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miR-21 modulates tumor outgrowth induced by human adipose tissue-derived mesenchymal stem cells *in vivo*

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ABSTRACT

Mesenchymal stem cells (MSCs) have generated a great deal of interest in clinical situations, due principally to their potential use in regenerative medicine and tissue engineering applications. However, the therapeutic application of MSCs remains limited, unless the favorable effects of MSCs on tumor growth *in vivo*, and the long-term safety of the clinical applications of MSCs, can be more thoroughly understood. In this study, we determined whether microRNAs can modulate MSC-induced tumor outgrowth in BALB/c nude mice. Overexpression of miR-21 in human adipose-derived stem cells (hADSCs) inhibited hADSC-induced tumor growth, and inhibition of miR-21 increased it. Downregulation of transforming growth factor beta receptor II (TGFBR2), but not of signal transducer and activator of transcription 3, in hADSCs showed effects similar to those of miR-21 overexpression. Downregulation of TGFBR2 and overexpression of miR21 decreased tumor vascularity. Inhibition of miR-21 and the addition of TGF-β increased the levels of vascular endothelial growth factor and interleukin-6 in hADSCs. Transplantation of miR-21 inhibitor-transfected hADSCs increased blood flow recovery in a hind limb ischemia model of nude mice, compared with transplantation of control oligo-transfected cells. These findings indicate that MSCs might favor tumor growth *in vivo*. Thus, it is necessary to study the long-term safety of this technique before MSCs can be used as therapeutic tools in regenerative medicine and tissue engineering.

1. Introduction

Mesenchymal stem cells (MSCs) not only support hematopoiesis, but have also been shown to differentiate along a variety of mesodermal lineages, thereby generating osteoblasts, chondrocytes, and adipocytes [1–3]. Recent studies have also reported that MSCs are more plastic than was initially thought, and are capable of developing into diverse cell lineages, including myoblasts, cardiomyocytes, and neural cells [4–6]. Therefore, MSCs have been considered to represent ideal sources of cell therapy in a variety of disease processes, including cardiovascular disease [7], as well as in the treatment of human malignancies [8].

On the basis of previous studies implicating stem cells and stromal support cells in the neoplastic process [9–16], transplanted MSCs can be expected to accelerate tumor growth *in vivo*. Fierro et al. reported some changes in the proliferative capacity of MCF-7 cells following co-culture with MSCs [17]. Djouad et al. showed that MSCs cause side effects associated with systemic immunosuppression, favoring melanoma growth *in vivo* [18], and Tsai et al. reported that mesenchymal stem cells promote the formation of colorectal tumors in mice [19]. Our previous study showed that human adipose-derived stem cells (hADSCs) increased tumor growth in a subcutaneous transplantation model of nude mice [20].

Mechanisms of MSC-induced tumor growth include MSC-derived exosome-induced vascular endothelial growth factor (VEGF) expression in human gastric and colon cancer cells [21], the formation of a microenvironment favorable for tumor growth [22], and the secretion of paracrine factors by MSCs [23]. Therefore, there is a possibility that the modulation of MSC functions may

Abbreviations: MSC, mesenchymal stem cell; hADSCs, human adipose-derived stem cells; TGFBR2, transforming growth factor beta receptor II; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay.

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affect MSC-induced tumor outgrowth. Understanding this process is important to ensure the safety of MSC transplantation in clinical applications.

MicroRNAs (miRNAs) have been implicated in many processes including cell proliferation and apoptosis [24,25], fat metabolism [24], neuronal patterning [26], and tumorigenesis [27]. Recent evidence indicates that miRNAs influence stem cell functions, including differentiation, by negatively regulating gene expression at the post-transcriptional level [28,29]. Our recent studies showed that miRNAs control the proliferation and differentiation of hADSCs [30–32]. In this study, we determined whether the control of miRNA activity in hADSCs can modulate hADSC-induced tumor growth *in vivo*, and elucidated the mechanism of action of this process.

2. Materials and methods

2.1. Culture of hADSCs and H460 cells

All protocols involving human subjects were approved by the Institutional Review Board of Pusan National University. Superfluous materials were collected from individuals undergoing elective abdominoplasty after informed consent was given by each individual. The hADSCs were isolated according to methods described in previous studies [33]. We used hADSCs of 3–5 passages for each experiment. H460 cells, that were derived from a large cell carcinoma of the human lung, were commercially obtained (ATCC) and were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum.

2.2. Genetic modification of hADSCs

Overexpression of miR-21 and miR-486 was induced by lentivirus transduction [31,32]. Lentivirus preparation and transduction procedures were performed as previously described [31]. We used virus titers ranging from 5×10^5 to 1×10^7 transducing units (TU)/ml. miRNA inhibitors (anti-miRs) and a scrambled RNA oligomer were purchased from Ambion (Austin, TX). These were transfected into hADSCs at a final concentration of 50 nM using the DharmaFECT Transfection Reagent (Dharmacon, Lafayette, CO), as per the manufacturer's instructions. Small interfering RNA (siRNA) duplex oligos (on-TARGET plus SMART pool, Dharmacon) targeting STAT3 or TGFBR2 mRNA, or a non-targeting duplex oligo (negative control) were transfected into cells using the DharmaFECT Transfection Reagent.

2.3. RT-PCR

Total cellular RNA was isolated from hADSCs and reverse transcribed using conventional protocols. The primer sequences used in the experiment were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5′-TCC ATG ACA ACT TTG GTA TCG-3′, 5′-TGT AGC CAA ATT CGT TGT CA-3′; signal transducer and activator of transcription 3 (STAT3): 5′-TGC CTT ATC AGG GCT GGG ATA C-3′, 5′-GGG ACC TTT AGA CAC GCA AGG A-3′; transforming growth factor beta receptor II (TGFBR2): 5′-ACG TGT TGA GAG ATC GAG G-3′, 5′-CCC AGC ACT CAG TCA ACG TC-3′. All primer sequences were generated from established GenBank sequences.

2.4. Xenotransplantation of tumor cells

BALB/c nude mice at 7 weeks of age were purchased from the Laboratory Animal Center of Seoul, Korea. The animals were subcutaneously injected with H460 cells (5×10^5) alone, or mixed with hADSCs (2×10^5) into the backs of the mice, or mixed with hADSCs (2×10^5) into the brain. The time course of tumor growth after subcutaneous injection was estimated by the surface area of skin elevation at the injection site. Tumor samples were collected 10 days after the subcutaneous injection of H460 cells.

2.5. Histopathology and immunohistochemistry

Ten days after transplantation, the animals were sacrificed via CO_2 inhalation, and tissue sections from paraffin-embedded tumors were stained with hematoxylin and eosin. For immunohistochemistry, the sections were permeabilized with 0.3% Triton X-100 for 10 min, then blocked for 1 h with 8% bovine serum albumin (BSA) at room temperature. Primary CD31 antibodies (Abcam, Cambridge, MA) were diluted to 1:500 in phosphate-buffered saline (PBS) with 2% BSA, and were then treated overnight at 4 °C. The primary antibody-conjugated anti-rabbit secondary antibodies (Abcam) were diluted to 1:100 in PBS with 2% BSA and incubated for 1 h at room temperature. Imaging was conducted with a confocal microscope (Leica, Solms, Germany).

2.6. Quantitation of cytokines

At 90% confluence, the culture media of hADSCs were switched to serum-free α-MEM and incubated for 24 h. The expression levels of cytokines in conditioned media was assayed using the Human Angiogenesis enzyme-linked immunosorbent assay (ELISA) Strip I for Profiling 8 Cytokines (Signosis, Sunnyvale, CA, USA) according to the manufacturer's instructions. The absorbance of each well was measured with a microplate reader (VICTOR™ X3 Multilabel Plate Reader, PerkinElmer, Inc., USA) at 450 nm within 30 min. Protein standards provided with the Human Angiogenesis ELISA strip were used for quantification.

2.7. Determination of proangiogenic action in hindlimb ischemia

The animal protocol used in this study was reviewed and approved by the Pusan National University Institutional Animal Care and Use Committee (PNU-IACUC) for ethical procedures and scientific care. The neovascularization capacity of the cells was investigated in a murine model of hind limb ischemia that was generated by the ligation of the proximal femoral artery in 8-week-old BALB/c nude mice (Sam: TacN (SD) fbr) as previously described [34]. Briefly, animals were anesthetized with pentobarbital sodium (0.5 mg/g), and to produce hind limb ischemia, the proximal portion of the femoral artery, including the superficial and deep branches, was ligated twice using a 7-0 silk suture. After 24 h, 1×10^6 hADSCs (180 μl of cell suspension in PBS) were injected intramuscularly at 3 different sites into the ischemic legs. After 2 weeks, a Laser Tissue Blood Flowmeter (Omegawave, Inc., Japan) was used to measure cutaneous blood flow.

2.8. Statistical analysis

Data were analyzed using the paired t test, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. MicroRNAs affect hADSC-induced tumor growth in vivo

To examine if microRNAs affect hADSC-induced tumor growth, we overexpressed microRNAs that are either involved in hADSC proliferation [35] or highly expressed in hADSCs [32]. To overexpress them, we used miR-21, miR-29a, miR-196, and miR-486 lentivirus. Among the miRNAs tested in this experiment, overexpression of miR-21 in hADSCs inhibited hADSC-induced tumor growth. To

confirm these findings, we also examined the effects of miR-21 inhibitor transfection on hADSC-induced tumor growth. Transfection of miR-21 inhibitor increased tumor growth compared with control oligo-transfected cells, in contrast to the results of cotransplantation of miR-21-overexpressing hADSCs (Fig. 1A and B).

To determine how miR-21 affects hADSC-induced tumor growth, we examined the effects of modulation of miR-21 targets on hADSC-induced tumor growth. Our previous studies demonstrated that miR-21 targets STAT3 [35] and TGFBR2 [31]. Therefore, to determine which target plays a role in the inhibition of tumor growth enhancement by hADSCs, we inhibited STAT3 or TGFBR2 expression with their specific siRNAs. RT-PCR analysis demonstrated that siRNA-transfection inhibited STAT3 or TGFBR2 expression effectively (Fig. 2A). Although siSTAT3-hADSCs caused an increase in tumor growth similar to that seen in control oligo-transfected cells, cotransplantation with siTGFBR2-transfected cells significantly decreased the hADSC-induced tumor-growth effect, similar to observations in hADSCs overexpressing miR-21 (Fig. 2B).

3.2. miR-21 affects the hADSC-induced increase in tumor vascularity

To examine whether miR-21 overexpression affects the hADSC-induced increase in tumor vascularity, we performed immunohistological examination of tumor tissues using the CD31 monoclonal antibody. Immunohistochemical analysis showed that hADSC cotransplantation increased the vascular density of tumor tissues, and that inhibition of TGFBR2 expression in hADSCs suppressed the hADSC-induced increase in tumor vascularity, similar to miR-21 overexpression (Fig. 3A and B).

To determine whether miR-21 affects the production of angiogenic factors in hADSCs, we measured the levels of secretory factors. Inhibition of miR-21 by transfection of the miR-21 inhibitor increased the expression levels of VEGF and interleukin (IL)-6 in hADSCs, as demonstrated by real-time PCR analysis (Fig. 4A and B). We further investigated the effect of TGF-β signaling on the production of angiogenic cytokines in hADSCs. The addition of TGF-β (1 ng/ml) increased the expression of IL-6 and VEGF mRNA in hADSCs (Fig. 4C).

To determine the angiogenic action of miR-21 inhibition *in vivo*, we examined the effect of miR-21 inhibition on hADSC-induced blood flow recovery in a hind limb ischemia model in nude mice. Transplantation of miR-21 inhibitor-transfected hADSCs resulted in significantly greater blood flow recovery in hind limb ischemia induced by femoral artery ligation in nude mice, than did transplantation of control oligo-transfected hADSCs (Fig. 4D).

4. Discussion

miR-21 has been identified as the miRNA most commonly and strongly up-regulated in human brain tumor glioblastoma [36]. Since then, miR-21 has been studied in the contexts of development, oncology, stem cell biology, and aging [37,38]. In this study, we showed that miR-21 overexpression decreases the enhancement of tumor growth caused by hADSC cotransplantation. Our previous studies demonstrated that miR-21 affects adipogenic differentiation through the inhibition of TGF-β signaling [31] and inhibits proliferation through the inhibition of STAT3 [35]. Papagiannakopoulos et al. [39] also showed that miR-21 targets genes related to the TGF-β pathway in glioblastoma cells [39]. The data in this study indicate that inhibition of TGFBR2 by siRNA transfection mimics the effect of miR-21 overexpression on tumor formation, indicating that the modulation of TGF-β signaling by miR-21 is partly responsible for this effect.

Multiple mechanisms may be responsible for the MSC-induced increase in tumorigenesis, as described in the introduction. Among them, one of the most important is the increase in tumor angiogenesis. In this study, we showed that miR-21-overexpression and TGFBR2 siRNA transfection in MSCs suppress hADSC-induced

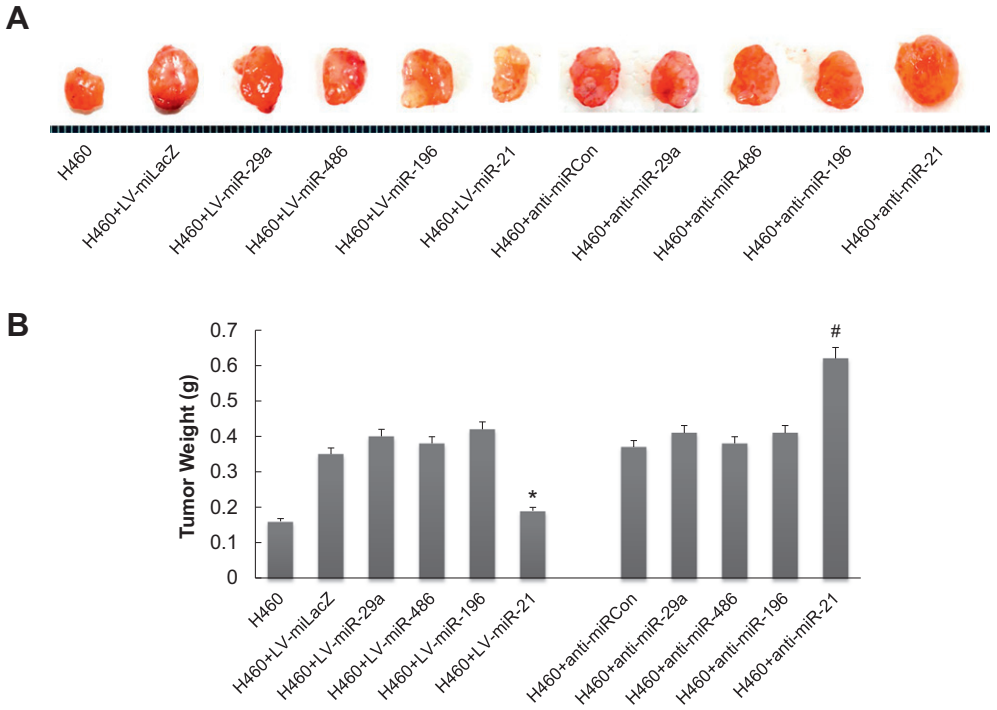

Fig. 1. Effect of microRNAs on hADSC-induced tumor growth *in vivo*. (A) Effect of hADSC co-transplantation on tumor formation due to subcutaneous transplantation of H460 cells. H460 cells (5×10^5) were subcutaneously injected with or without hADSCs (2×10^5) into the posterior left and right flanks, respectively. Representative photograph of a H460 cell-derived tumor in a nude mouse 10 days after transplantation. (B) Quantification of H460 cell-derived tumor weight. The data are expressed as the means ± SEM ($n = 4$). $^*p < 0.05$, compared to data from cotransplantation of LV-miLacZ-transduced hADSCs. $^\#p < 0.05$, compared to data from cotransplantation of control oligo-transfected hADSCs.

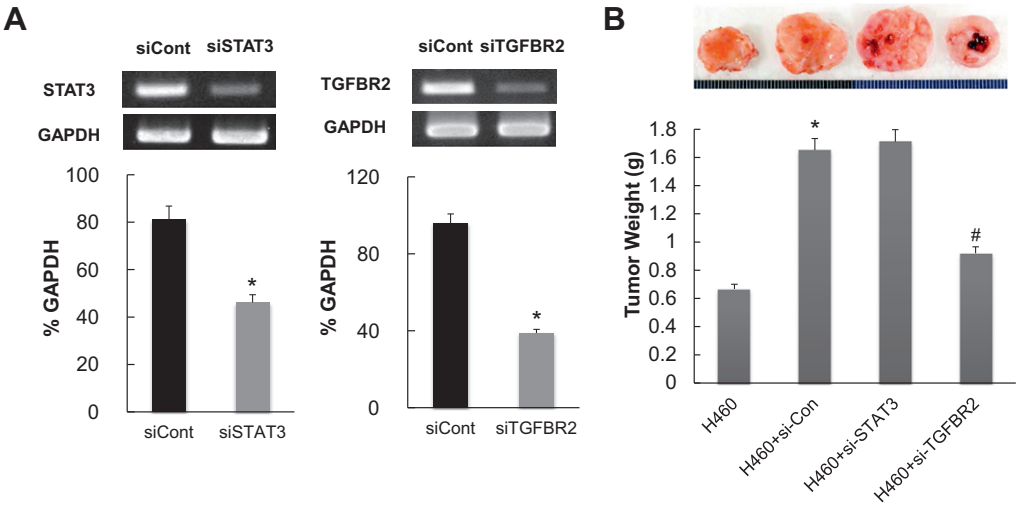

Fig. 2. Effect of TGFBR2 or STAT3 siRNA oligonucleotides on hADSC-induced tumor growth. (A) RT-PCR analysis of TGFBR2 or STAT3 expression in siRNA oligonucleotide-transfected hADSCs. Total cellular RNA was isolated from siRNA oligonucleotide-transfected hADSCs after 72 h. The relative quantities of amplified products were analyzed by an image analyzer. Data represent the mean ± SEM of the relative ratio to the GAPDH signal in the corresponding samples ($n = 3$). $^{*}p < 0.05$, compared to data from control siRNA-transfected hADSC. (B) Effect of TGFBR2 or STAT3 siRNA on hADSC-induced tumor growth. Data represent mean ± SEM ($n = 4$). $^{*}p < 0.05$, compared to data from transplantation of H460 cells alone. $^{\#}p < 0.05$, compared to data from cotransplantation of control siRNA-transfected hADSCs.

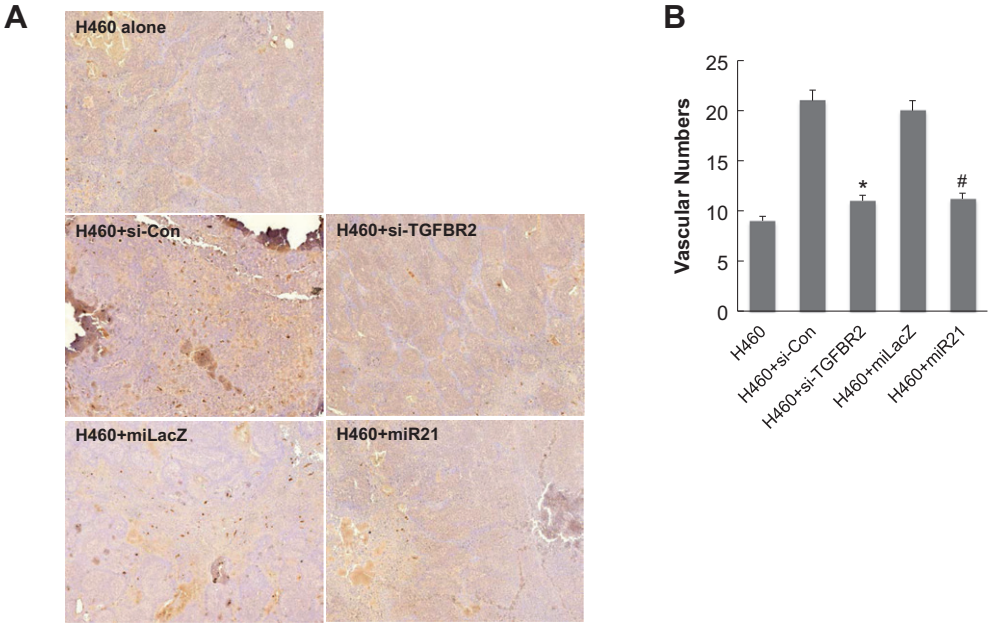

Fig. 3. Effect of hADSC cotransplantation on vascular density within tumor masses. (A) CD31 immunohistochemistry on the paraffin sections of tumor tissues. Tissue sections were incubated with CD31 antibody-labeled secondary antibody. (B) Quantification of microvessel numbers in tumor masses. The numbers of immunostained microvessels in the tissue sections were counted at a magnification of 200×. The data are expressed as the number per microscopic field (mean ± SEM, $n = 4$). $^{*}p < 0.05$, compared to data from compared to data from cotransplantation of control siRNA-transfected hADSCs. $^{\#}p < 0.05$, compared to data from cotransplantation of LV-miLacZ-transduced hADSCs.

tumor angiogenesis. Our data also reveal that transfection of miR-21 inhibitors or TGF-β treatment in hADSCs increase protein and mRNA levels of VEGF and IL-6 expression and increased hADSC-induced proangiogenic action in a hind limb ischemia model. These findings indicate that the modulation of TGF-β signaling by miR-21 affects MSC-induced angiogenic actions and tumor promoting effects. IL-6 and VEGF are well-known angiogenic cytokines in tumor tissues [40], and are secreted in MSCs [41–44]. Recent studies [45,46] showed that TGF-β increases VEGF secretion in rat and mouse MSCs, supporting our conclusion.

Several recent studies showed that inflammatory cytokines or cancer-secreted factors can induce the modification of MSCs and potentiate MSC-induced tumor growth [47], and cancer cell-derived lysophosphatidic acid-induced angiogenesis [48,49]. Therefore, we cannot exclude the possibility that the control of tumor outgrowth by the modulation of MSC functions may be partly due to changes in the tumor or microenvironment-derived phenotypic changes in MSCs.

miR-21 has been reported to increase tumorigeneity of lung cancer [50]. Although we genetically modified hADSCs with miR-21 lentivirus or miR-21 inhibitor, miR-21 or microRNA inhibitor oligonucleotides can be transferred to cancer cells through hADSC-derived exosomes, and then may affect the proliferation of cotransplanted H460 cells. Zhu et al [21] reported that exosome derived from human MSCs promote tumor growth *in vivo*. However, to show the tumor-promoting effect by the cotransplan-

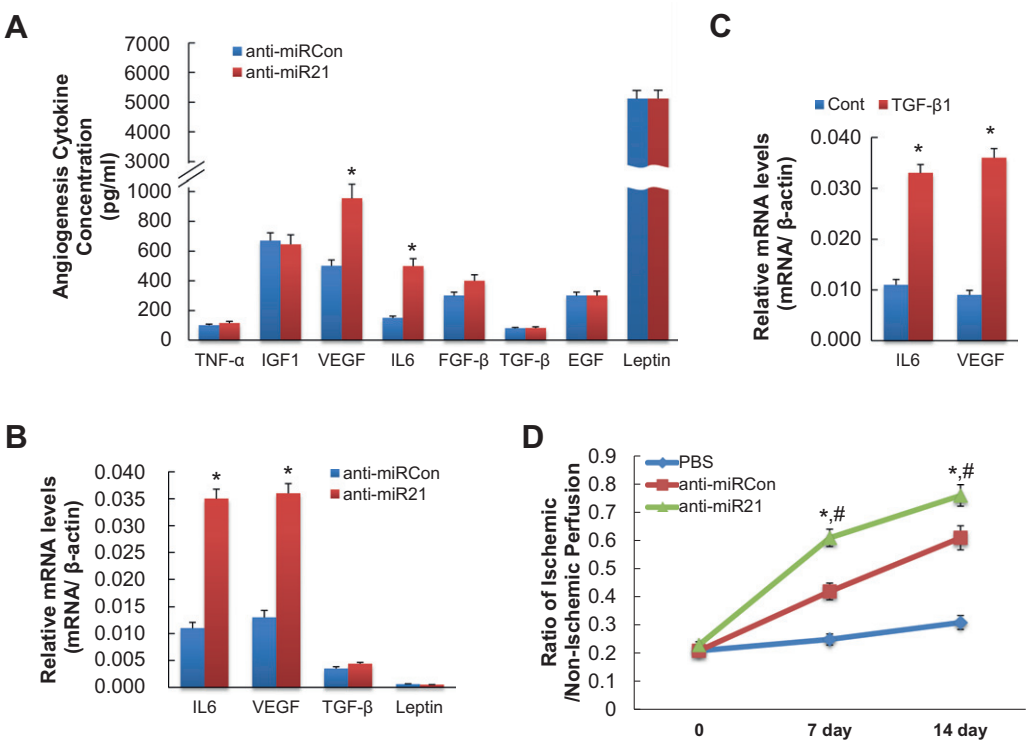

Fig. 4. Determination of angiogenic cytokines and hindlimb ischemia. (A) Profiling of angiogenic cytokines. Conditioned media was obtained from anti-miRCon-transfected hADSCs or anti-miR21-transfected hADSCs and the cytokine concentrations were determined. $^{*}p < 0.05$, compared to data from anti-miRCon-transfected hADSCs. (B) Real-time PCR analysis of angiogenic cytokine expression. Values were normalized using β-actin as an internal control. Data represent mean ± SEM ($n = 4$). $^{*}p < 0.05$, compared to data from anti-miRCon-transfected hADSCs. (C) Effect of TGF-β treatment on VEGF and IL-6 levels. Values were normalized using β-actin as an internal control. Data represent mean ± SEM ($n = 4$). $^{*}p < 0.05$, compared to data from anti-miRCon hADSCs. (D) *In vivo* proangiogenic action of hADSC in hindlimb ischemia model. Hindlimb ischemia was induced by femoral artery ligation in nude mice, and animals were treated with either PBS (Cont.) or hADSCs 1 day after ligation. Quantitative analysis with laser Doppler was performed, and data are presented as ratio of perfusion in ischemic leg compared with nonischemic leg over time on day 14. Data represent mean ± SEM ($n = 5$). $^{*}p < 0.05$, compared to data from the PBS injected group. $^{\#}p < 0.05$, compared to data from anti-miRCon-transfected hADSC group.

tation of MSC-derived exosomes, they used a large amount of exosome (40 μg). In our experiment, we cotransplanted 2×10^5 hADSCs with 5×10^5 H460 cells. Therefore, the possibility that the amount of exosomes derived from transplanted hADSCs at our experimental condition would be enough for modifying the proliferation of cotransplanted cancer cells is quite low.

The control of hADSC-induced tumor outgrowth by modulating microRNA function, demonstrated in this study, provides a useful tool for controlling adverse effects of MSC transplantation in clinical applications as well as for enhancing their therapeutic efficacy.

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