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Adenoviral Expression of Vascular Endothelial Growth Factor Splice Variants Differentially Regulate Bone Marrow-Derived Mesenchymal Stem Cells

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Bone marrow-derived mesenchymal stem cells (MSCs) are being explored for clinical applications, and genetic engineering represents a useful strategy for boosting the therapeutic potency of MSCs. Vascular endothelial growth factor (VEGF)-based gene therapy protocols have been used to treat tissue ischemia, and a combined VEGF/MSC therapeutics is appealing due to their synergistic paracrine actions. However, multiple VEGF splice variants exhibit differences in their mitogenicity, chemotactic efficacy, receptor interaction, and tissue distribution, and the differential regulatory effects of multiple VEGF isoforms on the function of MSCs have not been characterized. We expressed three rat VEGF-A splice variants VEGF120, 164, and 188 in MSCs using adenoviral vectors, and analyzed their effects on MSC proliferation, differentiation, survival, and trophic factor production. The three VEGF splice variants exert common and differential effects on MSCs. All three expressed VEGFs are potent in promoting MSC proliferation. VEGF120 and 188 are more effective in amplifying expression of multiple growth factor and cytokine genes. VEGF164 on the other hand is more potent in promoting expression of genes associated with MSC remodeling and endothelial differentiation. The longer isoform VEGF188, which is preferentially retained by proteoglycans, facilitates bone morphogenetic protein-7 (BMP7)-mediated MSC osteogenesis. Under serum starvation condition, virally expressed VEGF188 preferentially enhances serum withdrawal-mediated cell death involving nitric oxide production. This work indicates that seeking the best possible match of an optimal VEGF isoform to a given disease setting can generate maximum therapeutic benefits and minimize unwanted side effects in combined stem cell and gene therapy.

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Vascular endothelial growth factors (VEGFs) are potent effectors of vascular development, normally expressed in the mesenchymal tissue during development, and inducible by hypoxia (Ferrara, 1996; Neufeld et al., 1999; Greenberg et al., 2002). In humans, alternative splicing from the single VEGF-A gene gives rise to multiple VEGF splice variants encoding 121, 145, 165, 189, and 206 amino acids (Ferrara, 1996). In rodents, three homologous splice variants of 120, 164, and 188 amino acids have been demonstrated (Breier et al., 1992).

Much interest has been placed on the role of the various VEGF isoforms in vascular biology. These VEGF variants exhibit differences in their mitogenicity, chemotactic efficacy, receptor interaction, and tissue distribution (Keyt et al., 1996; Cheung et al., 1998; Soker et al., 1998; Neufeld et al., 1999). For instance, VEGF165 preferentially interacts with neuropilin-1 (NRP1), bridging and stabilizing the NRP1–VEGFR2 complex (Pan et al., 2007). Brain injury is associated with differential expression of VEGF isoforms (Dore-Duffy et al., 2007). Although all VEGF variants contain a signal peptide sequence, only the shorter isoforms VEGF121, 145, and 165 are secreted and readily diffusible presumably due to the absence of a putative heparin-binding domain. Zhang et al. (2000) demonstrated that VEGF121 was more tumorigenic than the other VEGF splice variants. Notably, using the Cre/loxP-mediated exon deletion strategy, Carmeliet et al. (1999) demonstrated impaired myocardial angiogenesis and ischemic cardiomyopathy in mice expressing the VEGF120 splice variant exclusively, suggesting that these multiple VEGF splice variants are not functionally redundant or equivalent. Consistent with this demonstration, embryonic lethality was observed in heterozygous VEGF-deficient (VEGF+/−) embryos, which is indicative of a tight dose-dependent regulation of embryonic vessel development by VEGFs (Carmeliet et al., 1996).

Bone marrow-derived mesenchymal stem cells (MSCs) have recently received much attention in the field of regenerative medicine in part due to their abilities to produce a wide range of growth factors and cytokines (Darland et al., 2003; Wieczorek et al., 2003; Kinnaird et al., 2004). The ease of isolation, capacity for large-scale expansion, and alleged immunoprivileged status of MSCs have prompted the development of clinical trials aimed at assessing the safety and efficacy of MSC transplantation for a variety of pathological conditions such as heart failure, bone fractures, and neurological diseases (Devine, 2002; Giordano et al., 2007). Interestingly, the cardiovascular therapeutic effects of MSCs have been largely attributed to their paracrine actions (Gnecchi et al., 2005; Tang et al., 2005), although the

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identity of the effective growth factors remains to be determined. VEGF165, in particular, has been used to enhance the function of MSCs and endothelial progenitor cells (EPCs) for stem cell therapy (Iwaguro et al., 2002; Wang et al., 2006). Although this combined stem cell and gene therapy approach may amplify the endogenous stem cell growth factor network and further boost therapeutic efficacy, the optimal VEGF isoform(s) and its regulatory effect on stem cell function have not been addressed. We note that clinical applications of VEGFs invariably used VEGF121 or VEGF165, assuming that they have similar therapeutic effects (Yla-Herttuala et al., 2007). In this work, we tested the hypothesis that isoform-specific functions of VEGFs could be identified that differentially regulate the behavior and phenotype of MSCs. Toward this end, we cloned multiple VEGF-A splice variants, expressed the cDNA sequences using recombinant adenoviral vectors, and examined their regulatory effects on porcine MSCs. These studies reveal that the growth, differentiation, growth factor/cytokine secretion capacity, and survival of MSCs are differentially regulated by VEGF-A splice variants. The implication of these findings in relation to stem cell/gene therapy is discussed.

Materials and Methods

Cell culture

Porcine bone marrow-derived MSCs were isolated as described (Vacanti et al., 2005). MSCs were maintained in Advanced-DMEM (ADMEM; Invitrogen, Grand Island, NY) supplemented with 4% fetal bovine serum (FBS), 2 mM GlutaMax, and 50 μg/ml gentamycin and incubated in a humidified 5% CO_2 atmosphere at 37°C. MSC cultures used typically received less than 10 passages. Neonatal rat cardiomyocytes and fibroblasts were prepared as previously described (Kositprapa et al., 2000), and the cells were maintained in MEM supplemented with 10% horse serum and 50 μg/ml gentamycin. P19 embryonal carcinoma stem cells and HEK293 cells were maintained in MEM supplemented with 5% FBS and 50 μg/ml gentamycin. Cells were trypsinized using a 0.05% trypsin-0.5 mM EDTA solution upon confluency. For VEGF addition experiments, freshly reconstituted recombinant mouse VEGF120 and VEGF164 proteins (R&D Systems; Minneapolis, MN) were added to culture medium after overnight cell plating.

Hypoxia and VEGF cloning

Neonatal rat cardiomyocytes were subjected to hypoxia for 8–16 h as described previously (Kositprapa et al., 2000), and total RNA was isolated using the guanidinium thiocyanate method. cDNA was prepared as detailed (Kositprapa et al., 2000), and full-length VEGF sequences were amplified by PCR using sequence information derived from GenBank NM-003376: GAAACCATGAACTTT-CTGCTCT (5′ primer) and GACAAGCCAAGGCGGTGAGCC (3′ primer). PCR products were cloned using the TOPO TA cloning system (Invitrogen, Carlsbad, CA), and sequenced in both directions by RPCI DNA sequencing facility (Buffalo, NY). VEGF sequence information was deposited into GenBank (accession numbers: AY033506-AY033508).

Construction and use of VEGF adenoviruses

Adenoviral production protocols were as described previously (Lin et al., 2007). The recombinant adenovirus Ad-LacZ was provided by Kirk Hammond (UC San Diego), which encodes β-galactosidase. VEGF cDNA inserts were excised from the TOPO vector using *Hin*dIII-*Not*I (for VEGF164 and 188) or *Kpn*I-*Xho*I (for VEGF120), and inserted into digested pShuttle-CMV vector. The recombinant shuttle DNA and pAdEasy-1 DNA were recombined in HEK 293 cells. The virus was amplified three rounds in HEK293 cells to yield $\sim 10^9$ viral particles/ml culture medium. Recombinant virus was confirmed by RT-PCR and/or Western blotting. For cell infection, viral lysates were diluted 10-fold with serum-free MEM, and added to adherent cells for 3 h with occasional agitation, following which cells were maintained in the growth medium containing 10 μl viral lysate per ml of medium. Cells were harvested at various time points after infection as indicated in each experiment. Recombinant adenovirus expressing bone morphogenetic protein-7 (BMP7), fibroblast growth factor-5 (FGF5), or β-galactosidase (LacZ) was each described in our previous work (Suzuki et al., 2005; Vacanti et al., 2005).

Chromogenic β-galactosidase (LacZ) staining

Cells plated on 35-mm dishes were infected with LacZ adenovirus after overnight plating, and fixed 2 days after viral infection by 4% paraformaldehyde. X-gal staining of β-galactosidase was performed by immersing fixed cells in the substrate solution (60 mM Hepes pH7.4, 100 mM NaCl, 3 mM $MgCl_2$, 3 mM K-ferricyanide, 3 mM K-ferrocyanide, and 0.5 mg/ml X-gal) at 37°C for several hours until the blue color appeared. Digital imaging was performed using a Nikon E600 fluorescence microscope.

In situ immunostaining

Porcine MSCs plated on cover slips were fixed with 4% paraformaldehyde at room temperature for 5 min and blocked in phosphate-buffered saline (PBS) containing 10% mouse or rabbit serum, following which cells were incubated at 4°C with VEGFR-1 antibody (#ab2350; Abcam, Cambridge, MA), VEGFR-2 antibody (#MAB3571; R&D Systems), or NRP1 antibody (#ab16786; Abcam) each diluted in an antibody incubation solution (PBS supplemented with 1% bovine serum albumin and 0.2% Triton X-100). An isotype control antibody was used in each case as negative staining control. Cells were washed and stained with a TRITC-conjugated secondary antibody for 1 h at room temperature. Washed cells were mounted in a mounting medium containing DAPI (Vector Laboratories, Burlingame, CA), and digitally imaged using an epifluorescence microscope (Nikon Eclipse E600).

BMP treatment and alkaline phosphatase (ALP) assay

Recombinant human BMP7 was purchased from R&D Systems. MSCs 1-day after adenoviral infection were incubated with 0.5 μg/ml BMP7 in ADMEM + 1% FBS for 2 weeks, following which cells were rinsed once with HBSS and lysed with PBS supplemented with 0.1% TX-100. Lysates were clarified briefly by centrifugation. Protein concentrations and ALP activities of the lysates were determined using the BCA protein assay kit (Pierce Biotech, Rockford, IL) and the ALP assay kit (Biomedical Research Service, Buffalo, NY). ALP activities were expressed as optical density ($OD_{412\ nm}$) units per mg proteins.

RNA preparation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Cells were lysed with RLT buffer supplied with the Qiagen's RNeasy RNA isolation kit, and RNA was isolated according to the manufacturer's instructions. qRT-PCR was performed as follows. One microgram RNA was used for reverse transcription using Superscript III reverse transcriptase (Invitrogen, Carlsbad) according to the manufacturer's instructions. PCR primers were designed using MIT's Primer3 software. Each PCR amplification product was verified by agarose gel electrophoresis followed by DNA sequencing confirmation. Real-time PCR was performed using the MyIQ machine with the SYBR green kit (Bio-Rad, Hercules, CA). Amplification conditions after an initial denaturation step for 3 min at 95°C were: 45 cycles of 95°C 10 sec for denaturation and 55°C 30 sec for annealing and elongation. Melting curve analysis was performed to check for a single amplicon. MyIQ analysis software was used for determining crossing points. Data were analyzed by the $2^{\Delta\Delta CT}$ method, and presented as percent gene expression in control cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used

as the reference gene for calculations. Primer sequences for porcine cDNAs are: GAPDH-TGCCCAGAACATC (5′) and GGATGACCTTGCC (3′); β2-microglobulin-AAACGGAA-AGCCAAATTACC (5′) and ATCCACAGCGTTAGGAGTGA (3′); TGFβ1-AAGCGGCAACCAAATCTATG (5′) and CACGT-GCTGCTCCACTTTTA (3′); IGF2-ACACCCTCCAGTTTG-TCTGC (5′) and AAGCAGCACTCTTCCACGAT (3′); bFGF-GGAGTGTGTGCAAACCGTTA (5′) and TCGTTTCAGTGCC-ACATACC (3′); FGF7: GCATGCAATGACATGACTCC (5′) and TGACTTTGCCTCGTTTGTCA (3′); FGF9-GGACTCTACCT-GGGCATGAA (5′) and TCCAGTGTCCACGTGTTTGT (3′); GDF8-CTGTAACCTTCCCAGGACCA (5′) and TGCTCA-TCACAGTCGAGTCC (3′); VEGF-CTACCTCCACCA-TGCCAAGT (5′) and ACACAGGACGGCTTGAAGAT (3′); NGFβ-CAAGGGAGCAGCTTTCTGTC (5′) and CTGTGTCAA-GGGAATGCTGA (3′); VEGFR1-GAAAGGCCAAGATTTGT-GGA (5′) and GGGAGTGGAGTACGTGAAGC (3′); VEGFR2-CTTTGTGCGAGGTATCCAGA (5′) and GCTGATCATGTA-GCTGGGAAT (3′); neuropilin-GGCTAAGAATGGAGCTGCTG (5′) and CCTCCTGTGAGCTGGAAGTC (3′); PECAM1-TCAAT-GCTCCGTGAAAGAAG (5′) and CCTGGGTGTCATTCAAA-GTG (3′); vWF-CTTCTGGACCTGGTCTTCCT (5′) and ATGT-GCAGGTGTTCCATCAT (3′); β-actin-TCTGGCACCACACC-TTCT (5′) and TGATCTGGGTCATCTTCTCAC (3′); aFGF-AGAGGCTGGAGGAAAACCAT (5′) and GGCTTTCTGGCCA-TAGTGAG (3′); Angpt1-GGAAACCGAGCCTATTCACA (5′) and GCTCTGTTTTCCTGCTGTCC (3′); LIF-GTCACCCATG-TCACAGCAAC (5′) and CCCCTGGGCTGTGTAGTAGA (3′); BDNF-GACGAGGACCAGAAAGTTCG (5′) and AGAAGAGG-AGGCTCCAAAGG (3′); IL6-TTCACCTCTCCGGACAAAAC (5′) and TCTGCCAGTACCTCCTTGCT (3′); MCP1-CACCAG-CAGCAAGTGTCCTA (5′) and TCCAGGTGGCTTATGGA-GTC (3′); SDF1-CCTTGCCGATTCTTTGAGAG (5′) and CAATTTTGGGTCAATGCACA (3′). Oligonucleotides were synthesized by Invitrogen (Carlsbad, CA).

MTT cell proliferation assay

Cells were plated on 24-well plates ($1–2 \times 10^4$ cells/well) in the growth medium for the assays. Protocols for MTT assays were as described previously (Chen et al., 1998). In brief, growth medium containing 0.25 mg/ml MTT was added to each well, and cells were further incubated at 37°C for 20 min, following which the medium was replaced by 0.2 ml DMSO per well. MTT dye conversion was determined by measuring $OD_{540\ nm}$ of the DMSO extracts using DMSO as blank.

LDH release assay

Cells were plated onto 24-well plates (2×10^4 cells/well) in regular growth media for the assays. Protocols for LDH release assays were as described previously (Chen et al., 1998). In brief, cell death as determined by LDH release was performed by mixing 20 μl of collected culture medium with 50 μl of LDH assay solution in a 96-well plate. The plate was incubated at 37°C for 30–60 min, following which the assay was terminated by addition of 50 μl of 5% acetic acid to each well. LDH activities in the media were measured at $OD_{492\ nm}$. Some error bars were too small to appear on the graph. The NO synthase inhibitor N^G-Monomethyl-L-arginine (L-NMMA) was purchased from EMD Biosciences (San Diego, CA).

Western blotting

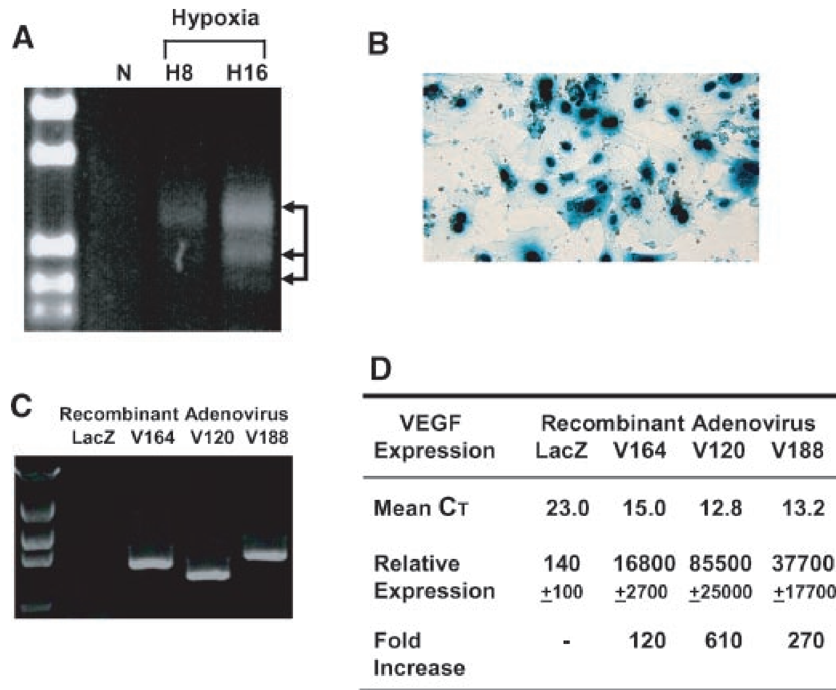
Total cellular proteins (typically 20–30 μg per lane) were resolved by SDS–PAGE and electrotransferred to Immobilon-P membrane as described (Walowitz et al., 1998). The membrane was first incubated with a 1,000-fold diluted primary antibody solution for at least 3 h or overnight at 4°C followed by washing with a PBS solution supplemented with 0.025% TW-20. Secondary antibodies conjugated with horse radish peroxidase were used to probe blotted membranes, and signals were developed using the chemiluminescent substrate luminol (Pierce Biotechnology) and imaged by Fuji imager.

Results

Combined gene and stem cell applications have been found to boost the therapeutic potential of adult stem cells (Iwaguro et al., 2002; Gnecchi et al., 2006; Wang et al., 2006). Although the therapeutic potential of MSCs has been demonstrated in both preclinical and clinical studies, we are interested in enhancing the therapeutic potency of MSCs by virally expressed VEGF. However, multiple VEGF splice variants exist, and no study has been performed to examine whether the VEGF variants may affect MSCs differentially. Thus, we first attempted to isolate multiple VEGF splice variants by cDNA cloning. VEGF is synthesized and secreted by many differentiated cells in response to a variety of stimuli including hypoxia. We previously characterized hypoxia response of cultured neonatal rat cardiomyocytes (Kositprapa et al., 2000; Lin et al., 2004), and this hypoxic cell system was used to isolate multiple rat VEGF-A splice variants. RT-PCR analysis shown in Figure 1A reveals multiple transcripts induced by 8–16 h of hypoxia. These putative VEGF-A transcripts were cloned and sequenced. Screening/sequencing of more than 20 clones revealed six different VEGF splice variants encoding five different VEGF species (GenBank accession #:AY033503-033508). Approximately half of the clones analyzed encode only a 23-amino acid signal peptide. Three mature VEGF splice variants were identified, encoding 120, 164, and 188 amino acids (corresponding to human VEGF121, 165, and 189), as documented for rodent VEGFs (Ferrara, 1996).

Recombinant adenoviruses were constructed expressing each of the three VEGF splice variants. MSCs were first infected with LacZ and VEGF adenoviruses to assess infection efficiency and expression level. Chromogenic X-gal staining of LacZ adenovirus-infected MSCs shows that 100% of the plated cells were infected by the adenovirus and expressed the reporter gene as indicated by the presence of dark blue X-gal deposits inside the cells (Fig. 1B). Non-quantitative RT-PCR followed by gel electrophoresis reveals overexpressed full-length transcripts encoded by VEGF120, 164, and 188 (Fig. 1C). Real-time quantitative qRT-PCR analysis (Fig. 1D) shows a low level of endogenous VEGF expression in MSCs with a mean threshold cycle (C_T) of 23. Calculation of relative VEGF expression in infected MSCs using the $2^{\Delta\Delta CT}$ method reveals that VEGF120, 164, and 188 adenoviruses caused a mean 610-, 120-, and 270-fold increase in VEGF expression. We believe that high-level VEGF expression mediated by the adenoviral vector system could potentially modify the phenotype of MSCs. Further, differential levels of VEGF isoform expression may cause differential effects on MSCs.

We next examined the VEGF signaling system by determining whether VEGF receptors including VEGF-R1, VEGF-R2, and neuropilin might be expressed by cultured MSCs. Since limited information indicates that MSCs express little or no VEGF-R1 and VEGF-R2 (Okuyama et al., 2006; Ball et al., 2007), we used the more sensitive qRT-PCR method for the analysis. Threshold cycles (C_T) of the three receptor genes were compared to those of three house-keeping genes β-actin, β2-microglobulin (β2-M), and GAPDH genes, which showed mean C_T values of 19–21 (Fig. 2, left part). VEGF-R1 and neuropilin genes were expressed at low levels with mean C_T values of ~25 whereas VEGF-R2 was barely detected with a mean C_T of ~34. At the protein level, although Western blotting failed to detect all three receptor proteins (data not shown), in situ immunostaining detected faint but specific VEGF-R1 and neuropilin antibody staining (Fig. 2, right parts). VEGF-R2 protein was again undetectable. Thus, both qRT-PCR



VEGF Expression	Recombinant Adenovirus LacZ	V164	V120	V188
Mean C_T	23.0	15.0	12.8	13.2
Relative Expression	140 $\pm$100	16800 $\pm$2700	85500 $\pm$25000	37700 $\pm$17700
Fold Increase	-	120	610	270

Fig. 1. Cloning and expression of multiple VEGF-A splice variants. A: Neonatal rat cardiomyocytes were subjected to hypoxia for 8 and 16 h. RNA samples isolated from normoxic cardiomyocytes (N) and those subjected to 8-h (H8) and 16-h (H16) hypoxia were analyzed by RT-PCR. cDNA products were fractionated by 1% agarose gel. B: Colorigenic X-gal staining of MSCs infected with LacZ adenovirus showing 100% viral infection efficiency. C: Gel analysis of RT-PCR products from MSCs infected LacZ, VEGF120, 164, and 188 adenoviruses showing overexpressed full-length VEGF transcripts. D: Real time quantitative qRT-PCR analysis of VEGF expression in infected MSCs showing threshold cycles and fold induction in VEGF expression.

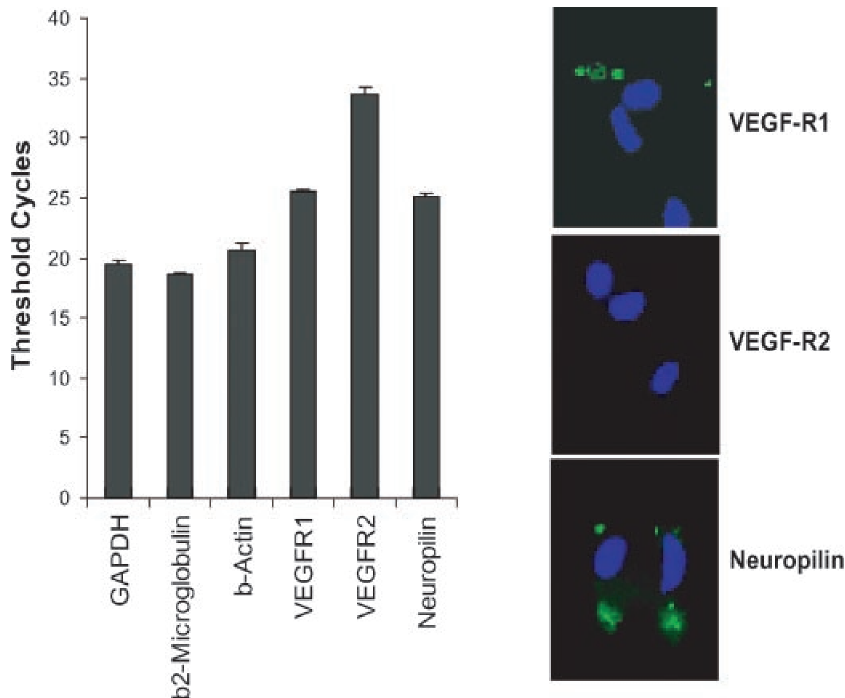


Fig. 2. Analysis of expression of VEGF receptors in cultured MSCs. Left part: Real time qRT-PCR analysis of house-keeping (GAPDH, β2-microgloblulin, and β-actin) and VEGF receptor (VEGF-R1, VEGF-R2, and neuropilin) genes showing their mean threshold cycles (C_T). Right parts: In situ immunostaining of the three VEGF receptors using FITC-conjugated secondary antibodies. A proper isotype control antibody was used in each case as negative control. Images shown were digitally acquired.

and in situ immunostaining assays indicate a low level of VEGF-R1 and neuropilin expression in cultured porcine MSCs.

Growth factors such as BMP2, EGF, TGFβ1, and PDGF have been shown to stimulate the growth of MSCs (Lou et al., 1999; Jiang et al., 2002; Jian et al., 2006; Tamama et al., 2006). Although VEGFs are known potent mitogens for endothelial cells, whether they may exert an effect on the growth of MSCs has not been characterized. The adenoviral expression system was therefore used to determine whether multiple VEGF isoforms upon overexpression might be mitogenic for MSCs. MTT cell proliferation assays were performed 6 days after adenoviral infection of MSCs. Figure 3 shows that expression of LacZ had no effect on MSC proliferation. Expression of each of the VEGFs caused a ~20% increase in MSC proliferation. All three VEGF splice variants exhibited a similar MSC growth stimulatory effect although VEGF120 was expressed at the highest level (see Fig. 1D). Interestingly, adenoviral expression of BMP7 and FGF5 exerted a similar MSC growth-promoting effect (Fig. 3). The specificity of this action of VEGFs is also revealed in the figure showing that proliferation of the P19 mouse embryonal carcinoma stem cells and primary rat fibroblasts were not stimulated by VEGFs. Interestingly, all three VEGF variants exhibited a modest but significant inhibitory effect on the proliferation of the fibroblasts. These results indicate that adenoviral expression of each of the three VEGF-A splice variants similarly promotes the growth of MSCs in culture.

Since VEGFs produced from virally infected MSCs might act through autocrine and/or paracrine mechanisms, we next examined whether conditioned media from virus-infected MSCs might exhibit the MSC mitogenic effect. As expected, VEGF-virus conditioned media also stimulated MSC proliferation (Fig. 4, left part). However, the three VEGF isoforms were found to differ in this assay in that VEGF188 was significantly more potent than VEGF164, which appeared more potent than VEGF120 in promoting MSC growth. To further validate the data, we examined purified recombinant mouse VEGF 120 and 164 proteins, which are commercially available. Figure 4 (right part) shows that both exogenously added VEGF120 and 164 proteins stimulated the growth of MSCs in a dose-dependent manner, and again VEGF164 was significantly more potent than VEGF120. In repeated experiments, we also note that VEGF proteins are highly unstable and tend to lose activity rapidly after reconstitution from dried powder. Since this issue of differential protein stability can complicate data interpretation, the following experiments were performed using the adenoviral expression system, which is suitable for applications involving combined gene and stem cell therapeutics.

Proliferating MSCs are known to produce multiple cytokines, chemokines, growth factors involved in a plethora of cellular and physiological processes (Dexter et al., 1997; Majumdar et al., 1998; Wieczorek et al., 2003; Vacanti et al., 2005). Paracrine actions of these trophic factors are thought to play an important role in MSC-mediated cell therapy (Gnecchi et al., 2005; Tang et al., 2005). Since VEGFs can regulate multiple signaling transduction pathways and often act in concert with other trophic factors (Ferrara, 2004), we next determined whether MSC production of trophic factors might be differentially regulated by the VEGF isoforms. Multiple trophic factor genes encoding chemokine/cytokines and growth factors were analyzed by qRT-PCR to establish a gene expression profile associated with proliferating MSCs. Threshold cycles of these genes were compared to that of the house-keeping gene GAPDH, which showed a mean C_T value of ~19 (Fig. 5). This analysis also demonstrates that MCP1, FGF2 (or bFGF), and FGF7 were expressed at high levels with C_T values of 18–21, which were comparable to that of GAPDH, while GDF8 (myostatin) was the least abundantly expressed gene with a mean C_T of 31. The remaining growth factor/ cytokine genes were moderately expressed with mean C_T values of 23–27.

Having established the gene expression profiles, we went on to analyze the effect of VEGF expression on trophic factor gene expression using the qRT-PCR method. The chemokine/ cytokine gene category shown in Figure 5 includes stromal cell-derived factor-1 (SDF-1 or CXCL12), interleukin-6 (IL-6), and monocyte chemotactic protein-1 (MCP-1), which showed a mean C_T of 27, 24, and 20, respectively, in control MSCs. Expression of these genes was stimulated two to threefold by VEGF120 and/or VEGF188 three days after adenoviral infection (Fig. 6). Interestingly, V164 exerted no effect on the expression of these genes. Similar results were obtained for the four

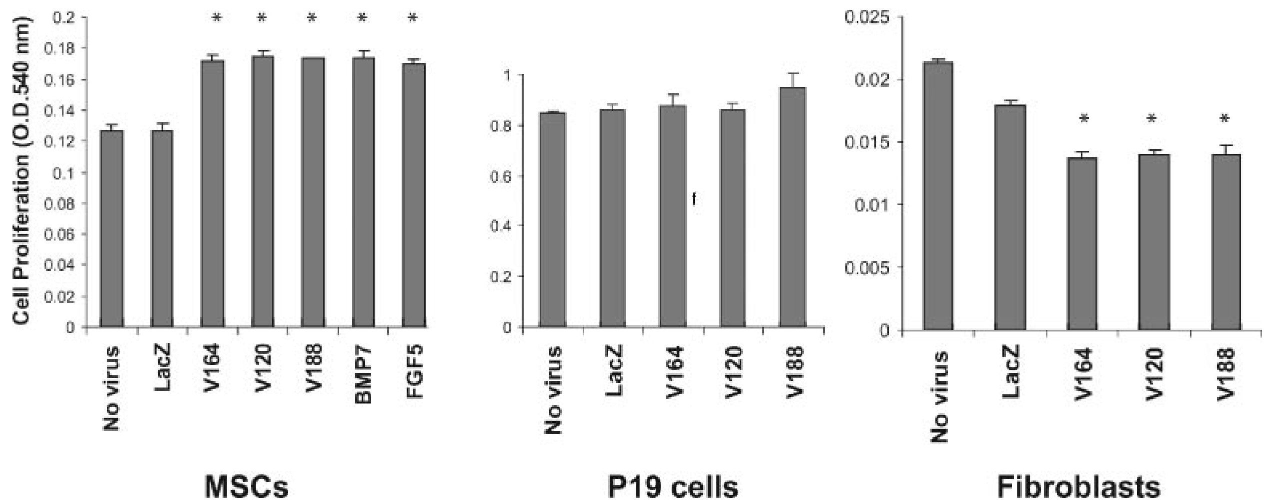


Fig. 3. Stimulation of MSC proliferation by adenoviral expression of VEGFs. MSCs, P19 embryonal carcinoma stem cells, and primary rat fibroblasts were plated on 24-well plates in triplicates. Cells were infected with adenovirus on the next day, and maintained in a growth medium for 5–6 days, after which MTT assays were performed. Results shown were representatives of two independent assays, and were mean $OD_{540\ nm}$ ± standard errors. **$P < 0.05$ **compared to LacZ.

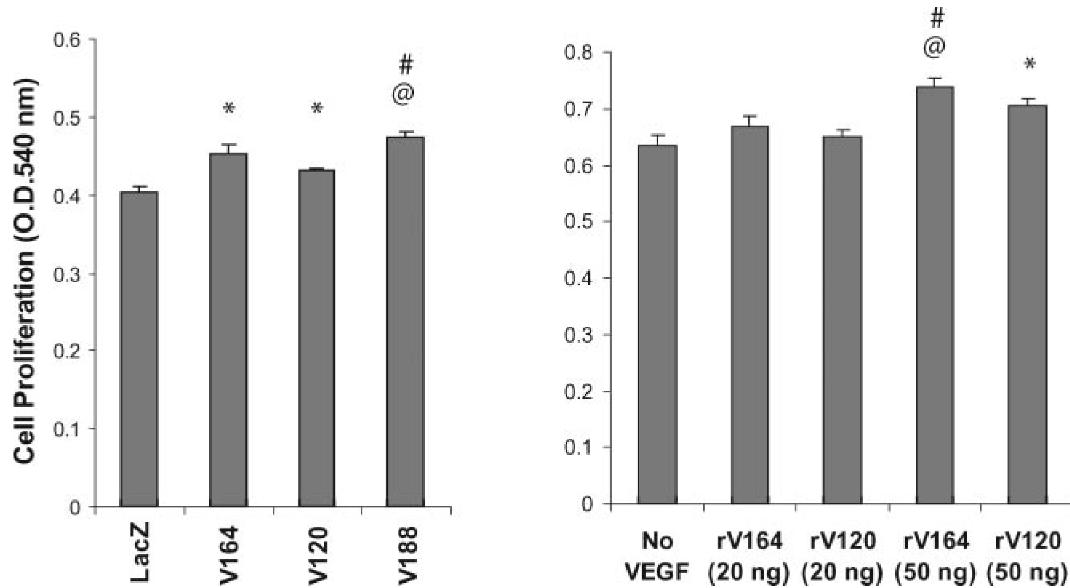


Fig. 4. VEGF-conditioned medium and purified VEGF proteins also promote MSC growth. Left part: MSCs were infected with LacZ and VEGF adenoviruses, and conditioned media collected 3 days after infection were added to MSCs plated in a 24-well plate after mixing 1:1 with fresh growth medium. MTT assays were performed after 4 days. $^{*}P<0.05$ compared to LacZ; $^{\#}P<0.005$ compared to LacZ; $^{@}P<0.01$ compared to VEGF120. Right part: Commercial purified recombinant VEGF proteins (rVEGF120 and rVEGF164) were added to MSCs at 20 and 50 ng/ml. MTT assays were performed after 4 days. $^{*}P<0.01$ compared to LacZ; $^{@}P<0.005$ compared to LacZ; $^{\#}P<0.05$ compared to rVEGF120.

growth factor genes: FGF1 (or aFGF), angiopoietin-1 (Angpt-1), leukemia inhibitory factor (LIF), and brain-derived neurotrophic factor (BDNF), which showed a mean C_T of 26, 25, 26, and 26, respectively, in control MSCs. Expression of these growth factor genes was stimulated two to fivefold by VEGF120 and/or VEGF188 (Fig. 6). VEGF164 again showed slight or no effect. Thus, although multiple VEGF splice variants expressed by adenoviral vectors indiscriminately promote the growth of MSCs, only VEGF120 and VEGF188 significantly stimulate the expression of trophic factor genes in virus-infected MSCs.

Limited studies suggest that MSCs exposed to VEGF can be induced to express endothelial markers such as VEGF receptor-2 (VEGFR-2), platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31), and von Willebrand factor (vWF) (Al-Khaldi et al., 2003; Oswald et al., 2004). qRT-PCR analysis of RNA samples isolated from proliferating MSCs revealed slight expression of PECAM-1 (mean $C_T = 26$) and vWF (mean $C_T = 29$) whereas expression of VEGFR-2 was negligible (mean $C_T = 34$). After VEGF adenoviral infection, expression of PECAM-1 and vWF, but not that of VEGFR2, were stimulated two to threefold by VEGF164 in the

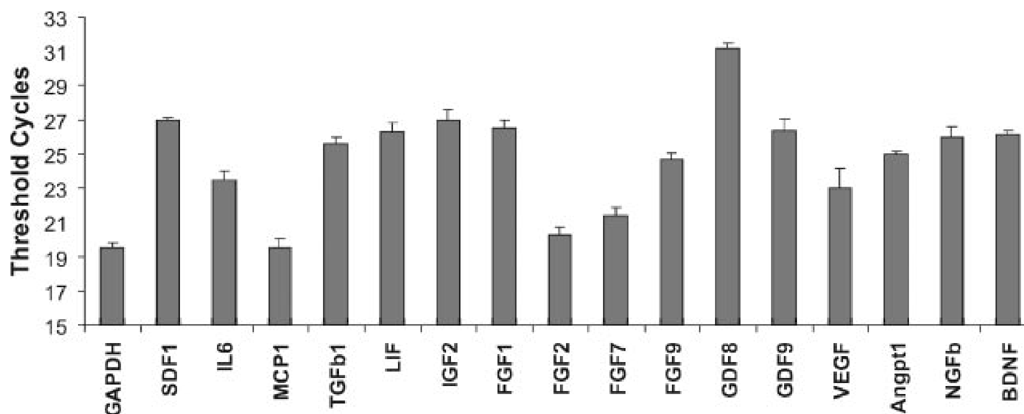


Fig. 5. Real time qRT-PCR analysis of MSC growth factor and cytokine gene expression. Gene abbreviations are glyceraldehyde-3-phosphate dehydrogenase (GAPDH), stromal-derived factor 1 (SDF1), interleukin 6 (IL6), MCP1, and transforming growth factor-β1 (TGFβ1), leukemia inhibitory factor (LIF), insulin-like growth factor 2 (IGF2), fibroblast growth factor 1 (FGF1), FGF2, FGF7, FGF 9, growth and differentiation factor 8 (GDF8), GDF9, vascular endothelial growth factor-A (VEGF), angiopoietin 1 (Angpt1), nerve growth factor β (NGFβ), and brain-derived growth factor (BDNF). Triplicate RNA samples were isolated from proliferating MSCs, and each RNA sample (1 μg) was analyzed in duplicates. Results shown were representatives of two independent experiments, and were mean $C_T \pm$ standard errors.

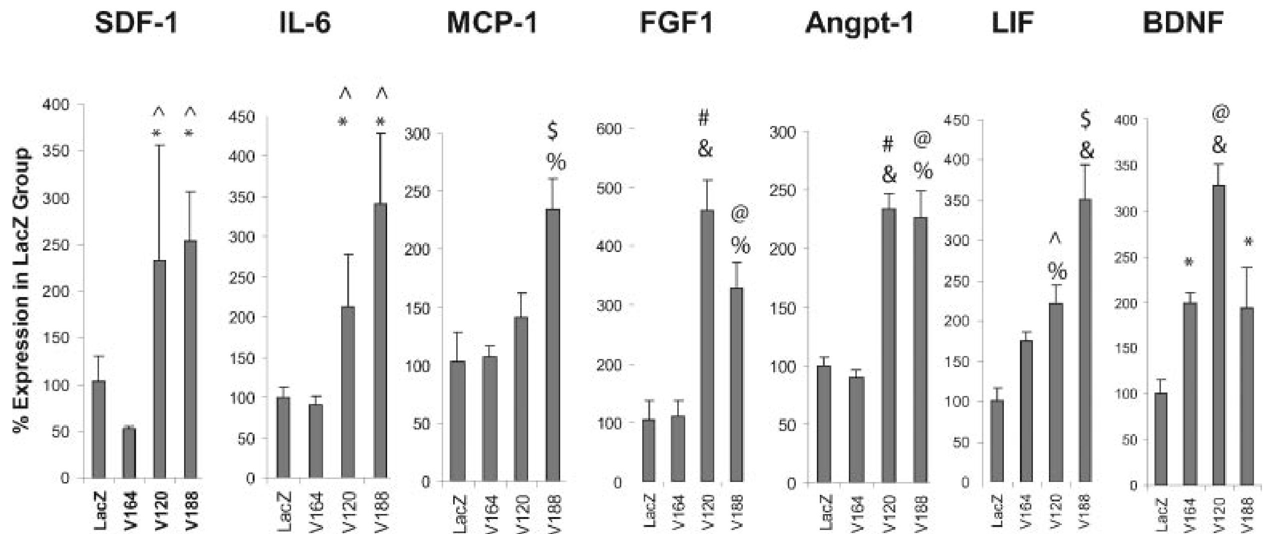


Fig. 6. VEGF120 and 188 are mediators of trophic factor gene expression. MSCs plated on 100-mm dishes were infected with the indicated adenovirus in triplicates. RNA was isolated 3 days after infection, and subjected to qRT-PCR analysis using primer sets for each of the genes listed in Figure 5. Data were calculated by the $2^{\Delta\Delta CT}$ method, and presented as percent gene expression in control cells (LacZ virus infection). GAPDH was found not to be affected by VEGFs, and was used as the reference gene for calculations. Each of the triplicate RNA samples (1 μg) was analyzed in duplicates. Results shown were representatives of two independent experiments, and were mean expression ± standard errors. $^{*}P < 0.05$ compared to LacZ; $^{\wedge}P < 0.05$ compared to V164; $^{\%}P < 0.005$ compared to LacZ; $^{\$}P < 0.005$ compared to VEGF164; $^{\&}P < 0.001$ compared to LacZ; $^{\#}P < 0.001$ compared to VEGF164; $^{@}P < 0.005$ compared to VEGF164.

proliferating MSCs (Fig. 7). The increased expression of PECAM-1 and vWF were accompanied by increased expression of the β-actin gene, again mainly mediated by VEGF164 and to a lesser extent by VEGF188. Since actin mediates cytoskeletal remodeling, and is a major component of endothelial cells (Lamalice et al., 2007), this finding suggests that VEGF164 is a more potent inducer of cellular remodeling and endothelial marker expression in proliferating MSCs. In contrast, VEGF120

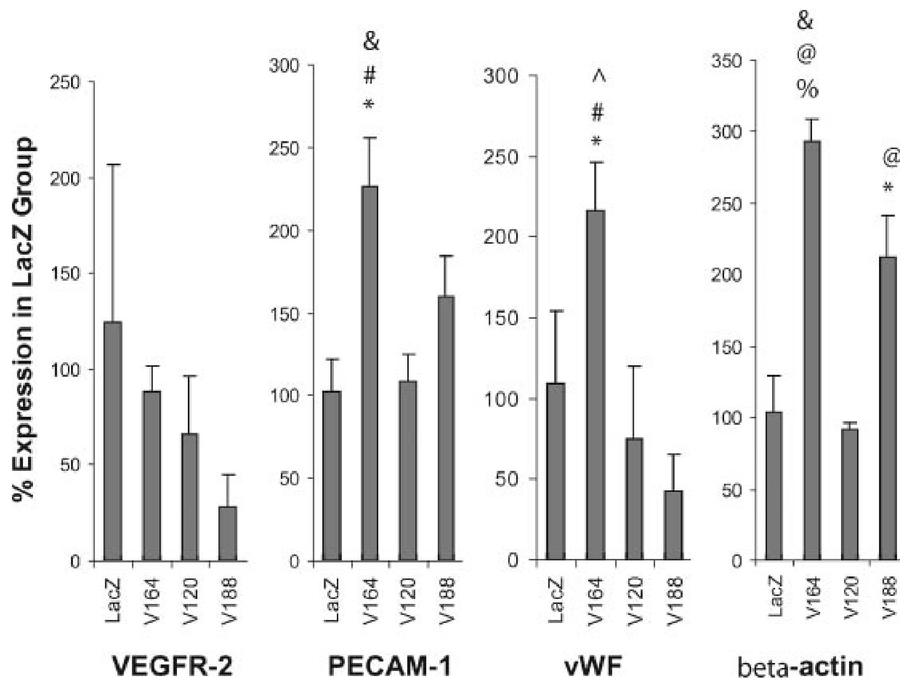


Fig. 7. VEGF164 induces expression of endothelial genes. Real time qRT-PCR analysis of RNA samples isolated from infected MSCs as described in Figure 6. Each of the triplicate RNA samples (1 μg) was analyzed in duplicates. Results shown were representatives of two independent experiments, and were mean expression ± standard errors. $^{*}P < 0.05$ compared to LacZ; $^{\#}P < 0.05$ compared to VEGF120; $^{\&}P < 0.05$ compared to VEGF188; $^{\wedge}P < 0.001$ compared to VEGF188; $^{\%}P < 0.001$ compared to LacZ; $^{@}P < 0.001$ compared to VEGF120.

is ineffective in promoting these cellular events, indicating that VEGF-mediated MSC proliferation and gene expression can be uncoupled.

VEGF is also a known mediator of osteoblast activity and this action of VEGF can be regulated by BMPs (Midy and Plouet, 1994; Street et al., 2002; He and Chen, 2005). Synergy between VEGF and BMP is further revealed by the demonstration that BMP4 enhances expression of VEGF (He and Chen, 2005; Vogt et al., 2006). Since the ability of BMPs in promoting MSC osteochondral differentiation is well documented (Lou et al., 1999; Nochi et al., 2004; Sekiya et al., 2005), we next determined whether there might be a differential cross-talk between the VEGF splice variants and BMP7. Figure 8 (left parts) shows that MSCs maintained for 2 weeks in a medium containing BMP7 underwent osteochondral differentiation as revealed by Alcian blue staining of proteoglycans-secreting MSC nodules (Vacanti et al., 2005). Activity of the osteoblast marker enzyme ALP was measured (Fig. 8, right part), which shows prominent BMP7-mediated induction of ALP activities. The enzyme assay also shows that VEGF188, but not the two shorter VEGF isoforms, further enhanced BMP7 activity along the osteogenic pathway. Thus, VEGF188 and BMP7 act in synergy in promoting MSC osteogenesis.

VEGF is known to promote the proliferation, differentiation, and survival of endothelial cells (Gerber et al., 1998; Gratton et al., 2001). In particular, VEGF acting as a survival factor can rescue endothelial cells maintained in serum-free medium. Studies presented above have demonstrated that multiple VEGF splice variants can differentially affect the proliferation, differentiation, and growth factor expression of MSCs. We examined whether virally expressed VEGFs might act as a survival factor for MSCs in serum starvation condition. Cell death caused by serum starvation was monitored by release of the cytosolic enzyme lactate dehydrogenase (LDH) into the culture medium. Figure 9 (left part) shows that MSCs expressing each of the VEGF isoform exhibited higher levels of cell death (LDH release) than those expressing LacZ. In addition, VEGF188 appeared to trigger the most extensive cell death after 3 days of serum starvation. Since VEGF causes endothelial nitric-oxide synthase (NOS) activation and nitric oxide release from endothelial cells (Fulton et al., 1999; Blanes et al., 2007), we further measured accumulation of nitrite, the major breakdown product of nitric oxide, in the medium. Figure 9 (middle part) shows that VEGF188, which caused the highest level of cell death, also significantly elevated the amount of nitrite after 3 days of serum starvation, demonstrating a correlation between VEGF-mediated cell death and nitrite accumulation. To demonstrate that the observed nitrite-cell death connection is mediated by NOS, we repeated the assay in the presence of the NOS inhibitor L-NMMA. Figure 9 (right part) shows that VEGF 188-mediated cell death was attenuated by 5 μM L-NMMA, indicating the involvement of NOS in the pathway.

Discussion

The importance of VEGFs and their signaling systems in the proliferation, differentiation, survival, and function of endothelial cells have been well documented (Ferrara, 2004; Coultas et al., 2005). However, clinical applications of VEGF gene therapy in cardiovascular medicine have not provided convincing evidence of therapeutic efficacy (Yla-Herttuala et al., 2007). On the other hand, encouraging results have been obtained from recent clinical trials of MSCs for various cardiovascular diseases (Giordano et al., 2007). Further,

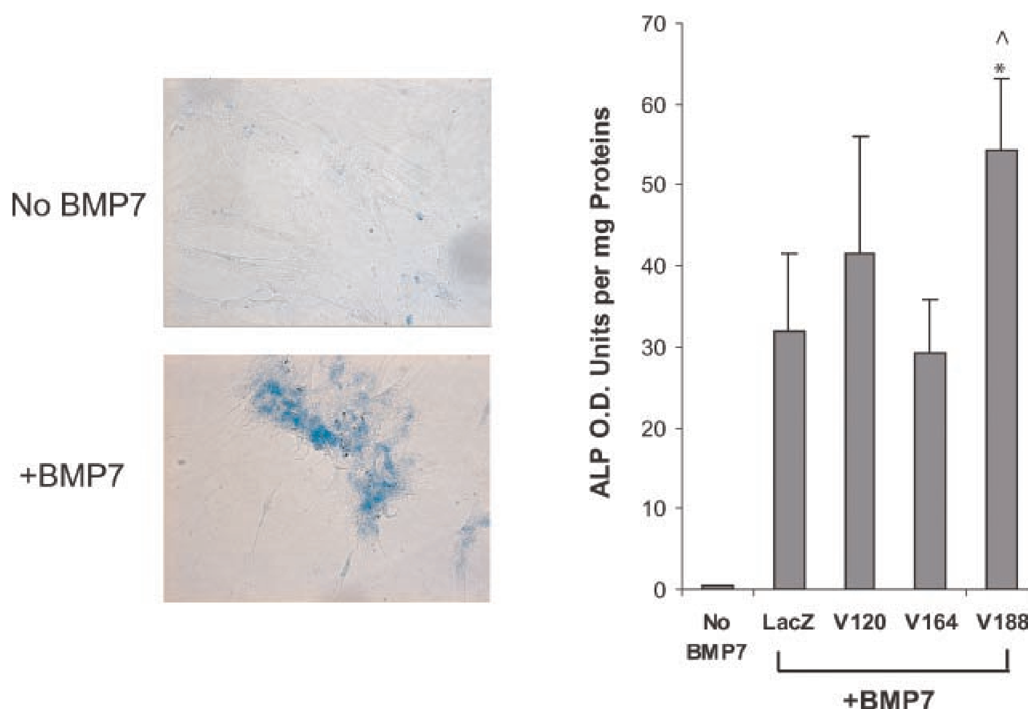


Fig. 8. VEGF188 cooperates with BMP7 in MSC osteochondral differentiation. Left parts: Phase contrast images of control MSCs and those exposed to BMP7 for 2 weeks. BMP7 treatment induced cell aggregation and osteochondrocytic nodule formation as revealed by Alcian blue staining. Right part: Activities of the osteogenic enzyme alkaline phosphatase (ALP) presented as $OD_{412\ nm}$ per mg proteins. Results shown were representatives of two independent experiments, and were mean ± standard errors. Protein lysates were prepared after 2 weeks. *$P < 0.05$ compared to LacZ; ^$P < 0.05$ compared to VEGF164. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

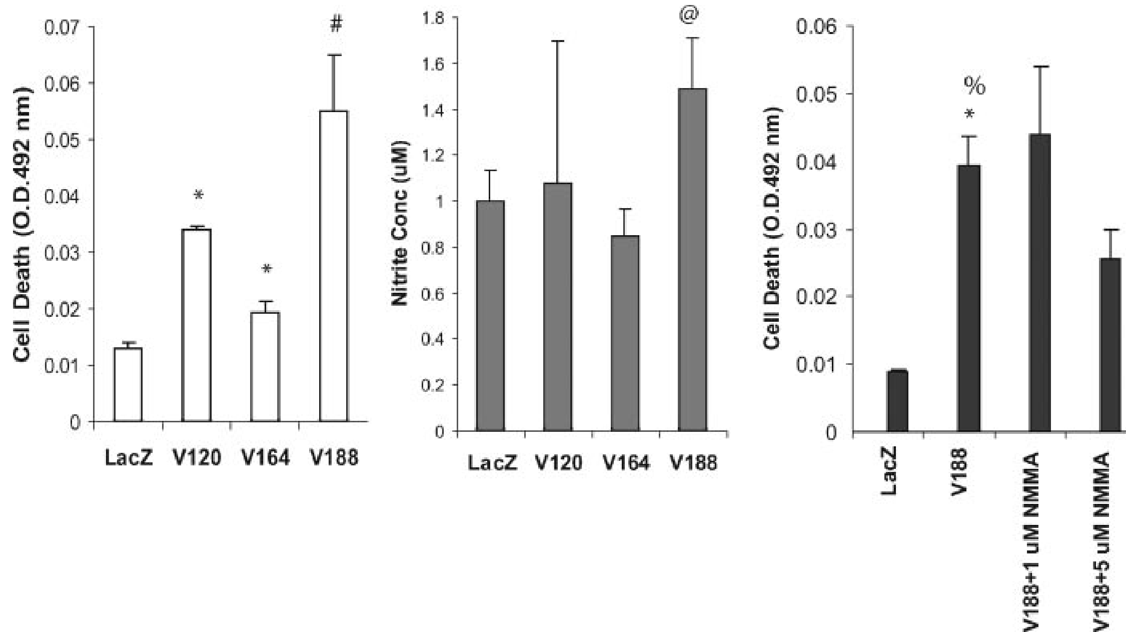


Fig. 9. Virally expressed VEGF enhances serum starvation-mediated cell death. MSCs plated on 24-well plates were infected with the adenoviruses, and maintained in serum-free ADMEM after infection for 3 days. Medium samples were collected and assayed for LDH activities (left and right parts) and nitrite (middle part). Results shown were representatives of two independent experiments, and were mean ± standard errors. *$P < 0.001$ compared to LacZ; #$P < 0.005$ compared to LacZ; @$P < 0.05$ compared to LacZ; %$P < 0.05$ compared to 5 μM L-NMMA. No protective effect was seen with 1 μM L-NMMA.

genetically modified MSCs have been found to offer marked protection of ischemic heart (Gnecchi et al., 2005). In this regard, VEGF165 has been used to boost the function of MSCs and EPCs for stem cell therapy (Iwaguro et al., 2002; Wang et al., 2006). Thus, a combined VEGF/MSC-based cell therapy could offer a synergistic therapeutic effect on tissue repair. However, the use of a combined VEGF/MSC therapeutic approach would require knowledge of specific effects of each of the VEGF splice variants on MSCs. Indeed, we show here that MSCs can be differentially regulated by adenoviral expression of multiple VEGF-A isoforms. It is acknowledged however that the artificially achieved high levels of VEGFs make it difficult to determine whether the observed effects may be due to specific or non-specific actions of VEGFs.

Asahara et al. have shown that ex vivo gene transfer of VEGF augments the proliferative activity EPCs (Iwaguro et al., 2002). Our work here shows that proliferation of MSCs is also augmented by VEGFs. The signaling mechanisms underlying this effect of VEGF on MSC and endothelial cell proliferation may be different since VEGF165 was previously found to be a more potent mitogen for endothelial cells than VEGF121 (Keyt et al., 1996), which lacks the 44-amino acid domain involved in differential VEGF receptor interaction (Soker et al., 1996). Indeed, using VEGF-conditioned medium and exogenously added VEGF proteins, we found that VEGF164 was more effective in promoting MSC growth than VEGF 120. On the other hand, the three VEGF species when expressed by the adenoviral vector system appeared equally potent in promoting MSC proliferation. This difference may be attributed to differential stability of VEGF proteins. In addition, adenoviral expression of VEGF in MSCs is expected to achieve higher levels of VEGF in a more sustained fashion. Since our work shows that MSC proliferation is also enhanced by FGF5 and BMP7, MSC proliferation may represent a point of convergence for multiple growth factor signaling. Notably, we found that VEGF164 signaling in proliferating MSCs is accompanied by moderate expression of endothelial genes such as PECAM-1 and vWF, suggesting promotion of the endothelial lineage by VEGF164. This dual effect of VEGF164 on MSCs resembles that mediated by BMP, which has been shown to promote both MSC proliferation and chondrogenic differentiation (Lou et al., 1999; Nochi et al., 2004). Since VEGF165 preferentially interacts with the VEGFR co-receptor NRP1, bridging and stabilizing the NRP1-VEGFR2 complex (Pan et al., 2007), the NRP1 signaling pathway may play a dominant role in promoting the endothelial phenotype of MSCs. Consistent with this notion, MSCs were shown to express low levels of NRP1 by us and others (Ball et al., 2007).

In contrast to the pro-endothelial function of VEGF164, we found that VEGF188 is more effective in cooperating with BMP in promoting osteogenic differentiation of MSCs. VEGF has previously been shown to stimulate osteoblast differentiation (Midy and Plouet, 1994; Street et al., 2002). Osteochondrogenesis is associated with accumulation of proteoglycans, which are highly negatively charged, and are known to preferentially retain the longer VEGF isoforms (Houck et al., 1992; Neufeld et al., 1999). Retention of VEGF188 by proteoglycans may thus underlie the observed cooperation between BMP and VEGF188. Cooperation between these two growth factors has also been demonstrated in several cell systems (Dai et al., 2004; Vogt et al., 2006). This finding indicates that VEGF188/189-based stem cell therapy may be better suited for treating disease such as osteogenesis. On the other hand, since VEGF164/165 appears more effective in promoting the endothelial phenotype (and therefore blood vessel growth), this isoform may be best used for treating disease conditions caused by tissue ischemia. We found that the shortest VEGF isoform is expressed most efficiently, and capable of amplifying the endogenous growth factor network. This feature of VEGF120/121 in conjunction with its high

diffusibility may render it ideal for systemic growth factor therapeutics.

MSCs are capable of producing a wide range of growth factors and cytokines (Majumdar et al., 2000; Darland et al., 2003; Imabayashi et al., 2003; Wieczorek et al., 2003; Kinnaird et al., 2004), and as such their behavior and phenotype can be expected to be influenced by these factors. For instance, growth factors such as BMP2, EGF, TGFβ1, and PDGF have been shown to stimulate the growth of MSCs (Lou et al., 1999; Jiang et al., 2002; Jian et al., 2006; Tamama et al., 2006). Insulin-like growth factor-1 (IGF-1) and bFGF can control the migratory capacity of MSCs (Schmidt et al., 2006; Li et al., 2007). Heregulin can protect MSCs from serum deprivation and hypoxia-induced cell death (Gui et al., 2007). Prolonged treatment of MSCs with hepatocyte growth factor (HGF) can induce the expression of cardiac-specific markers (Forte et al., 2006). Cross-talks between VEGFs and other trophic factors may result in growth factor synergism and further amplify their expression and/or effect (Maity et al., 2000; Neuhaus et al., 2003; Kryczek et al., 2005). Along this line, we found that VEGF120 and/or VEGF188 are more effective in promoting expression of trophic factor genes including SDF-1, IL-6, MCP-1, FGF1, Angpt-1, LIF, and BDNF, and this effect of VEGFs may be mediated in part by VEGF-R1 but not VEGF-R2. That SDF-1 expression is induced by VEGF is consistent with the demonstration that VEGF stimulates chemotactic migration of primary human osteoblasts (Mayr-Wohlfart et al., 2002). This finding raises the possibility that VEGF-modified MSCs may home to the site of tissue injury more effectively. Angpt-1 contributes to blood vessel maturation and stability, and increased expression of Angpt-1 by VEGF suggests a potentially useful VEGF/MSC strategy in combating tissue ischemia. Induction of BDNF by VEGF is consistent with a cross-talk between VEGF signaling and neural stem cells as shown recently (Li et al., 2006).

That expression of SDF-1, IL-6, and MCP-1 were augmented by VEGF120/188 is worth noting since the three cytokines are mediators of inflammatory reactions. This demonstration is consistent with the notion that VEGF-A can promote the progression of atherosclerosis (Celletti et al., 2001), and suggests that VEGF165 should be used for gene and stem cell therapy where tissue inflammation is the major underlying cause of pathologic manifestation. Indeed, there is evidence that VEGF121 may be less efficacious for treating myocardial ischemia, which is associated with tissue inflammation, despite the fact that both the 121 and 165 isoforms have been used indiscriminately for gene therapy protocols (Carmeliet et al., 1999).

VEGF is not only a mitogen but also a potent survival factor for endothelial cells. In particular, VEGF can rescue serum-starved endothelial cells (Gerber et al., 1998). We found that although VEGF is mitogenic for MSCs, it failed to rescue serum-starved MSCs. Further, cell death triggered by serum starvation was exacerbated by VEGFs with VEGF188 causing the most extensive cell death and accumulation of nitrite, a breakdown product of nitric oxide, 3 days after serum starvation. Although VEGF is known to possess a cytoprotective effect (Gratton et al., 2001; Siner et al., 2007), this action of VEGF is most likely mediated by complex extracellular cues, and may be cell type dependent. For instance, VEGF can act in concert with TGFβ1 to induce endothelial cell death whereas VEGF activates p38MAPK and promotes endothelial cell survival in the absence of TGFβ1 (Ferrari et al., 2006). In serum-starved MSC, VEGF signaling can similarly be modified, diverting a pro-survival signal toward a pro-death pathway. Cell death-inducing property of VEGF is also evident from the study of Wada et al., showing that VEGF causes the cell death of primitive neural stem cells but promotes the survival of definitive neural stem cells (Wada et al., 2006). Since we observed a correlation between the extent of cell death and nitrite accumulation, production of peroxynitrite, which is a potent inducer of cell death (Espey et al., 2000), might be involved in mediating the cell killing effect of VEGF under the serum starvation condition. Since MSCs have been found to express eNOS and iNOS (Damoulis et al., 2007; Sato et al., 2007), it would be of interest to determine whether the expression and activity of these two NOS isoforms may be differentially affected in VEGF-expressing MSCs.

In summary, we show that multiple VEGF splice variants exert common and differential effects on MSCs. All three isoforms are equally potent in promoting MSC proliferation when expressed by the adenoviral vector system. VEGF120 and 188 are more effective in promoting expression of trophic factor genes. VEGF164 on the other hand is more effective in promoting expression of endothelial genes and cellular remodeling. The longer isoform VEGF188 preferentially facilitates BMP7-mediated MSC osteogenesis. Under serum starvation condition, VEGF188 enhances serum withdrawal-mediated cell death. These differential effects of VEGFs on MSCs are most likely caused by their differential receptor interactions. The VEGF ligand-receptor interaction appears highly dynamic. In addition to its interaction with VEGF-R1 and neuropilin, VEGF165 has recently been shown to interact with PDGF receptors and induce receptor tyrosine phosphorylation (Ball et al., 2007). Although it remains elusive how VEGFs achieve the observed differential effects on MSC, this work illustrates that the best possible match of an optimal VEGF isoform to a given disease setting should be considered for VEGF-based stem cell therapy.

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