

# HIF-2 $\alpha$ -ILK Is Involved in Mesenchymal Stromal Cell Angiogenesis in Multiple Myeloma Under Hypoxic Conditions

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## Abstract

Mesenchymal stromal cells are proven to be likely induce the angiogenic response in multiple myeloma and thus represent an enticing target for antiangiogenesis therapies for multiple myeloma. Substantial evidence indicates that angiogenesis in multiple myeloma is complex and involves direct production of angiogenic cytokines by abnormal plasma cells and these B-cell neoplasia generated pathophysiology change within the microenvironment. In this study, we demonstrated that mesenchymal stromal cells cultured with U266/Lp-I under hypoxic conditions resulted in an increased  $\alpha$ -smooth muscle actin expression and high productive levels of both hypoxia-inducible factor-2 $\alpha$  and integrin-linked kinase proteins. Moreover, inhibition of hypoxia-inducible factor-2 $\alpha$  by Small interfering RNA (siRNA) in mesenchymal stromal cells decreased the protein levels of both  $\alpha$ -smooth muscle actin and integrin-linked kinase after mesenchymal stromal cells cultured with U266 under hypoxic conditions. We further demonstrated that transfection of integrin-linked kinase-siRNA reduced the protein level of  $\alpha$ -smooth muscle actin and attenuated angiogenesis *in vitro* by decreasing the attachment of Q-dot labeled cells and secretion of angiogenic factors. In conclusion, our research showed that mesenchymal stromal cells cultured with myeloma cells under hypoxia participated in the angiogenesis of multiple myeloma, which is regulated by the hypoxia-inducible factor-2 $\alpha$ -integrin-linked kinase pathway. Thus, targeting integrin-linked kinase may represent an effective strategy to block hypoxia-inducible factor-2 $\alpha$ -induced angiogenesis in the treatment of multiple myeloma.

## Keywords

myeloma, HIF-2 $\alpha$ -ILK, angiogenesis, hypoxia

## Abbreviations

bFGF, basic fibroblast growth factor; BM, bone marrow; EMT, epithelial–mesenchymal transition; HIF, hypoxia-inducible factor; HUVEC, human umbilical vein endothelial cell; ILK, integrin-linked kinase; MM, multiple myeloma; MSC, mesenchymal stromal cell; NT-siRNA, nontarget siRNA; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin

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## Introduction

Multiple myeloma (MM), a virtually fatal plasma cell neoplasm, is characterized by the production of large amounts of monoclonal immunoglobulins and destructive bone lesions.<sup>1</sup> Although we have witnessed remarkable progress in the understanding of the pathogenesis of MM coupled with rapid development of novel therapeutic agents in the past decade, further progress is still urgently required for this incurable disease. In particular, unbalanced regulation of cytokines and chemokines in the bone marrow (BM) microenvironment can result in rapid development of drug resistance or relapse of the disease, which

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is perhaps still the major hurdle in the cure of this malignant disease.<sup>2,3</sup> The interaction between MM cells and the BM microenvironment regulates the growth and resistance of MM cells by altering the expression of relevant cytokines, growth factors, and chemokines, which play a critical role in angiogenesis and invasion.<sup>4</sup>

Mesenchymal stromal cells (MSCs) are nonhematopoietic, multipotent cells. They exert multiple effects on tumor development and progression, by increasing the stemness of tumor cells, coordinating migration, and promoting angiogenesis.<sup>5</sup> Our previous study indicated that growth medium conditioned by MSCs derived from MM significantly promoted the proliferation, chemotaxis, and capillary formation of human umbilical vein endothelial cells (HUVECs) by elevating the concentration of angiogenic factors.<sup>6</sup> Thus, MSCs may serve as a platform for the delivery of biological agents to the tumor microenvironment.

Hypoxic microenvironments facilitate and trigger major molecular and immunological processes necessary to drive progression of tumors to malignancy.<sup>7</sup> Hypoxia is one of the most significant environmental factors affecting cells and plays an important role in different aspects of cell biogenesis such as metabolism, angiogenesis, innate immunity, and stemness induction and maintenance.<sup>8,9</sup> The response to hypoxia in human cells is mainly regulated by hypoxia-inducible factors (HIFs), a family of transcription factors that orchestrate signaling events leading to angiogenesis and tumorigenesis. Under hypoxic conditions (1.5% O<sub>2</sub>), the signal transducer and activator of transcription3 (STAT3) and mitogen-activated protein kinase (MAPK) signaling pathways in breast cancer cells are activated, resulting in increased secretion of IL-6, which serves to activate and recruit MSC homing and then facilitate tumor progression.<sup>10</sup>

Failure to cure MM is multifactorial and can be attributed to the underlying genetic heterogeneity of the cancer and to the surrounding microenvironment. Hence, understanding the mutual interaction between MM cells and microenvironment may lead to the development of novel treatment strategies able to eradicate this disease. Hypoxia may be a consequence of the distance of the growing tumor cells from existent capillaries or inefficiency of the new vessels.<sup>11</sup> Hypoxia-inducible factors contain 2 subunits: O<sub>2</sub>-sensing  $\alpha$ -subunits (HIF-1 $\alpha$ , HIF-2 $\alpha$ , and HIF-3 $\alpha$ ) and a stable  $\beta$ -subunit (HIF-1 $\beta$ ).<sup>12</sup> Hypoxia-inducible factor- $\alpha$  subunits are critical for vasculogenesis and hematopoiesis during development and are hallmarks of the hypoxic tumor microenvironment. Expression of the endogenous marker of hypoxia for the HIF-1 and HIF-2 pathways is strongly associated with radiotherapy failure in head and neck squamous cell carcinoma.<sup>13</sup> Several studies have shown that HIF-1 $\alpha$ , but not HIF-2 $\alpha$ , enhances the transcription of glycolytic pathway genes to increase energy uptake. For example, a recent study showed that integrin-linked kinase (ILK) and nuclear factor-kappaB (NF- $\kappa$ B)-mediated HIF-1 $\alpha$ -dependent (but not HIF-2 $\alpha$ ) vascular endothelial growth factor (VEGF) expression in response to hypoxia could ultimately control breast cancer progression and angiogenesis.<sup>14</sup> In contrast, the

role of HIF-2 $\alpha$  is seldom invested in cancer. Covello *et al*<sup>15</sup> reported that HIF-2 $\alpha$  could regulate stem cell function and differentiation through activation of octamer-binding transcription factor 4 (Oct-4), a transcription factor essential for maintaining stem cell pluripotency, which in turn contributes to HIF-2 $\alpha$ 's tumor-promoting activity. Hypoxia-inducible factor-2 $\alpha$ , which is highly expressed in tumor-initiating and stem cell-like cells, acts to directly increase the activity of octamer-binding transcription factor 4, which maintains stem cells in an undifferentiated state.<sup>16</sup> Notably, recent study showed that HIF-2 $\alpha$  contributes to antiestrogen resistance in breast cancer cells and downregulation or inhibition of EGFR can lead to decreased HIF-2 $\alpha$  levels, suggesting HIF2-EGFR regulatory crosstalk will be useful for counteracting antiestrogen resistance in the clinic.<sup>17</sup>

Several studies have indicated that ILK is overexpressed in various types of cancer and is correlated with poor patient prognosis. Our team confirmed that the inhibition of ILK promoted apoptosis of MM cells, decreased the invasion of MM cells, and inhibited IL-6 and VEGF secretion from MSCs.<sup>18</sup> The link between hypoxia and MSC-mediated MM angiogenesis has until now remained unclear. In this study, we report that HIF-2 $\alpha$  upregulates ILK, which potentiates MSC-mediated angiogenesis in MM. Moreover, our study found that a previously undiscovered HIF-2 $\alpha$ -ILK pathway is involved in the angiogenesis of MSCs in MM under hypoxic conditions. The results indicate that targeting HIF-2 $\alpha$  and ILK may provide a potential target for MM therapy.

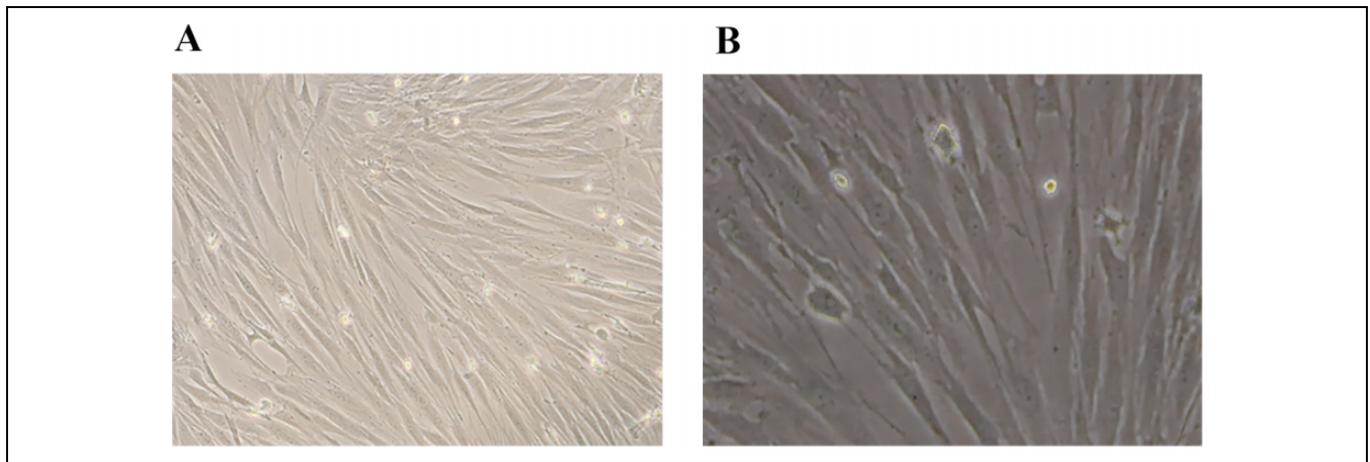
## Materials and Methods

### Cell Culture

The U266 and LP-1 MM cell lines were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Science. The cells were grown in suspension in RPMI 1640 medium (Gibco-BRL, Grand Island, New York) supplemented with 15% fetal bovine serum (Gibco-BRL), penicillin (100 mg/mL), streptomycin (100 mg/mL), and 2 mM L-glutamine (Gibco-BRL). Cells were maintained at 37°C in 5% carbon dioxide and 95% air and underwent passage twice weekly.

### Isolation and Culture of MSCs

Bone marrow samples were collected from patients recently diagnosed with MM who had not received any treatment at the Cancer Hospital of Tianjin. Bone marrow was collected by an iliac crest puncture. Samples of 10 to 20 mL were collected and placed in heparinized tubes. The collection of samples was approved by the Cancer Hospital of Tianjin Review Board. Informed consent was written from all donors. Mononuclear cells were separated by Ficoll-Paque gradient centrifugation (specific gravity 1.077 g/mL; Nycomed Pharma AS, Oslo, Norway) and cultured in expansion medium at 37°C with 5% CO<sub>2</sub> in a fully humidified atmosphere. Expansion medium



**Figure 1.** Morphology of bone marrow mesenchymal stem cells under inverted microscope (A: magnification  $\times 100$ , B: magnification  $\times 200$ ).

contained 60% Dulbecco's modified Eagle medium/F12 (Gibco Life Technologies, Paisley, United Kingdom), 40% MCDB-201 (Sigma, St Louis, Missouri), 2% fetal calf serum (Gibco),  $1\times$  insulin transferrin selenium (Gibco),  $1\times$  linoleic acid bovine serum albumin, 100 U/mL penicillin, and 1000 U/mL streptomycin (Gibco). After culture for 24 to 48 hours, the culture medium was replaced and nonadherent cells were removed. Once cells were more than 80% confluent, they were detached with 0.25% trypsin-EDTA (Sigma). All separated MSCs were used at passage 2 in our study.

### Immunophenotype of MSCs

Mesenchymal stromal cells were stained with antibodies directed against CD11a, CD11b, CD14, CD29, CD31, CD34, CD44, CD45, CD105, CD106, human leukocyte antigen DR (HLA-DR), CD73, CD90, or a matched isotype control (BD Biosciences Pharmingen, San Diego, California). Then they were incubated at room temperature for 30 minutes. The stained cells were washed with phosphate buffer saline (PBS) and then treated with a FITC (fluoresceine isothiocyanate)-labeled secondary antibody in the dark for 15 minutes. Immunofluorescence analysis was performed using a 5-parameter flow cytometer (FACSCalibur; Becton Dickinson, San Jose, California).

### Coculture of MSCs With U266

Mesenchymal stromal cells were cultured at a starting concentration of  $1 \times 10^5/\text{cm}^2$ , with or without  $5 \times 10^5/\text{mL}$  U266 cells by Transwell coculture system in serum-free conditions. The 2 groups were kept under normoxia at  $37^\circ\text{C}$  in a humidified atmosphere containing 95% air and 5%  $\text{CO}_2$  or under hypoxia at  $37^\circ\text{C}$  in a humidified atmosphere containing 93% nitrogen, 2% oxygen, and 5%  $\text{CO}_2$ . Cocultured MM cells were separated from monolayer MSCs by careful pipetting with ice-cold PBS (repeated twice). The remaining MSC monolayer was then trypsinized, and cells were counted using a hemocytometer.

**Table 1.** The Immunophenotypes of Cultured MSCs.<sup>a</sup>

Antigen	MSCs %
CD11a	3.6 (1.6-5.1)
CD11b	2.5 (0.6-3.2)
CD14	3.1 (1.3-4.5)
CD29	96.7 (92.6-98.3)
CD31	1.9 (0.8-3.3)
CD34	1.7 (0.6-2.2)
CD44	98.5 (96.5-99.1)
CD45	2.4 (1.6-4.3)
CD105	97.1 (95.3-99.1)
CD106	96.6 (93.2-98.7)
HLA-DR	2.5 (1.5-4.3)
CD73	97.1 (95.2-99.3)
CD90	95.4 (94.7-98.1)

Abbreviation: HLA-DR, human leukocyte antigen DR; MSC, mesenchymal stromal cell.

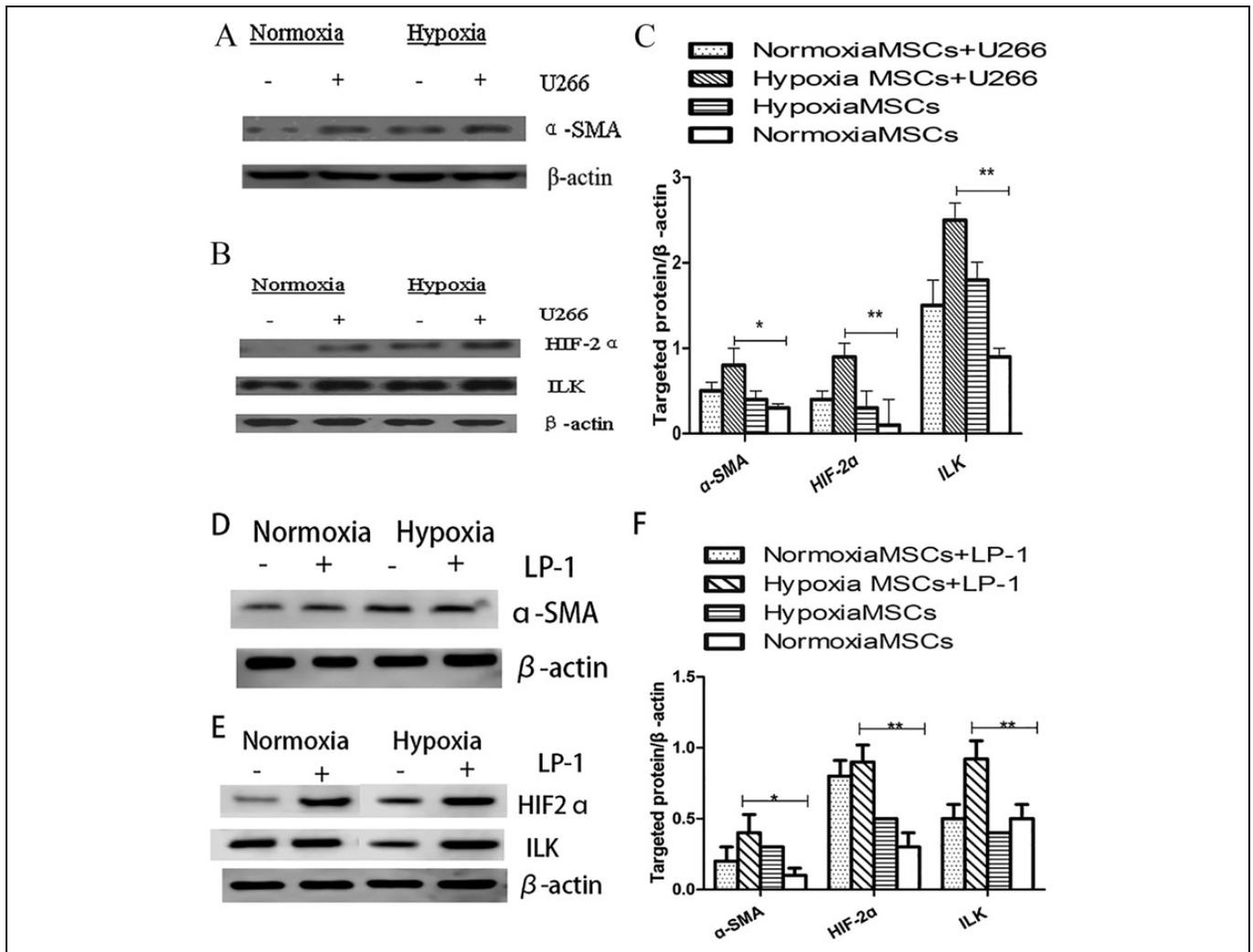
<sup>a</sup>Flow cytometry analysis was performed to determine the immunophenotype of isolated MSCs and ensure the homogeneity of extraction: Nearly all the MSCs expressed CD106, CD105, CD29, CD44; they did not express CD11a, CD11b, CD14, CD31, CD34, CD45, and HLA-DR.

### Cell Transfection

We used validated small interfering RNA (siRNA) directed against HIF-2 $\alpha$  and ILK messenger RNAs (mRNAs), respectively, from Ambion (Carlsbad, California). Mesenchymal stromal cells were trypsinized and  $10^6$  cells were counted into 100 mL of Nucleofector solution (Amaxa/Lonza, Allendale, New Jersey) with 100 nM siRNA. Transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, California). Nontarget siRNA (NT-siRNA) or control siRNA was used as a control. Twenty-four hours post-transfection, the transfected cells were seeded into 6-well plates for 48 hours and then prepared for further analysis.

### Quantification of Angiogenic Factors by ELISA

Mesenchymal stromal cells were cultured for 72 hours in the following 4 configurations with 2 variables: Coculture with



**Figure 2.** Mesenchymal stromal cells (MSCs) cultured with or without U266/LP-1 under normoxia (21% O<sub>2</sub>) and hypoxia (2% O<sub>2</sub>), respectively, for 72 hours. The proteins in MSCs encoded by  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; A), hypoxia-inducible factor (HIF) 2 $\alpha$ , and integrin-linked kinase (ILK; B) were detected by Western blot. An elevated expression of  $\alpha$ -SMA, HIF2 $\alpha$ , and ILK in MSCs was found in response to coculture and hypoxia. C, Corresponding histogram of targeted protein expression of  $\alpha$ -SMA, HIF2 $\alpha$ , and ILK/ $\beta$ -actin. D-F, Done in LP-1 cells. Similar results were obtained in 3 independent experiments (\* $P$  < .05).

U266, culture alone, under normoxia or hypoxia conditions. The secretions of angiogenic factors including VEGF, platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) were measured after 72 hours. Medias conditioned by MSCs which is dealt with the 4 different methods mentioned above were collected and analyzed using commercially available human VEGF, PDGF, and bFGF enzyme-linked immunosorbent assay (ELISA) Development Kits (Peprotech, CA, USA).

### Western Blot Analysis

After 48 hours of incubation at 37°C and 5% CO<sub>2</sub> under normoxic (21% O<sub>2</sub>) or hypoxic conditions (2% O<sub>2</sub>), MSCs ( $2 \times 10^6$  cells) cocultured with or without U266 cells were washed twice with ice-cold PBS and lysed in cell lysis buffer (10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 150 mM NaCl, 1 mM MgCl<sub>2</sub>,

1 mM CaCl<sub>2</sub>, 0.1% NaN<sub>3</sub>, 10 mM iodoacetamide, 3 mM phenylmethylsulfonyl fluoride, 1% Triton X-100) supplemented with a protease inhibitor cocktail (Roche Diagnostics, Pierce, USA). Equal amounts of lysate (equivalent to 30  $\mu$ g protein) were separated on 10% polyacrylamide gels (Bio-Rad Laboratories, Inc, Hercules, California). Proteins were transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom) and immunoblotted with primary antibodies to the following: HIF-2 $\alpha$  (Abcam, Cambridge, Massachusetts), ILK (Upstate, Lake Placid, New York),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; Abcam) and  $\beta$ -actin (Abcam). Membranes were then probed with a horseradish peroxidase-conjugated secondary antibody and reacted with electrochemiluminescence reagent (Amersham Biosciences, Baie-d'Urfé, Québec, Canada). Signals were detected using a luminescent image analyzer (LAS-100Plus; Fujifilm, Tokyo, Japan) and quantified by Image Gauge (Fujifilm).

Densitometry was performed using Quantity-One image analysis software.  $\beta$ -actin was used as an internal standard to normalize protein expression. Band intensities of  $\alpha$ -SMA, HIF2 $\alpha$ , and ILK are expressed as the percentage of the  $\beta$ -actin band intensity, which was set at 100%.

### In Vitro Angiogenesis and Binding Assay

Mesenchymal stromal cells were treated in advance with siRNAs directed against HIF-2 $\alpha$  and ILK mRNAs. After 24 hours posttransfection, the transfected cells were seeded into 6-well plates for further analysis. Approximately 80% confluent pre-treated MSCs were labeled with a Qtracker cell labeling kit (highly fluorescent Q-dot nanocrystals) obtained from Invitrogen (Molecular Probes, Kodak, USA). For 3D culture of MSCs, Matrigel (150  $\mu$ L) was polymerized in an 8-well chambered slide. Human umbilical vein endothelial cells and labeled MSCs (10 000 cells/well) were seeded into each well and incubated for approximately 24 hours, after which the binding efficiency of MSCs with capillary-like structure generated by HUVEC in regular media or different tumor cell-derived condition media was determined. Quantification of the number of capillary-like structures and attached Q-dots was carried out using the NIS Elements software program attached to the Nikon photographic fluorescence microscope.

### Statistical Analysis

For statistical tests, SPSS Statistics 17.0 software package (SPSS Inc, Chicago, Illinois) was used. All standards and samples were tested in triplicate. The Student *t* test was used to measure statistical significance among different treatment groups. Multiple comparisons were conducted using 1-way analysis of variance. A *P* value <.05 was considered statistically significant. Data are expressed as the mean  $\pm$  standard error of the mean.

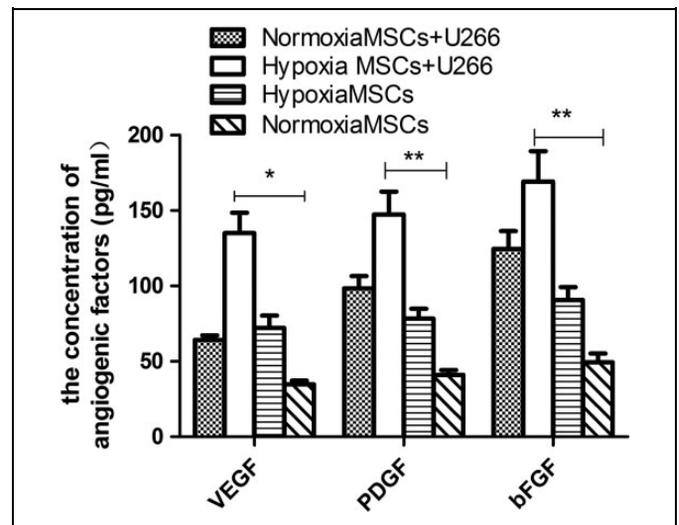
## Results

### Immunophenotype of MSCs

Results showed that we could get more adherent cells after 10 days and these cells showed typical morphological features of fibroblast cells (Figure 1). Then MSCs were characterized using flow cytometry to detect uniformity. The immunophenotypes of cultured MSCs are shown in Table 1. Nearly all MSCs expressed CD105 (97.1%) and CD44 (98.5%), while few expressed hematopoietic and endothelial cell markers (CD31: 1.9% and CD34: 1.7%).

### U266/Lp-1 Promotes MSC Differentiation Into Pericytes Under Hypoxic Conditions

Previous studies suggest 2 mechanisms by which MSCs participate in tumor angiogenesis. One is a direct contribution of MSCs to blood vessel formation by their differentiation into pericytes. As a result, we wanted to explore the role of MM cells in governing differentiation of MSCs to pericytes under

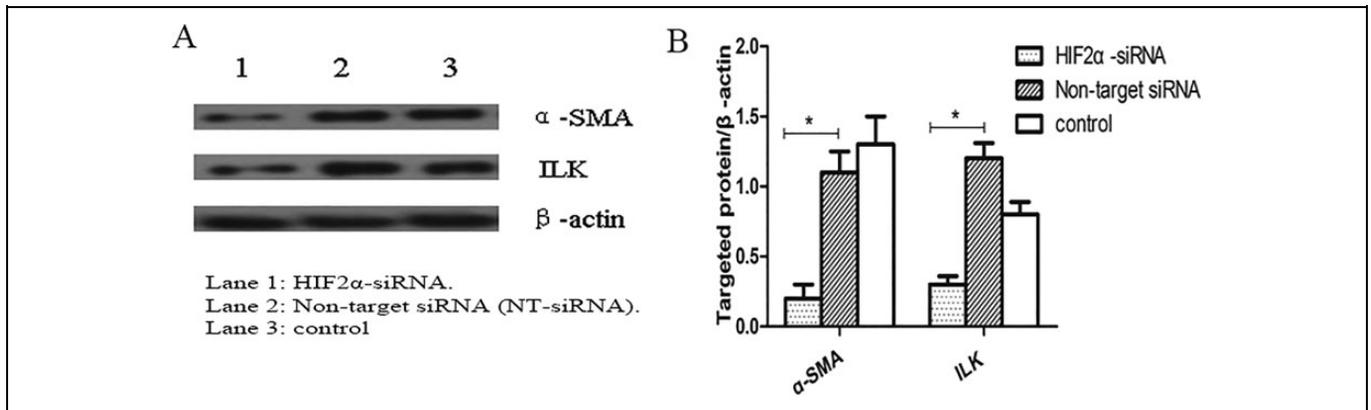


**Figure 3.** Angiogenic factors secreted by mesenchymal stromal cells (MSCs) were elevated under hypoxic conditions. Mesenchymal stromal cells cultured with or without U266 under normoxia (21% O<sub>2</sub>) and hypoxia (3% O<sub>2</sub>), respectively, for 72 hours. Enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of the secreted angiogenic factors (basic fibroblast growth factor [bFGF], vascular endothelial growth factor [VEGF], and platelet-derived growth factor [PDGF]) in the MSC-conditioned medium of each treatment condition listed (\**P* < .05).

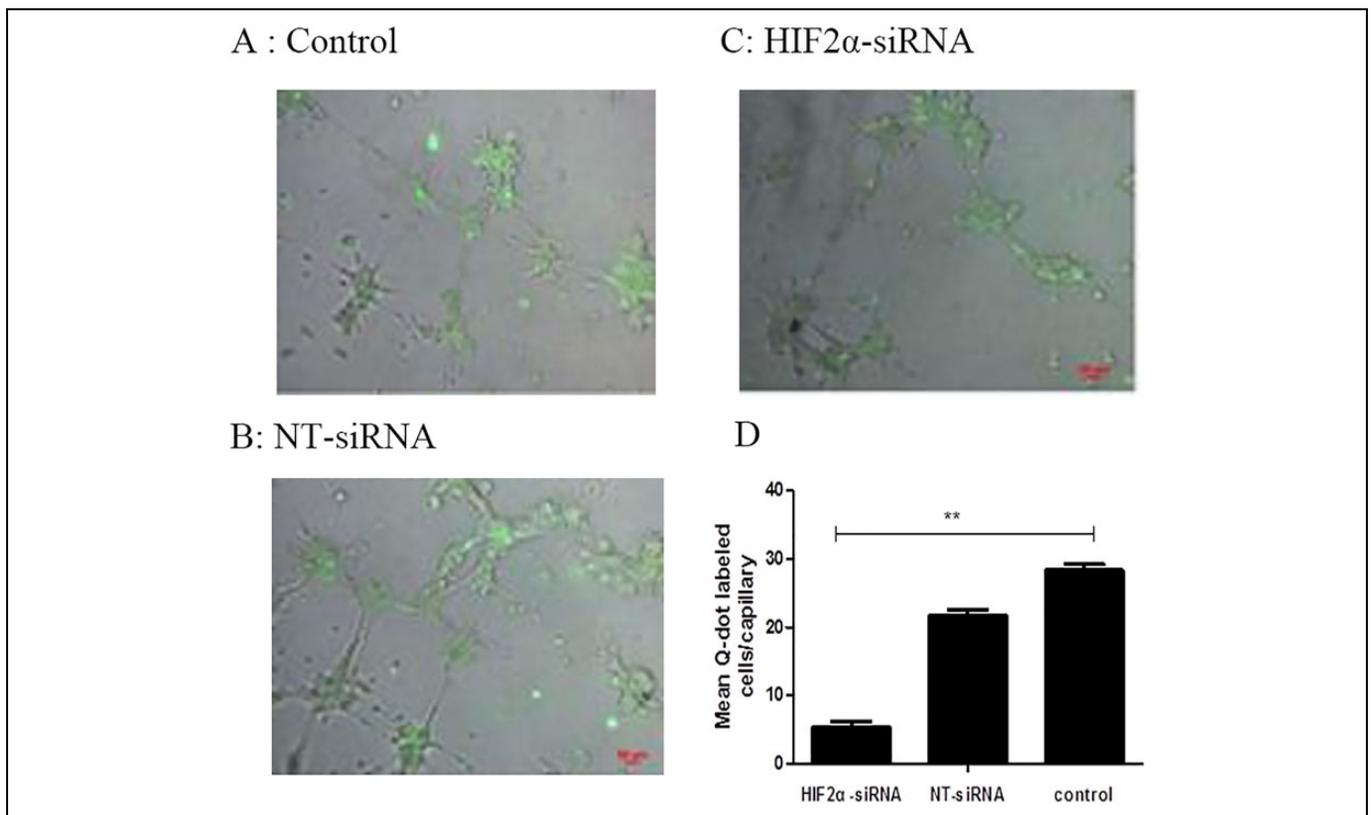
hypoxic conditions in the tumor angiogenesis process. First, we measured levels of  $\alpha$ -SMA, a positive marker of mural cells by Western blot. Mesenchymal stromal cells were cultured for 72 hours in the following 4 configurations with 2 variables: coculture with U266, culture alone, under normoxia or hypoxia conditions. We found that both U266/LP-1 and hypoxia promoted the expression of  $\alpha$ -SMA in MSCs, suggesting that both promote differentiation of MSCs to mural cells. Another way that MSCs may participate in angiogenesis of MM is through the activity of ILK, as suggested by our previous studies. Consistent with this hypothesis, Chou *et al*<sup>11</sup> reported that a HIF-1 $\alpha$ -ILK regulatory loop facilitates the epithelial-mesenchymal transition (EMT) in cancer cells. Therefore, we evaluated the expression of HIF-2 $\alpha$  and ILK in MSCs. We found that HIF-2 $\alpha$  and ILK were upregulated along with  $\alpha$ -SMA. Taken together, our results indicate that the expression levels of  $\alpha$ -SMA, HIF-2 $\alpha$ , and ILK were further elevated in MSCs cocultured with U266 under hypoxic conditions (Figure 2A and B). Figure 2C is the corresponding histogram. Figure 2D-F was done in LP-1 cells. All experiments were conducted in triplicate.

### Angiogenic Factors Secreted by MSCs Are Elevated Under Hypoxic Conditions

It is well established that another possible mechanism of MSCs participating in tumor angiogenesis is by secretion of angiogenic factors. To determine whether secretion of these factors is influenced by hypoxia or coculture with U266 cells, we



**Figure 4.** A, Transfection of hypoxia-inducible factor 2 $\alpha$  (HIF2 $\alpha$ )-small interfering RNA (siRNA) reduced the protein level of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and integrin-linked kinase (ILK) in mesenchymal stromal cells (MSCs). Lane 1: HIF2 $\alpha$ -siRNA. Lane 2: Nontarget siRNA (NT-siRNA). Lane 3: control. B, Corresponding histogram of targeted protein expression of  $\alpha$ -SMA and ILK/ $\beta$ -actin.



**Figure 5.** A, Hypoxia-inducible factor 2 $\alpha$  (HIF2 $\alpha$ )-siRNA attenuated the angiogenesis of mesenchymal stromal cells (MSCs) *in vitro*. Human umbilical vein endothelial cell (HUVEC) and Q-dot labeled MSCs were seeded on the Matrigel, and their associations were observed after 24 hours when *in vitro* capillary-like structures are formed. Photographs were taken of each well. A, Control. B, Nontarget small interfering RNA (siRNA) (NT-siRNA). C, Hypoxia-inducible factor 2 $\alpha$ -siRNA. Quantification of attachment of Q-dot-labeled cells using software provided with a Nikon fluorescent photomicroscope. D, The number of attached Q-dot-labeled newly created pericytes under HIF2 $\alpha$ -transfected conditioned media was decreased relative to the other 2 groups. The data are representative means  $\pm$  standard error of the mean (SEM) of 10 different capillary-like structures (compared with  $**P < .05$ ).

collected MSC-conditioned media under hypoxic conditions, coculture, or both. The levels of the angiogenic factors including VEGF, PDGF, and bFGF were measured by ELISA. The concentrations of VEGF, PDGF, and bFGF secreted by MSCs cocultured with U266 under hypoxic conditions were

135  $\pm$  13, 147  $\pm$  15, and 169  $\pm$  20 pg/mL, respectively, which were significantly higher than those of the control group (Figure 3;  $P < .05$ ). As a result, hypoxia significantly enhanced the secretion of angiogenic factors of MSCs, especially in the circumstances that MSCs cocultured with U266.

### Hypoxia-Inducible Factor-2 $\alpha$ -siRNA Reduced the Protein Level of ILK and $\alpha$ -SMA in MSCs

To investigate the role of HIF-2 $\alpha$  and the relationship with ILK in the angiogenesis of MSCs, we used siRNAs directed against HIF-2 $\alpha$  and NT-siRNA and a blank control to transfect MSCs cultured with U266 under hypoxic conditions (Figure 4). We found that inhibition of HIF-2 $\alpha$  dramatically decreased the protein level of ILK and  $\alpha$ -SMA in MSCs.

### Hypoxia-Inducible Factor-2 $\alpha$ -siRNA Attenuated the Angiogenesis of MSCs In Vitro

Given that HIF2 $\alpha$ -siRNA treatment reduced the expression of  $\alpha$ -SMA and ILK in MSCs, we wanted to determine whether it also attenuated MSC-mediated angiogenesis. To establish this, we used a 3-dimensional, Matrigel-based cell culture system seeded with HUVEC and observed attachment of Q-dot labeled differentiated MSCs. Human umbilical vein endothelial cell and MSCs were seeded on Matrigel and observed 24 hours later, after HUVEC formed capillary-like structures. The attachment of the newly formed induced pericytes was significantly decreased in the HIF2 $\alpha$ -siRNA group compared with the NT-siRNA and control groups (Figures 5A and B).

### Hypoxia-Inducible Factor-2 $\alpha$ -siRNA Treatment Significantly Decreases Secretion of Angiogenic Factors by MSCs

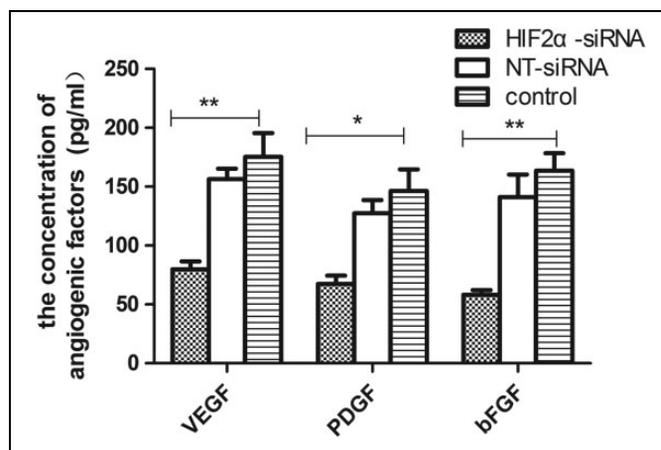
To confirm that HIF-2 $\alpha$  is a general regulator of MSC angiogenesis, we measured the concentration of bFGF, VEGF, and PDGF in MSC-conditioned media using ELISA. As expected, the concentration of MSC-secreted angiogenic factors decreased upon transfection with HIF-2 $\alpha$ -siRNA (Figure 6), compared with the other 2 control groups ( $P < .05$ ).

### Integrin-Linked Kinase-siRNA Treatment Decreases the Protein Level of $\alpha$ -SMA, Attenuates Angiogenesis, and Inhibits Secretion of Angiogenic Factors by MSCs

We speculated that HIF-2 $\alpha$  may control angiogenesis by regulating ILK. To test this, we knocked down ILK in MSCs with siRNA. We found that treatment with ILK-siRNA decreased the protein level of  $\alpha$ -SMA (Figure 7), suggesting decreased differentiation of MSCs into SMC. As expected, MSCs transfected with ILK-siRNA exhibited a markedly decreased ability to attach in our *in vitro* attachment assay (Figure 8). Consistent with decreased angiogenesis, MSCs transfected with ILK-siRNA displayed decreased secretion of angiogenic factors (Figure 9) compared with the other 2 control groups ( $P < .05$ ).

## Discussion

Multiple myeloma, a malignant and life-threatening plasma cell disease, remains incurable. Aberrant expression of signaling pathways and additional genetic abnormalities significantly

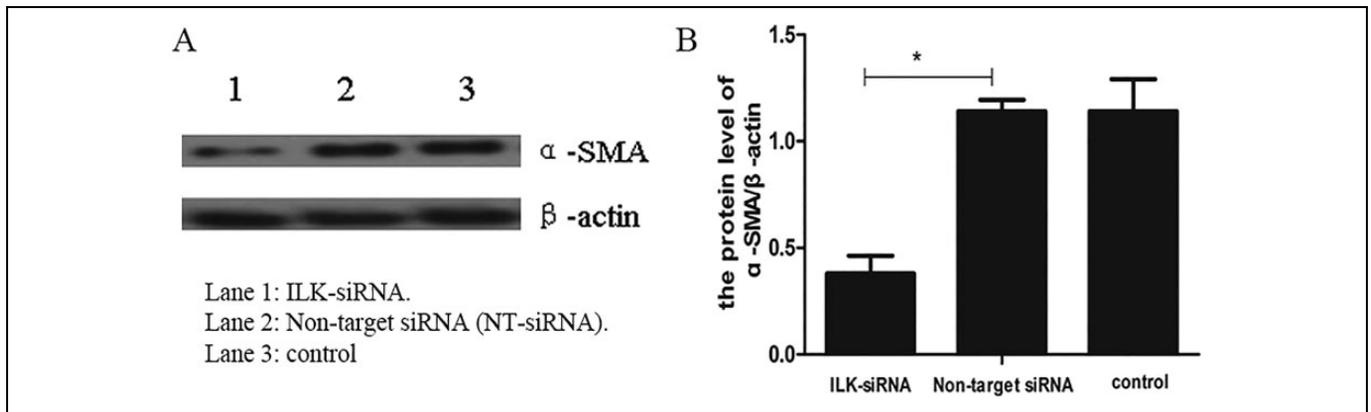


**Figure 6.** Hypoxia-inducible factor 2 $\alpha$  (HIF2 $\alpha$ )-small interfering RNA (siRNA) treatment significantly reduced the secretion of angiogenic factors in MSCs. Enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of angiogenic factors (basic fibroblast growth factor [bFGF], vascular endothelial growth factor [VEGF], and platelet-derived growth factor [PDGF]) in the CM of MSCs. The VEGF, PDGF, and bFGF secretion levels were decreased by 54%, 54%, and 64% in MSCs transfected with HIF2 $\alpha$ -siRNA compared with control siRNA, respectively ( $n = 3$  independent experiments; compared with  $*P < .05$ ).

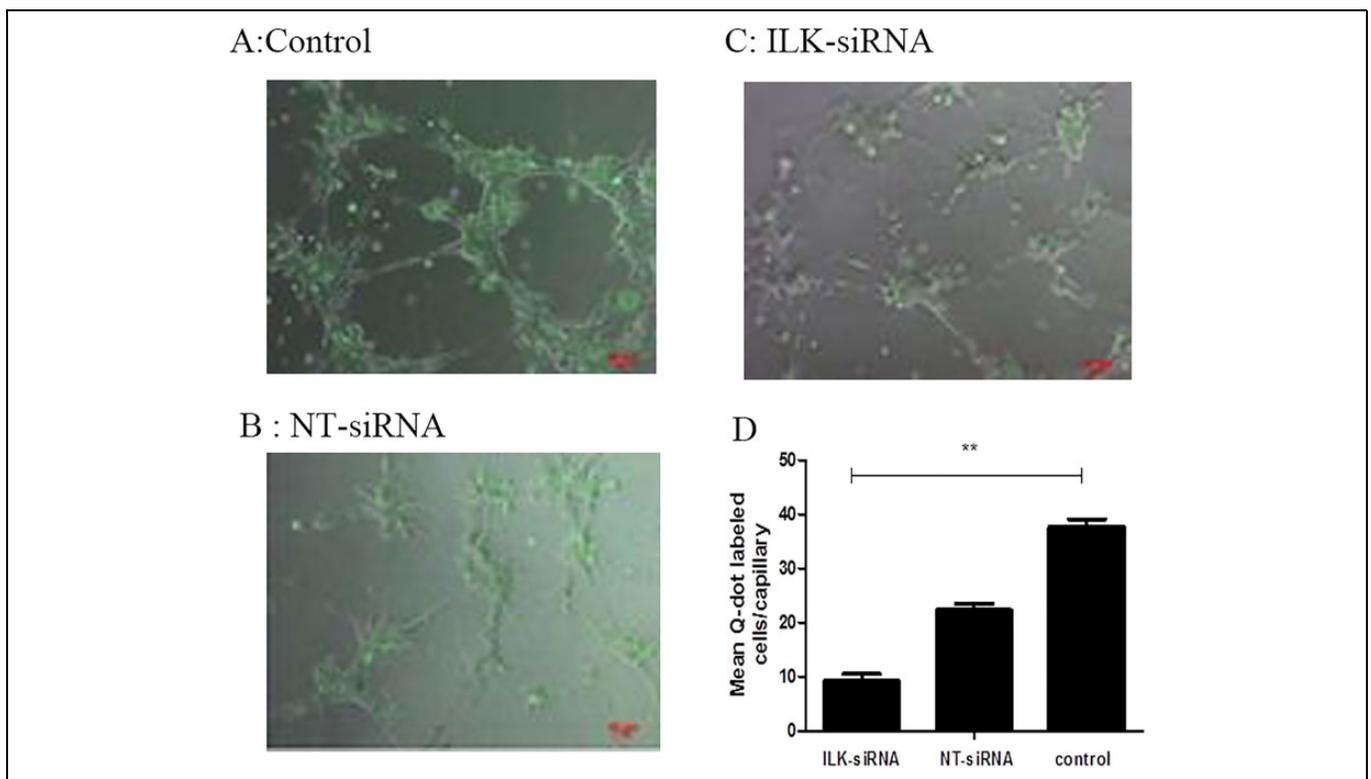
worsen poor prognosis in patients with MM.<sup>19,20</sup> As result, novel drugs are required to screen for novel therapeutic compounds to improve the prognosis of patients. It has been reported that MM cells must be in a dynamic interaction with their stromal environment to survive and expand.<sup>21</sup> A variety of angiogenic factors, proteases, reactive oxygen species, and inflammatory cytokines induce the formation of an extensive and suitable microenvironment, which is suitable for the growth, survival, and proliferation of malignant plasma cells in MM.<sup>22</sup> The role or roles that MSCs play in this complex process are unclear. One possible mechanism is that MSCs promote blood vessel formation required by endothelial cells by secreting cytokines. Another possible mechanism is that MSCs directly contribute to the formation of blood vessels.

In this study, we showed that the expression of  $\alpha$ -SMA was elevated in MSCs cultured with U266 under hypoxic conditions. Then, using ELISA, we found MSCs cultured with U266 under hypoxic conditions displayed an increased secretion of angiogenic factors. The above results indicated that culture with U266 under hypoxia conditions enhanced the angiogenesis of MSCs by differentiation into SMC and secretion of angiogenic factors.

Hypoxia is one of the most prominent hallmarks in the tumor microenvironment. Tumor cells can adapt to a low-oxygen environment by deploying a series of cellular responses, which confer aggressive, metastatic, and stem cell-like phenotypes to cancer cells.<sup>23,24</sup> Integrin-linked kinase, which links cell-adhesion receptors, integrins, and growth factors to a range of signaling pathways, is an attractive target for cancer therapeutics.<sup>25</sup> Chou *et al*<sup>11</sup> found a novel HIF-1 $\alpha$ -ILK regulatory loop that facilitates hypoxia-induced HIF-1 $\alpha$  expression and promotes EMT through modulation of Snail



**Figure 7.** A, Integrin-linked kinase (ILK)-small interfering RNA (siRNA) treatment decreased the protein level of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) of mesenchymal stromal cells (MSCs). Lane 1: integrin-linked kinase-siRNA (ILK-siRNA). Lane 2: Nontarget siRNA (NT-siRNA). Lane 3: Control. B, Corresponding histogram of targeted protein expression of  $\alpha$ -SMA/ $\beta$ -actin.

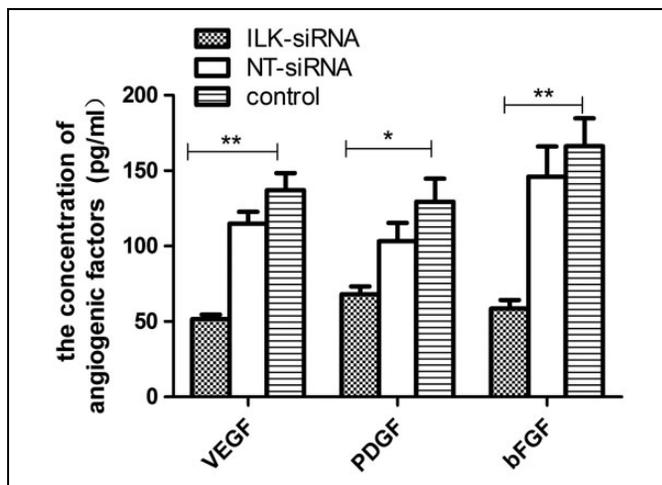


**Figure 8.** A, Integrin-linked kinase (ILK)-small interfering RNA (siRNA) treatment attenuated the angiogenesis of mesenchymal stromal cells (MSCs). Human umbilical vein endothelial cell (HUVEC) and Q-dot-labeled MSCs were seeded on Matrigel, and their associations were observed after 24 hours when *in vitro* capillary-like structures are formed. Quantification of attachment of Q-dot-labeled cells using software provided by Nikon fluorescent photomicroscope. A, control. B, nontarget siRNA (NT-siRNA). C, integrin-linked kinase-siRNA (ILK-siRNA). D, Quantification of attached Q-dot-labeled newly differentiated pericytes under ILK-transfected-conditioned media was decreased relative to the other 2 groups. The data are representative means  $\pm$  standard error of the mean (SEM) of 10 different capillary-like structures (compared with  $*P < .05$ ).

and Zeb1; moreover, ILK, in turn, stimulates HIF-1 $\alpha$  expression and an aggressive phenotype through cell type- and cell context-dependent pathways in breast and prostate cancer cells. Hypoxia-inducible factor-1 $\alpha$  is essential for hypoxia-induced MSC mobilization, possibly acting via its downstream genes VEGF and SDF-1 $\alpha$  Stromal cell-derived factor 1 (SDF-

1 $\alpha$ ).<sup>26</sup> Hypoxia-inducible factor-2 $\alpha$  has been identified in patients presenting with a cluster of paraganglioma, somatostatinoma, and polycythemia,<sup>27</sup> but HIF-2 $\alpha$  has been seldom investigated in patients with cancer.

Our next goal was to identify the role of the HIF-2 $\alpha$ -ILK pathway in angiogenesis of MSCs cocultured with U266 under



**Figure 9.** Transfection of integrin-linked kinase (ILK)-small interfering RNA (siRNA) significantly reduced the secretion of angiogenic factors by mesenchymal stromal cells (MSCs). Enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of angiogenic factors (basic fibroblast growth factor [bFGF], vascular endothelial growth factor [VEGF], and platelet-derived growth factor [PDGF]) in CM of MSCs. The VEGF, PDGF, and bFGF secretion levels were decreased by 62%, 47%, and 64% in MSCs transfected with integrin-linked kinase-siRNA (ILK-siRNA) compared with control siRNA, respectively (n = 3 independent experiments; compared with \* $P < .05$ ).

hypoxia conditions. We found that both HIF-2 $\alpha$  and ILK proteins in MSCs were elevated in response to coculture and hypoxic conditions. We used siRNA to investigate the role of HIF-2 $\alpha$  in the angiogenesis of MSCs. We found that siRNA-mediated knockdown of HIF-2 $\alpha$  decreased the protein level of  $\alpha$ -SMA and ILK in MSCs. Mesenchymal stromal cells transfected with ILK-siRNA exhibited a marked decrease in the attachment of Q-dot-labeled cells and secretion of angiogenic factors. Because of the oncogenic properties of ILK, we also wanted to determine the effects of ILK inhibition on angiogenesis and the relationship with HIF-2 $\alpha$ . We further found the transfection of ILK-siRNA decreased the protein level of  $\alpha$ -SMA and attenuated the ability of angiogenesis *in vitro* by downregulating the attachment of Q-dot-labeled cells and secretion of angiogenic factors.

Taken together, our results provide direct evidence that MSCs cultured with U266 under hypoxia participated in the angiogenesis of MM, the process of which is regulated and controlled by the HIF-2 $\alpha$ -ILK pathway. Thus, we propose that specific inhibition of HIF-2 $\alpha$  and ILK may provide an effective strategy for MM therapy.

#### Authors' Note

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