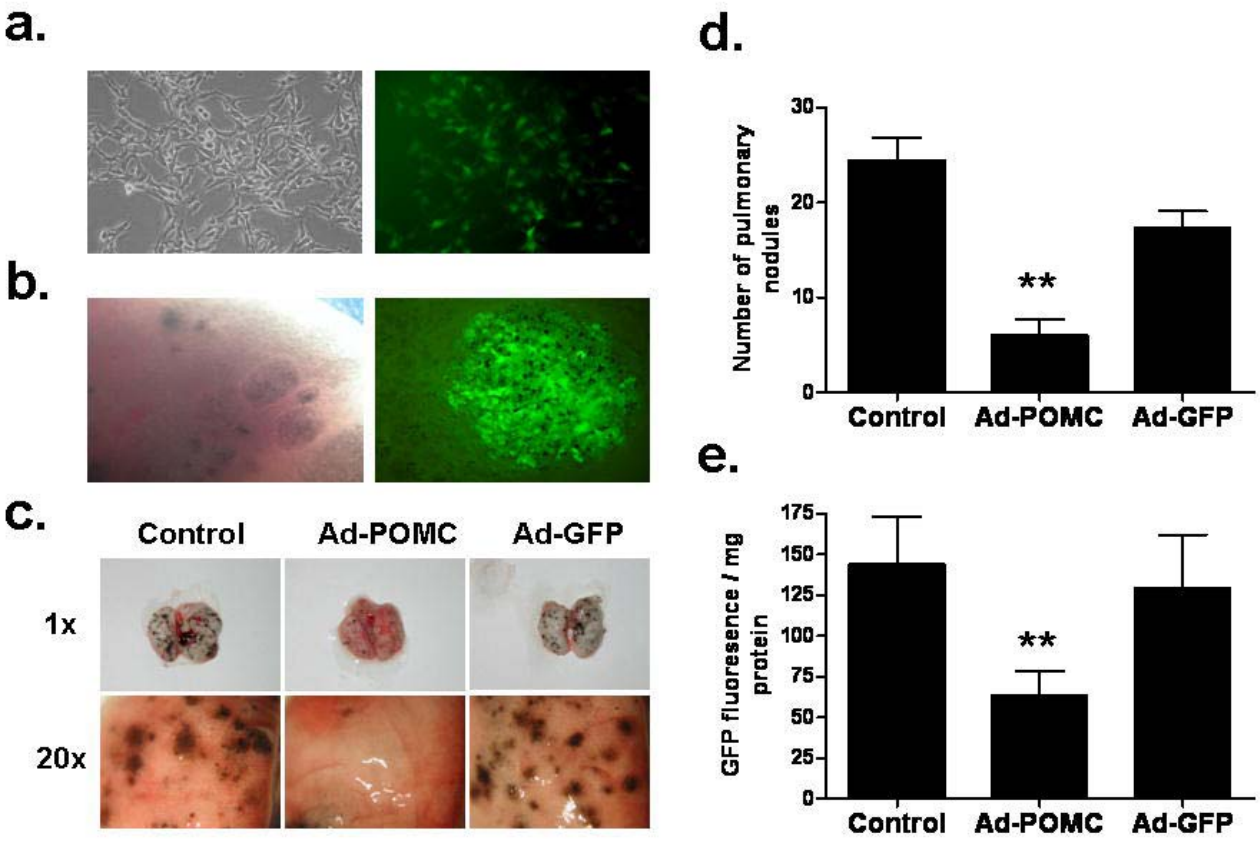

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Figure 4

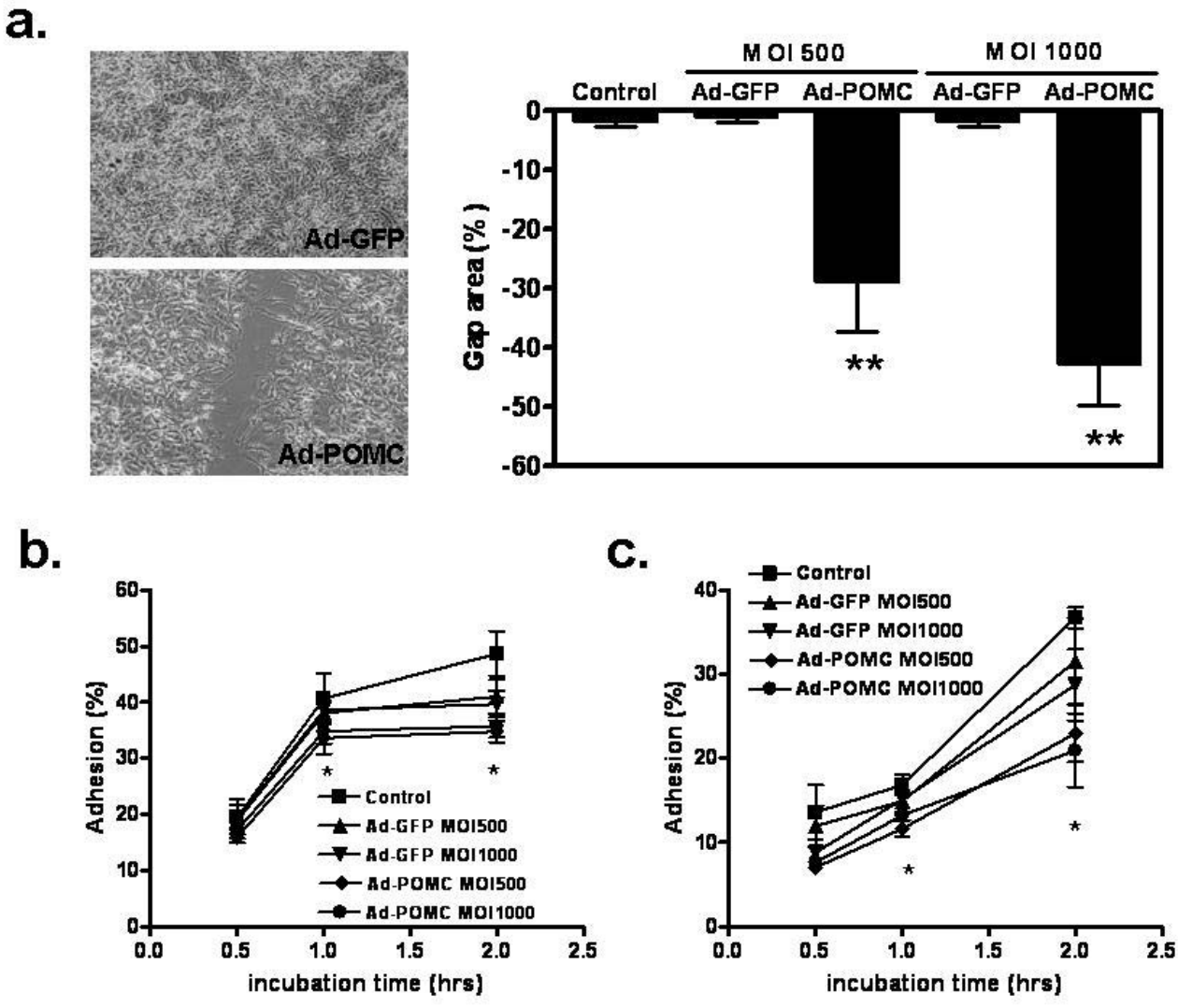

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Figure 5

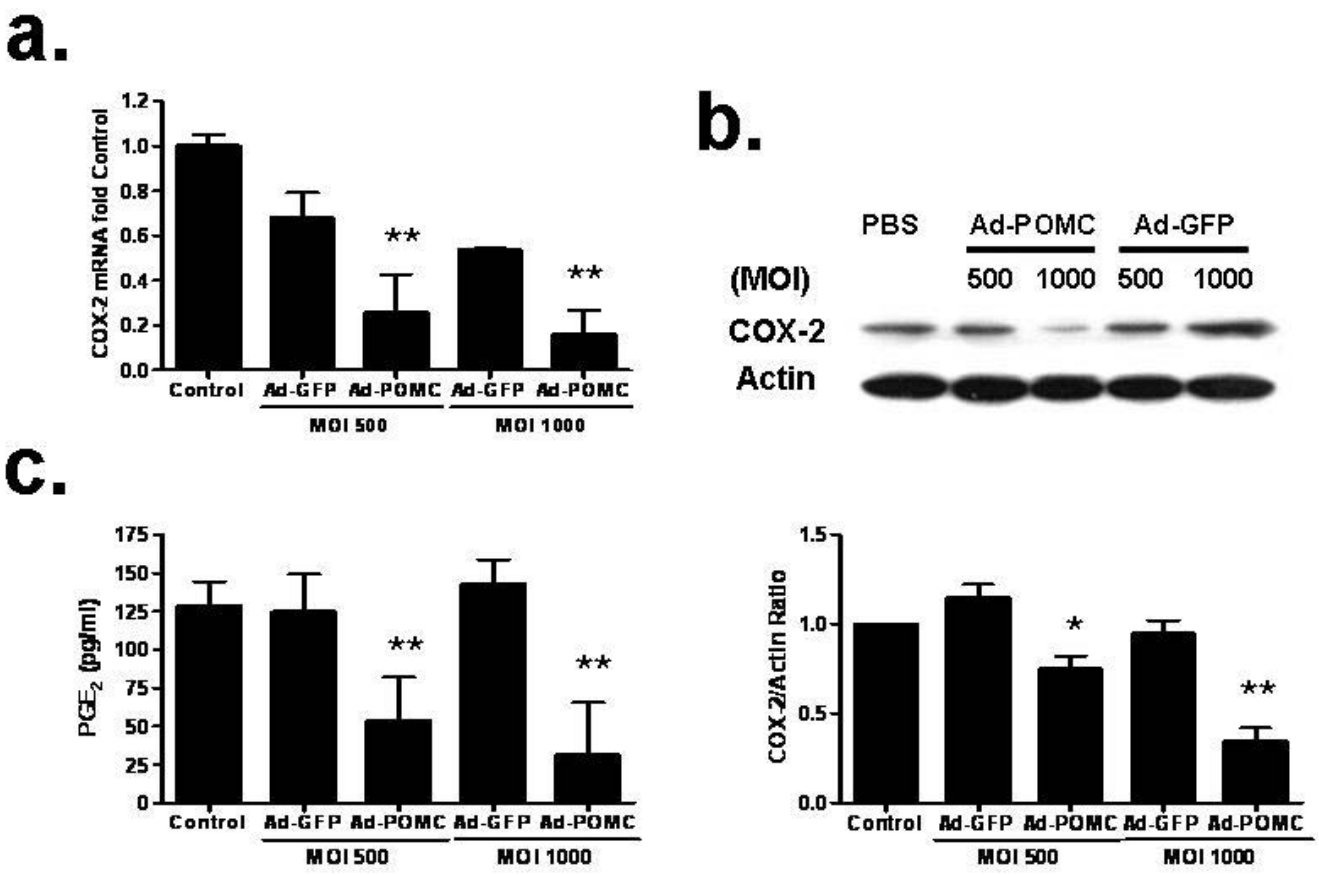

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Figure 6

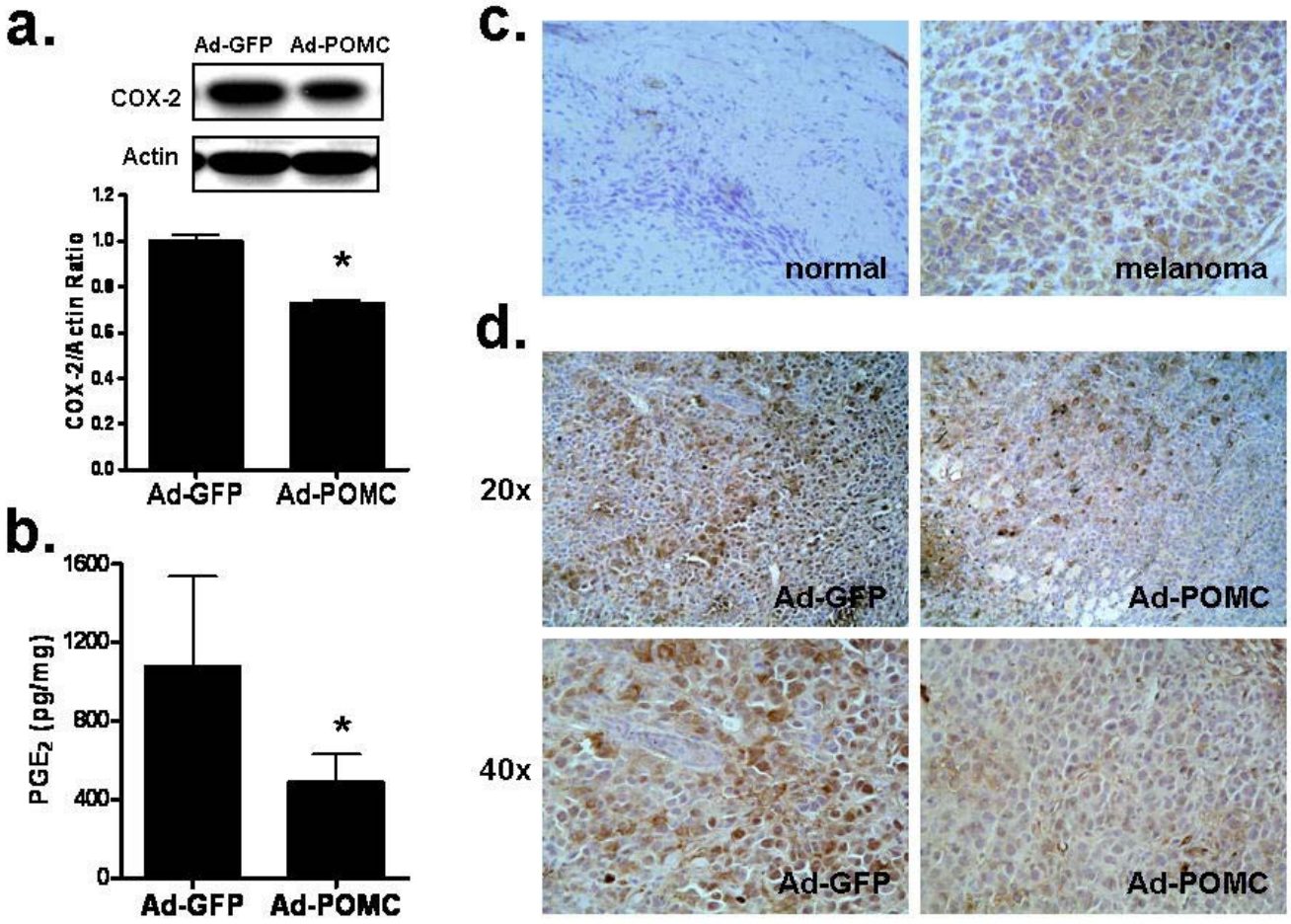

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Figure 7

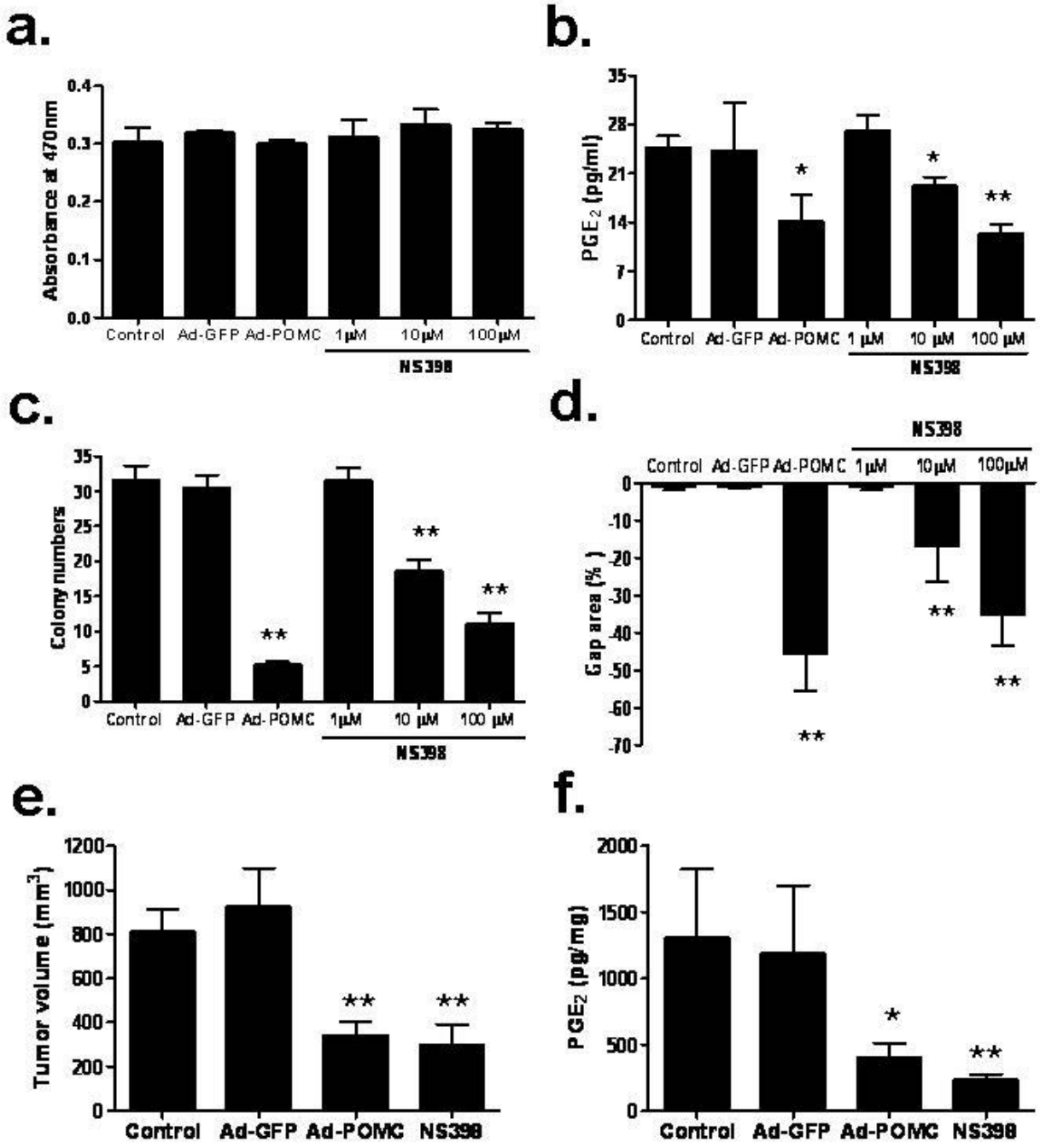

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Figure 8

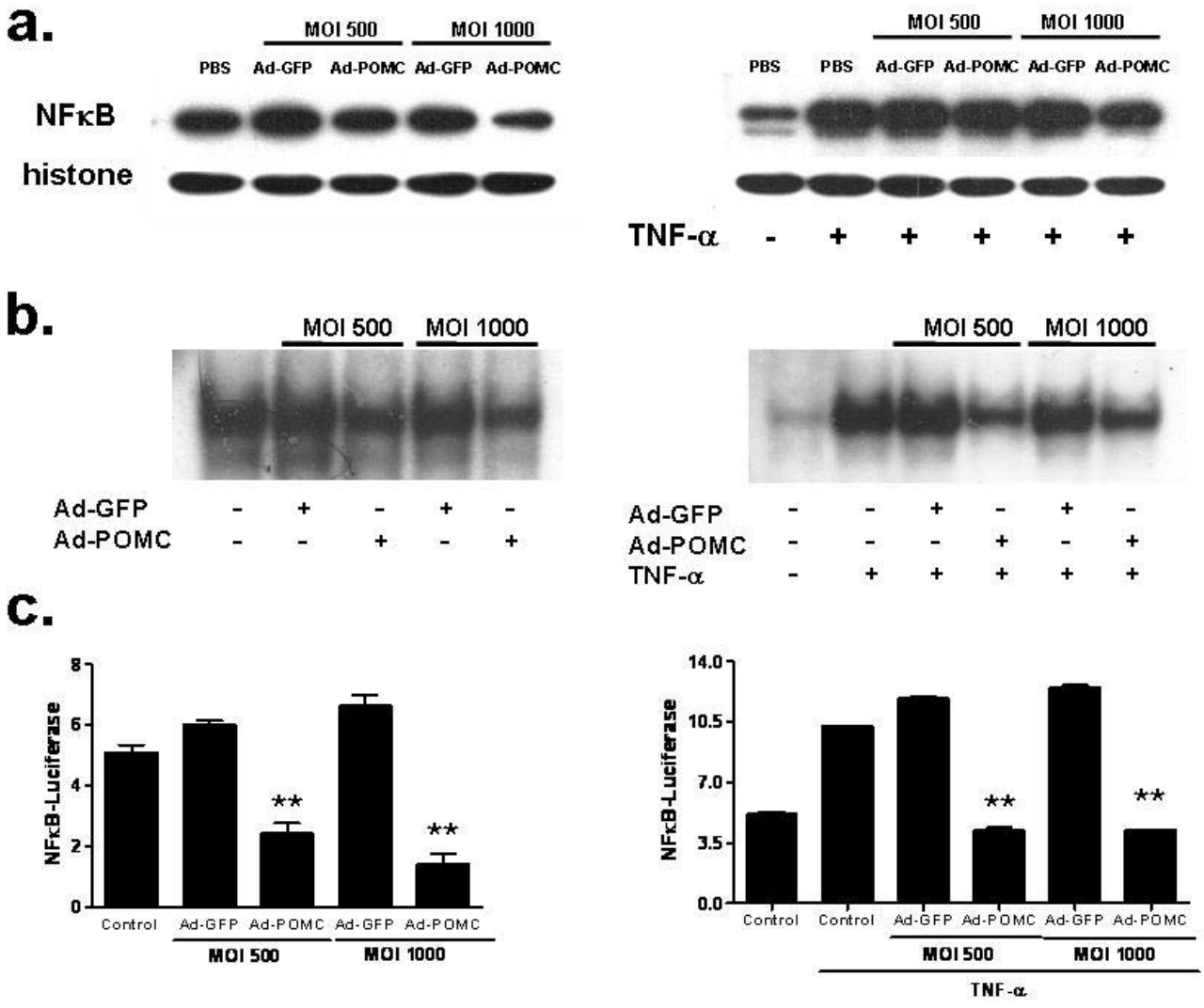

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Figure 9