Title: Transcriptional profiling reveals crosstalk between mesenchymal stem cells and endothelial cells promoting pre-vascularization by reciprocal mechanisms
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Abstract

Mesenchymal stem cells (MSCs) show great promise in blood vessel restoration and vascularization enhancement in many therapeutic situations. Typically, the co-implantation of MSCs with vascular endothelial cells (ECs) is effective for the induction of functional vascularization in vivo, indicating its potential applications in regenerative medicine. The effects of MSCs-ECs-induced vascularization can be modeled in vitro, providing simplified models for understanding their underlying communication. In this paper, a contact co-culture model in vitro and an RNA-seq approach were employed to reveal the active crosstalk between MSCs and ECs within a short time period at both morphological and transcriptional levels. The RNA-seq results suggested that angiogenic genes were significantly induced upon co-culture, and this pre-vascularization commitment might require the NF-κB signaling. NF-κB blocking and interleukin neutralization experiments demonstrated that MSCs potentially secreted interleukin factors including IL1β and IL6 to modulate NF-κB signaling as well as downstream chemokines during co-culture. Conversely, RNA-seq results indicated that the MSCs were regulated by the co-culture environment to a smooth muscle commitment within this short period, which largely induced myocardin, the myogenic co-transcriptional factor. These findings demonstrate the mutual molecular mechanism of MSCs-ECs-induced pre-vascularization commitment in a quick response.

1. Introduction

Stem cell transplantation has shown increasing therapeutic potential for treatment of pathological situations as well as tissue restoration [1-3]. Mesenchymal stem cells (MSCs), the stromal progenitor cells found in the bone marrow [3], were recently proved effective in vascular enhancement and protection [4, 5]. Since tissue engineering is currently limited by the inability to adequately
vascularize tissues surrounding the engineered constructs [6, 7], the co-implantation of MSCs with human endothelial cells (ECs) recently succeed in creation of fully functional blood vessels under different circumstances in vivo [8-10], which could serve as a good solution for controlled vascularization. However, the cellular and molecular bases of MSCs-ECs interaction and their joint influence on endothelial vascularization were not fully understood.

Previous research has revealed the active interaction between MSCs and ECs in different situations. Culture of MSCs in Human Umbilical Vein Endothelial Cell (HUVEC) conditioned media (CM) promoted the activity of the enhanced alkaline phosphatase (ALP), indicating that EC-secreted growth factors might prompt the osteogenic differentiation of MSCs in vitro [11, 12]. However, MSCs were also profoundly affected by ECs via direct cell-cell contact. The prolonged MSC-EC crosstalk in contact co-culture in vitro stimulated the proliferation of MSCs [13]. Under other circumstances, direct contact with vascular ECs resulted in an increased proportion of myogenic phenotypes in MSCs [14-17]. Tissue engineering-based research in both 2D and 3D co-cultures in vitro revealed that the active interaction between ECs and MSCs (or osteoprogenitor and fibroblast) induced the formation of tubelike cell aggregation structures. This result indicates that angiogenesis and pre-vascularization are involved in the cell-cell communication and remodeling of the functional blood vessel development in vivo [5, 18-26]. Moreover, MSCs could repress ECs by regulating cytokine-induced leukocyte recruitment [27], while the activated ECs could regulate the MSCs-to-ECs transmigration in a leukocyte-like mechanism [28, 29]. However, the innate mechanism of MSCs-ECs interaction-dependent vascularization remains elusive.

In this paper, therefore, we focus on revealing the internal molecular mechanisms in cell coupling and reciprocal ECs-MSCs interactions and thus on better understanding the functional vascularization effect induced by the MSCs-ECs co-culture environment. As long-term vascularization was responsible for the combinatorial effects of complex and nutrient micro-environment in-vivo, we simplified a 2D MSCs-ECs co-culture model in vitro to reason whether a simple direct MSCs-ECs interaction is sufficient to stimulate pre-vascularization at early period. Conditions for MSCs-ECs direct co-culture were preliminarily optimized, including respective seeding densities and culture medium formulation, which guarantee the suitable culture conditions. As we focused on the early interaction within MSC-EC co-culture, we comprehensively analyzed the transcriptome by RNA-seq for both cell types individually, followed by flow cytometer separation after 6, 12 and 24 h of co-culture. We aim to monitor the potential ECs-MSCs communications at molecular level. The results revealed that even during a relatively short period, the co-culture promoted the angiogenesis-related gene activation in HUVECs. Further analysis demonstrated that the angiogenesis-related NF-κB signaling was modulated mainly in HUVECs, which was at least partially stimulated by two interleukins (ILs) IL1β and IL6. Both IL1β and IL6 were mainly secreted by MSCs in a contact-dependent manner. Conversely, this short-period interaction also promoted the early smooth muscle commitment of MSCs partially by modulation of TGF-β. This was correlated with the physiological situations that recruited mural cell types support EC functionalization in the development and repair of blood vessels [8].

2. Materials and Methods

2.1. Cell culture conditions and separation by fluorescence activated cell sorter (FACS)

Human MSCs (HMSCs) with stable green fluorescent protein (GFP) expression from passage 5 (OriCell™ HMSC-GFP, Cyagen) were tested for CD29, CD44 and CD105 positive, and CD34, CD45 negative, and expanded in human MSC basal media with 10% fetal bovine serum, 1% v/v penicillin/streptomycin (HUXMX-90011, Cyagen); HUVECs (HUVEC, Lonza) obtained at passage
2-3, maintained and proliferated in EGM-2 basal medium (Lonza). The innate angiogenic potential of HUVEC were tested via Matrigel™ tube formation assay (Figure S1A). HMSCs within passage 8 and HUVEC within passage 5 were used during all experiments.

For co-culture, complete media for both cell types were mixed, and the most adequate medium ratio was pre-selected to finally set the ratio of HMSC basal medium to EGM-2 medium at 1:1. Both co-culture groups and mono-culture groups were maintained in this co-culture medium, and these media were changed every other day. Seeding density between HMSCs and HUVEC was optimized and applied at about 4x10^6 and 1.3x10^4 cells/cm^2 in all experiments based on a quick and obvious observation of previous reported cell aggregation structure formation. Controlled indirect co-culture of HMSCs on the plate bottom and HUVEC on the upside of the filter was conducted with a 24 mm Transwell (0.4 µm pore) Polyester Membrane Insert (Corning Corporation).

During FACS-based cell sorting, cells from each group were trypsinized and washed three times with phosphate buffer solution (PBS). GFP-positive HMSC populations were immediately isolated with a BD FACs cell sorter (Arl III, BD Biosciences). During CD31+ cell sorting, the GFP-negative populations were incubated with Cy3-conjugated anti-CD31 antibody (anti-CD31-Cy3, Sino Biologicals) and sorted with BD FACS. The sorted co-HMSCs and co-HUVEC were immediately collected and used in downstream RNA extraction or protein extraction.

### 2.2. Observation and quantification of cell aggregation structures

To observe cell aggregation structures, both static images and time lapse movies were captured by an Eclipse Ti microscope system (Nikon cooperation) with phase-contrast field and GFP fluorescence channels. We quantified angiogenic cell aggregation structures with a cell aggregate index calculated by ImageJ™. Co-culture-induced aggregation trend was quantified as follows:

\[
\text{Aggregation index} = \log_2 \left( \frac{\text{Cell Density}_\text{aggr.area}}{\text{Cell Density}_\text{est.rand}} \right) + \log_2 \left( \frac{\text{Cell Density}_\text{est.rand}}{\text{Cell Density}_\text{spar.area}} \right)
\]

Where Cell Density=cell counts/ spreading areas. Aggr.area means the aggregating area in which the co-cultured cells aggregate and tend to form cell aggregation structures, and the density is higher than the randomly distributed density; Spar.area mean the sparsely distributed area, in which cells are sparsely located in the co-cultured environment, and the density is lower than the randomly distributed density; Est.rand means estimated absolutely random distribution, or the estimated cell density for cells randomly distributed in the culture dishes for a defined seeding cell count.

### 2.3. Analysis of gene expression by high-throughput RNA sequencing and qPCR

For both high throughput transcriptome analysis and qPCR, RNA was isolated from at least three groups per experimental setting, including mono-cultures and FACS-sorted counterparts. RNA was extracted by a Total mRNA Isolation Kit (Tiangen). The quality and quantity of isolated RNAs were determined with a NanoDrop 2000 analyzer (NanoDrop Technologies) and a Bioanalyzer 2100 (Agilent).

During high throughput transcriptome analysis, the RNA integrity number in all cases ranged from 8.9 to 10, indicating minimal degradation. The Poly(A)-based mRNA was enriched with NEBNext™ Poly(A) mRNA Magnetic Isolation Module (New England Biolabs) following the manufacture’s instruction. CDNA libraries were constructed with a NEBNext™ Ultra™ Directional RNA Library Prep Kit for Illumina™ (New England Biolabs). The quantity of the constructed libraries was further confirmed by Bioanalyzer 2100. Pair-end RNA-seq procedures were conducted with Illumina™-2000 (Illumina) following the manufacturer’s instructions. RNA-seq data were analyzed as reported [30].
Heatmap constructions were synthesized by R 3.0.2 (R Foundation for Statistical Computing) with a package “gplots” as reported [30]. Gene ontology (GO) was analyzed by DAVID Bioinformatics Resources 6.7 as reported [31].

For qPCR analysis, cDNA was synthesized using a Fastquart RT Kit (Tiangen) and real-time PCR, and mRNA was analyzed by a SuperReal PreMix (SYBR Green) kit (Tiangen) and a Real-time 7500 Fast machine (Applied Biosystems). All primers used for real-time PCR are listed in Table S17. All data were analyzed by Comparative ΔΔCt.

2.4. Analysis of NF-κB activation by nuclear-cytoplasmic extraction and Western blot

The trypsinized cells and FACS-sorted cells were immediately washed with PBS, and the nuclear-cytoplasm was extracted by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) following the manufacture’s instruction. The extracted cytoplasmic and nucleus proteins were preserved immediately at -80°C until use.

Western blot for detection of NF-κB activation was performed based on the p65 protein cytoplasm-to-nuclei translocation following the standard Western blot protocol. Both cytoplasmic and nucleus p65 were detected by anti-p65 monoclonal antibody (Sino Biologicals) and were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Sigma-Aldrich). The secondary antibody conjugated with horseradish peroxidase (HRP) (Sigma-Aldrich) was used for blotting. The blots were visualized by electrogenerated chemiluminescence (ECL) reagents (Pierce Biotechnology, Thermo Scientific).

2.5. Co-culture environment interfered with chemical inhibitor, siRNA and neutralization antibodies

For PTDC-specific blockage of NF-κB signaling, a given concentration of PTDC (Sigma-Aldrich) was added into the culture medium 1 h after cell attachment. Control groups were treated with an equivalent volume of dimethyl sulfoxide (DMSO), which was used for PTDC dilution. For RNAi treatment, siRNA against target genes of interest and scramble siRNA were all purchased from GenePharma Company. SiRNA was transfected with Lipofectamine LTX and PLUS™ Reagent with 35 nM siRNA following the manufacture’s instruction for both cell types separately one day before co-culture. For IL1β, IL6 and TGF-β neutralization experiments, 50 - 200 ug/mL neutralizing antibodies against human IL1β, IL6 and TGF-β (Sino Biologicals) were added during the co-culture; control groups were treated with an equivalent amount of IgG (Sigma-Aldrich).

2.6. Evaluation of protein concentration in culture media by enzyme-linked immunosorbent assay (ELISA)

Cytokines IL1β and IL6 in the supernatants of media after different treatments were collected and preserved immediately at -80°C until use. Experiments were conducted using commercial ELISA kits (Boster Bioscience) following the manufacture’s instruction. Briefly, 100 uM of the supernatant of each sample was added to the anti-IL1β/IL6 antibody in the pre-coated 96-well plates, followed by incubation at 37°C for 90 min. Solutions were then discarded, and capture antibodies were added for another 60-min incubation. The plates were then washed by PBS and incubated with an avidin-biotin complex (ABC) working solution for 30 min. Chromogenic reaction was then performed with TMB, and the wavelength of 450 nm was read. Results were normalized to the total concentrations regarding to the standard sample curves.
2.7. Statistical Analysis

Data were shown as mean± standard deviation (SD). Experiments between two groups were analyzed using Student’s t test. Significance was evaluated using one-way analysis of variance (ANOVA). *p<0.01 or **p<0.05 were considered significant.

3. Results

3.1. Establishment of HMSCs-HUVECs co-culture system in vitro

To understand the potential of MSC-EC communication during vascularization, we simplified a 2D co-culture model. The primary HMSCs stably transfected with GFP and the primary GFP-negative HUVECs were used in the co-culture experiments. The seeding ratio of HUVECs to HMSCs was optimized at about 3:1, at the density around 4x10^5 and 1.3x10^5 cells/cm² respectively, and the culture media formulations were optimized to support both mono-cultures of two cell types and co-cultured conditions.

3.2. Co-culture promoted formation of cell aggregation structures

In the experimental conditions, we clearly observed and quantified cell aggregation structures caused by the HUVECs-HMSCs mutual recruitment (Figure 1A, B)[18, 22]. In this high density co-culture mode, cell aggregation structures were gradually formed within 24 h and stabilized for more than 4 days (Figure 1A), and then their clear morphology was gradually overwhelmed by the co-culture owing to the outgrowth of both types of cells. Real-time monitoring further confirmed that this structure was formed mainly between 12 and 24 h (Figure 1C). During this process, HUVECs behaved more actively than HMSCs, and these cell aggregation structures were mainly attributed to the active migration and rearrangement between HUVECs and MSCs (Video S1). This morphological arrangement strictly depended on the contact co-culture mode, since the indirect co-culture using Transwell™ or conditioned medium (CM) did not induce the formation of cell aggregation structures (Figure S1B).

3.3. RNA-seq revealed differential gene expression profiles and angiogenic genes induction

As angiogenic cell aggregation structure was spontaneously formed within an interval from 12 to 24 h, we next mainly performed the co-culture system within 24 h, and immediately separated the GFP-positive cells as a HMSC population and the GFP-negative cells as a HUVEC population via fluorescence activated cell sorting (FACS). The purity of the HUVEC population, was further validated by an EC surface marker CD31 (Figure 2A). As angiogenic cell aggregation structures were spontaneously formed within from 12 to 24 h, we used FACS to sort HMSCs and HUVECs (co-MSCs & co-HUVECs hereafter) after 12 and 24 h of co-culture, as well as their mono-culture controls (mono-MSCs & mono-HUVECs) for detailed transcriptome analysis through RNA-seq (Figure 2A, Figure S1). A total of > 36 million raw reads were obtained for each sample with ~95% integrity after filtering. Collectively, ~80% of the reads were mapped to >16,000 genes in each sample, and >60% of reads covered at least 80% of genes (Tables S1-S5). For co-MSCs, 8.27% and 13.88% of total genes were modified significantly compared to the mono-culture controls for 12 and 24 h respectively (fold change>2, P<0.001). For co-HUVECs, 7.63% and 9.36% of total genes were modified significantly compared to mono-culture control for 12 and 24 h respectively (Figure 2B, Tables S6-S9). Hierarchical clustering of RNA-seq data demonstrated that the morphological changes were accompanied by
dynamic changes in cell transcriptome (Figure 2C). Notably, among the significantly-induced expression profiles, a proportion of genes including CDH5, PECAM1, TGFB family and PDGF family (Tables S6-9) were well correlated with a previous report in a similar situation [32].

Consistent with our cell aggregation structure formation morphologically, the unbiased RNA-seq data analysis revealed that a large proportion of angiogenesis-related factors were expressed differentially and significantly (26/78 genes >2 fold induction on average; 5/78 > 2 fold suppression, Table S10) within 12 and 24 h in HUVECs (Figure 2D), including PLAUR [21] and fibroblast growth factor (FGF) [33], which were involved in the formation of cell aggregation structures in a similar context. However, unexpectedly classical angiogenic stimulators ANGPT and vascular endothelial growth factor (VEGF) family [28, 30, 41, 42] were not obviously induced in both cell types within this short period. The gene ontology (GO) analysis in differential gene expression revealed significant correlation between angiogenesis and vascularization (Figure 2E, Table S11, S12), indicating that direct co-culture of MSCs and ECs significantly induced the pro-angiogenic effect, even within a short period. As a large proportion of the angiogenesis-related genes encoded the secretion molecules, we then collected the culture media from 12- and 24-h co-culture groups. However, incubation of monocultured HUVECs with these CMs did not induce the formation of cell aggregation structures (data not shown), indicating that this pre-vascularization morphology was dependent on direct contact and might be associated with chemotaxis gradient.

3.4. NF-κB participated in co-culture-induced formation of cell aggregation structures

Since HMSCs-HUVECs co-culture could induce the early pro-angiogenic effect in a quick response, we further interrogated the potential evidence of signaling pathway activation from gene expression profiles. We first referred to two classic endothelial angiogenesis-related pathways both in vitro and in vivo: VEGF signaling [6, 7, 22] and Angiopoietin/TIE2 signaling [34, 35]. However, the RNA-seq results did not show significant elevation of upstream ligands or receptors for these pathways in co-HUVECs within 24 h. Alternatively, unbiased analysis demonstrated that a large proportion of experimentally validated NF-κB target genes (mainly based on http://www.bu.edu/NF-κB/gene-resources/target-genes/) were up-regulated upon co-culture for 12 and 24 h in both co-HUVECs and co-HMSCs (Figure 3A,B). NF-κB signaling was related to EC activation-induced cell-cell aggregation and angiogenesis [31, 32]. Notably, activation of NF-κB target genes was more significant in co-HUVECs (Table S13) compared to co-HMSCs (Table S14). Therefore, we hypothesized that the NF-κB signaling pathway might be stimulated upon our co-culture conditions and participate in angiogenic induction.

To validate the involvement of NF-κB signaling in the co-culture, we evaluated the NF-κB signaling activation based on the nucleus translocation of NF-κB large subunit P65 (RelA) within 24 h. Accordingly, the translocation of P65 nucleus was improved upon co-culture in HUVECs compared to the mono-culture counterparts within 6, 12 and 24 h separately (Figure 2C), although these translocation trends are not as significant as transient activation by tumor necrosis factor (TNF)-alpha (a classic positive control for NF-κB activation). Similarly, co-HMSCs shared similar but even moderate trends compared to co-HUVECs in the co-culture environment (Figure 2D).

We next interrogated whether the inhibition of NF-κB could block the co-culture-induced formation of angiogenic cell aggregation structures. A NF-κB signaling specific inhibitor pyrimidine dithiocarbamate (PTDC) could effectively block the P65 nucleus translocation raised by co-culture (Figure S3). Accordingly, attenuation of NF-κB signaling through a specific inhibitor pyrrolidine dithiocarbamate (PTDC) effectively suppressed the formation of cell aggregation structures in a dosedependent manner in the co-culture environment, but this suppression was reversible following the withdrawal of PTDC (Figure 3E,F). Collectively, the NF-κB signaling was activated and required for the co-culture-induced angiogenic process.
3.5. IL1β and IL6 were mainly elevated by HMSCs in a direct contact manner

As NF-κB signaling pathways can be activated by various external signals and maintained by feedback loops, we screened the candidates that may be responsible for NF-κB activation in RNA-seq data from both cell types. Among the known factors regulating NF-κB signaling, IL1β (Log2 ratio fold change:10.8/12.4) and IL6 (Log2 ratio fold change:5.3/4.6) were significantly inducive in HMSCs in both 12-h and 24-h co-culture groups (Table S6-7). Quantitative polymerase chain reaction (qPCR) validated that both IL1β and IL6 were significantly induced in co-HMSCs in a time-dependent manner (Figure 4A, B). In contrast, IL1β and IL6 were only moderately induced in co-HUVECs (Figure 4A, B), and their basal levels were significantly lower than in HMSCs. We then excluded the possibility that the elevation of the two ILs specifically in co-HMSCs was due to the innate signals of ECs or the indirect communication with ECs, since neither Transwell™-based indirect co-culture nor mono-HUVEC CM could achieve such significant induction (Figure S4A, B), demonstrating that the transcriptional induction of IL1β or IL6 was caused by direct contact with HUVECs in co-culture.

We further tested the secretion of both factors via standard sandwich ELISA, and they were induced in the supernatant of co-cultured medium (Figure 4C, D), which was correlated with their mRNA levels. To further elucidate whether the increased secretion of IL1β and IL6 was caused by co-HMSCs, we applied siRNAs against IL1β and IL6 separately to reduce their mRNA expressions. As a result, the high-level IL1β in the supernatant of co-culture groups was reduced significantly in the HMSCs, but not significantly in the HUVECs (Figure 4E). Similar results were found in IL6 RNAi tests (Figure 4F). These evidences suggested that IL1β and IL6 were mainly elevated by co-HMSCs.

3.6. IL1β and IL6 served as the NF-κB signaling potential regulators in co-culture environment

We then interrogated whether the co-HMSC-secreted IL1β and IL6 regulated the activation of NF-κB signaling for co-HUVECs in the co-culture environment. We used neutralizing antibodies against IL1B and IL6 to separately or simultaneously block their activities in the supernatant. As expected, blocking IL1β, IL6 or both effectively suppressed the NF-κB nucleus translocation induced by the co-culture (Figure 4G), and restricted the co-culture-induced formation of angiogenic cell aggregation structures (Figure 4H), which mimicked the effects of NF-κB attenuation by PTDC treatment.

Since some ILs including IL1β and IL6 are transcriptionally regulated by NF-κB, which may create a positive feedback loop to chronically activate the signaling pathway [36, 37], we further tested whether IL1β and IL6 could be self-regulated via the NF-κB signaling. Interestingly, the attenuation of NF-κB suppressed the expressions of IL1β and IL6 at both mRNA and protein levels in the supernatant (Figure S 5A-D); meanwhile, blocking their activity in the supernatant by neutralizing antibodies also decreased their mRNA transcription (Figure S 5A, B). Collectively, the co-HMSCs-elevated IL1β and IL6 served as the positive regulators for co-culture-induced early vascularization by modulating NF-κB signaling and they were regulated in a positive feedback manner.

3.7. NF-κB-dependent formation of cell aggregation structures was associated with angiogenic chemokines

The NF-κB signaling in the downstream targets at a series of genes, including a proportion of chemokines, surface receptors and secreted molecules that may promote angiogenesis both in vitro and in vivo [38]. Angiogenesis is commonly raised by HUVEC activation with complex chemokine regulation [39]. We thus screened the differentially-expressed genes that were overlapped with co-culture-stimulated NF-κB targets, and reported the angiogenic genes and the chemotaxics-related genes. To clarify the effects of these regulated genes during the co-culture- induced early
vascularization, we selected four significantly-induced candidates, P-selectin (SELP), CC chemokine ligand23 (CCL23), and CXC chemokine ligands 2/3 (CXCL2, CXCL3) for further study.

qPCR confirmed that the levels of SELP, CCL23, CXCL2 and CXCL3 significantly increased in a time-dependent manner (Figure 5A-D). Then we interrogated whether these genes were modulated by NF-κB signaling. Inhibition of NF-κB by 25 μM PTDC abolished the induction of these genes upon co-culture in co-HUVECs (Figure 5E-H); neutralizing antibodies against IL1β and IL6 in the supernatant also separately or simultaneously suppressed the induction of these genes (Figure 5E-H).

Collectively, these chemotaxis-related genes were potentially regulated by the interleukin-mediated NF-κB signaling.

To further demonstrate whether these NF-κB-modulated angiogenic chemotaxis-related genes might actively participate in early vascularization, we selectively or cooperatively knocked down the overlapped candidates and evaluated their potential contributions to the formation of cell aggregation structures. As expected, the selective or cooperative inhibition partially suppressed the formation of cell aggregation structures (Figure 5I), which further indicated that these chemotaxis-related genes might function as a cocktail in the downstream for regulation of early pre-vascularization.

3.8. HMSCs were endowed with early smooth muscle differentiation upon co-culture

Besides the effects of HMSCs on the angiogenic activation of HUVECs in the co-culture environment, we also analyzed the potential differentiation trends of HMSCs induced by co-culture with terminally differentiated HUVECs. As MSCs were multipotent and showed both osteoblastic and myogenic trends under the influence of ECs in different situations, we comprehensively evaluated the multiple expression patterns on osteoblastic and myogenic differentiation markers under our experimental conditions. The unbiased analysis revealed that a proportion of the smooth muscle markers were induced within 24 h, including master transcriptional factors and co-factors for myogenesis (Figure 6A, Table S15). However, the osteoblast differentiation master transcriptional factors were not significantly induced within this short period, although alkaline phosphatase (ALPL) was overexpressed (Figure 6B, Table S16), which was consistent with other literatures [12, 13, 26].

We selectively validated the most-induced myogenic markers, including serum response factor (SRF), myocardin (MYOC), calponin (CNN1), and smooth muscle 22α (SM22α) by qPCR (Figure 6C), and the results were consistent with the expression trend in RNA-seq data. The induction of these genes was also mainly contact-dependent in the co-culture (Figure S6A-D). CNN1 and SM22α were reported as smooth muscle-specific markers, but we did not detect any significant induction of skeleton or cardiac muscle markers (MyoD, MyoG, NKX2.5, a-MHC, b-MHC and cTnT) either from RNA-seq data or qPCR (data not shown). Thus, we supposed this co-culture tended to induce smooth muscle fate commitment within 24 h.

Noticeably, MYOC which encoded an SRF co-transcriptional factor was the most significantly induced among these markers (Figure 6C). As myogenic specific genes are transcriptionally activated by the SRF-MYOC complex predominately and this mechanism was governed by the RhoA/ROCK signaling in the upstream [40-42], we treated the co-culture environment with a specific RhoA/ROCK inhibitor, Y27632. The treatment with Y27632 did not obviously disturb the formation of cell aggregation structures, but suppressed the MYOC elevation during co-culture (Figure 6D), indicating that RhoA/ROCK might also participate in the co-culture-induced early myogenic differentiation of HMSCs. RhoA/ROCK signaling can be activated by various external signals, including TGF-β1 and TGF-β3 [43, 44] which were significantly upregulated in both co-MSCs and co-HUVECs (Figure S7). Neutralization of TGF-β1 or TGF-β3 during the co-culture partially repressed the induction of MYOC (Figure 6D), demonstrating that TGF-β1 and TGF-β3 might be two external signals induced during co-culture, and act on HMSCs to trigger the early myogenic differentiation commitment within short period.
4. Discussion

Contact co-culture of ECs with MSCs can induce vascularization both in vitro and in vivo [20, 45-47], and thus is a promising solution to controlled vascularization enhancement in therapeutic needs. Here, we uncovered the underlying communication mechanism in MSCs-ECs co-culture at molecular level by scrutinizing their transcriptional profiles separately. For the real therapeutic application, certain specific cell types such as endothelial colony-forming cell (ECFC), which can be readily isolated from human cord blood and have a higher proliferative potential than mature EC, may become more ideal EC sources [48]. Therefore, our data here may be also illustrative for MSC co-culture with these specific ECs.

To obtain precise gene expression profiles without mixing with the other type of cells after intensive contact co-culture, our preliminary studies focused on a strict separation of two cell types. In previous works studying the direct contact interaction between heterogenous cell types, cell types after co-culture were sorted by either labelling cell types with cell tracker dyes [49, 50] or using specific surface antigens [13]. Our preliminary test indicates that these separation methods might yield a small false positive or false negative rate in the separated cell populations, and in the downstream, might affect the mRNA profiling such as secreting factors and differentiation markers, in which the expression levels might be highly varied. To maximize the precision in this process, we applied GFP-stably-expressed primary HMSCs in the co-culture experiment for sorting HMSCs with FACS, and further chose the anti-CD31-positive (an EC-specific surface marker) subpopulation in the GFP-negative population as ECs. This separation procedure maximally guaranteed the sorting purity after intensive cell-cell interaction. During RNA sequencing, we also strictly excluded those differentially-expressed genes that were potentially caused by slight mixture of the other type of cells.

Based on the RNA-seq data, the unbiased analysis revealed that a proportion of angiogenesis-related genes including cytokines, chemokines and adhesion molecules were significantly upregulated, which was consistent well with the formation of angiogenic cell aggregation structures observed in our co-culture model in vitro and other similar experimental settings [13, 22, 32]. Although at least several weeks will be taken by general transplantation to form possible functional blood vessels in vivo [8-10, 17], evidence from this work suggested that direct contact communication between HMSCs and HUVECs even in a short period (within 24 h) was potent enough for commitment of early pre-vascularization. To determine the key regulators mediating this process, we first inspected the gene expression levels of the most common angiogenic factors such as VEGF [51], FGF [33] and ANGPT family [34] that were widely reported as critical for angiogenesis induction. As a result, none of these factors was significantly induced in the co-culture condition within this short time period. Alternatively, a proportion of most significantly induced genes were among the NF-κB targets, which remind us the activation of NF-κB signaling. Unbiased NF-κB target analysis based on a public database indicated that a significant number of NF-κB-regulated genes were upregulated from our RNA-seq data, which strengthened our hypothesis. We proved its activation by determining an increased p65 translocation into the nucleus upon co-culture, since phosphorylated IkB can liberate the p65 in the cytoplasm so that p65 will shut into the nuclear compartment upon the activation of NF-κB signaling [52]. NF-κB plays a key role in regulating a wide range of physiological processes, including inflammation, cancer progression, synaptic plasticity as well as angiogenic response [52-54]. NF-κB may regulate the angiogenic potential and directional cell migration via stimulating adhesion molecules and cytokines, especially in tumor models and immune response [27, 29, 54, 55]. In this work, we found NF-κB signaling was also involved in the pre-vascularization induced by ECs-MSCs co-culture.

We then found that HMSCs secreted two potent interleukins, IL1β and IL6, which were responsible for the activation of NF-κB in co-HUVECs (also moderately in co-HMSCs). These two factors were
among the reported NF-κB activators only in both types of cells that were significantly induced both at mRNA and secreted protein levels, and their induction was strictly regulated by a contact co-culture model. Considering that only direct contact co-culture (rather than Transwell or conditioned medium) induce the ILs expression as well as following process, it is likely that interplay with heterotypic cell junction communication attribute to the initial ILs expression in MSC. Literatures on co-culture experiments provided evidence for this hypothesis, for example gap junction channel which consisted of Cx43 was observed in contact HUVEC and osteoprogenitor cell co-culture situation, and a gap junction blocker could effectively block the contact co-culture induced cell differentiation [56].

Besides, as IL1β and IL6 belong to the important immune-regulators [52], it is also possible that initial raising of these IL factors may be caused by a quick immune response between heterogeneous cell interactions.

Neutralizing these two ILs significantly abolished the NF-κB activation as well as cell aggregation formation in the co-culture environment. Both IL1β and IL6 may be expressed in MSCs [27, 57, 58]. IL1β is regarded as a typical NF-κB activator for long time, and its elevation potently supports the NF-κB activation. In our context, IL1β was more potent in activation of NF-κB than IL6. IL6 may function in a more intricate manner, and was classically regarded as an activator of JAK/STAT3 signaling, which can also regulate angiogenesis parallel to NF-κB. In this work, we also demonstrated that IL6 could concomitantly function with IL1β to induce and maintain the NF-κB signaling as reported in intestinal epithelia [59]. One possible mechanism of the IL-6-participated NF-κB activation is attributed to the crosslink between STAT3 and NF-κB [59-61]. Interestingly, IL1β and IL6 are both NF-κB targets and activators, and there was a NF-κB positive feedback loop in this context, suggesting this loop might be essential for sustainable NF-κB activation during the co-culture-induced pre-vascularization. Our evidences collectively demonstrated that HMSCs could activate NF-κB signaling in neighbor HUVECs via a paracrine mechanism.

Notably, MSCs were increasingly recognized as capable of dampening allogeneic immune response during transplantation by weakening the recruitment of innate immune cells [2, 27]. Although NF-κB is widely regarded as the proinflammatory signaling in many other contexts[53], our high throughput data revealed that this co-culture-based vascularization model with NF-κB participation did not actually evoke most relevant proinflammatory cytokines, including vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM) and selectin E (SELE) [62] in ECs. On one hand, we inferred that NF-κB induced in this context was relatively mild compared to TNF-α-mediated transient activation and thus might not be deleterious to both cell types or enhance inflammation. On the other hand, IL6 was also immunosuppressive via modulating JAK/STAT3 signaling. In the circumstance of MSC-controlled immunosuppressive machinery, MSCs secreted IL6 as anti-immunflatory effect through activating SOCS3 via JAK/STAT3 in ECs and thereby alleviated the recruitment of neutrophils or lymphocytes [27]. Therefore, we supposed that IL6 was likely involved in both JAK/STAT3 and NF-κB signaling as dual functions: IL6 participates in angiogenesis via NF-κB and safeguards immunosuppression in the co-culture.

Since angiogenic cell aggregation structures were formed by cell-cell recruitment, we focused on those significantly induced NF-κB targets that may be responsible for chemotaxis. We selected four chemotaxis-related molecules for further study, as all these genes were tightly regulated by IL1β- and IL6-potentiated NF-κB signaling. The results showed that these molecules might cooperatively contribute to the dynamic cell-cell recruitments. However, incubation of mono-cultured HUVECs in the co-culture conditioned medium failed to induce the formation of cell aggregation structures, indicating the complexity of contact-dependent communication and the importance of chemotaxis machinery, even though this conditioned medium might contain higher concentrations of angiogenic molecules as well as IL1β/IL6.

Many co-culture models indicated that MSC differentiation can be affected by various heterogenous cells, while MSC differentiation induction by ECs is intricate [11-14, 18, 49]. In this work,
Transcriptional profiling reveals crosstalk between mesenchymal stem cells and endothelial cells promoting pre-vascularization by reciprocal mechanisms. (doi: 10.1089/scd.2014.0330)

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References:


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**Figure Legends:**
Transcriptional profiling reveals crosstalk between mesenchymal stem cells and endothelial cells promoting pre-vascularization by reciprocal mechanisms. (doi: 10.1089/scd.2014.0330)

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Transcriptional profiling reveals crosstalk between mesenchymal stem cells and endothelial cells promoting pre-vascularization by reciprocal mechanisms. (doi: 10.1089/scd.2014.0330)
Figure 1. Co-culture promoted cell aggregation structure formation. (A): Representative images of HMSC-GFP and HUVEC co-culture within 96 h. Cell aggregation structure was clearly observed at 24 h and further stabilized. Images at 24, 48, and 96 h were enlarged. Cell aggregation structures were indicated as yellow dot-dash lines. Scale bars were indicated. (B): Cell aggregation structure formation trends were quantified by the cell aggregation in co-culture and mono-culture of HMSCs.
and HUVECs at indicated time points. >3 independent fields were randomly selected for quantification, and data were expressed as mean+SD (C): Representative time lapse snapshots showed gradual formation of the cell aggregation structure from the indicated time intervals in fixed filed. Early cell aggregation trends were indicated as arrow; Cell aggregation structures formation process was indicated as white dot dash lines. Scale bar were indicated.
Transcriptional profiling reveals crosstalk between mesenchymal stem cells and endothelial cells promoting pre-vascularization by reciprocal mechanisms. (doi: 10.1089/scd.2014.0330)
Figure 2. Co-culture dynamically regulated the transcriptome alteration in both MSCs and HUVECs and raised angiogenic gene expression in HUVECs. (A): Schematic of FACS-based cell sorting after co-culture and RNA-seq analysis. Co-HMSC was sorted based on GFP+ and co-HUVEC was sorted based on GFP- and CD31+. Transcriptome alteration was compared between co-cultured MSC/HUVEC and their mono culture counterparts. (B): Statistical analysis of gene differential expression after co-culture for 12 and 24 h separately in HMSCs and HUVECs. (C): Hierarchical clustering of RNA-seq data within genes both significantly altered in 12 and 24 h co-culture for HMSCs and HUVECs. (D): Significant proportion of angiogenic related genes were elevated upon co-culture in HUVECs. Heatmap was ordered via the fold of induction in log2 scale. Significantly induced genes and repressed genes were indicated. (E): Gene ontology (GO) analysis on vascularization and angiogenesis-related process. Correlation of listed relevant process in 12 and 24
hour co-culture in HUVECs was evaluated by P value.
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Figure S3:

Figure 3. NF-κB participated in co-culture induced cell aggregation structure formation. (A, B): NF-kB target genes differential expression in co-HMSCs and co-HUVECs within 12 and 24 h. Heatmap was ordered via the fold of induction in log2 scale. Fold of expression was indicated as Blue tracer in Heatmap. Genes with >2 folds of induction were indicated. (C): Top: Western Blot of p65 (RelA) nucleus translocation in co-HUVECs and mono-HUVECs. GAPDH was used as a loading
control for each group and TNF-α (10 ng/mL) treated HUVEC was used as a positive control; Bottom: quantification of relative nucleus/cytoplasmic ratio of p65 via signal intensity (the ratio in co-culture groups were normalized to the relative ratio in mono-culture control groups, and hereafter). (D): Top: Western Blot of p65 (RelA) nucleus translocation in co-HMSCs and mono-HMSCs after co-culture within 24 h; Bottom: quantification of nucleus/cytoplasmic ratio of p65 via signal intensity. (E): Representative images of PTDC treatment (25–100 μM and 25 μM withdrawal) on the influence of cell aggregation structure formation in co-culture for 24 h. (F) Quantification of cell aggregation structure formation after PTDC treatment and withdrawal.
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Figure 4. HMSCs secreted IL1β and IL6 to regulate cell aggregation structure formation.
potentially through regulating NF-κB in HUVECs. (A, B): Relative mRNA expressions of IL1β and IL6 in HMSCs and HUVECs after co-culture within 24 h. (C, D): Secretion levels of IL1β and IL6 in culture medium after co-culture within 24 h. (E, F): Effect of siRNA targeting IL1β and IL6 on secretion of IL1β and IL6 in culture medium. MSCs and ECs were pretreated with siRNA targeting IL1β, IL6 or negative scramble siRNA as indicated. (G): Effect of neutralizing IL1β, IL6 and both on co-culture induced NF-kB activation in co-HUVECs. (Left): Representative Western Blot analysis of p65 (RelA) nucleus translocation in co-HUVEC at 24 h after treatment of scramble IgG, Anti-IL1β, Anti-IL6 or both; (Right): quantification of nucleus/cytoplasmic ratio of p65 via signal intensity. (H): (Left): Representative images of co-culture induced cell aggregation structure formation at 24 h after neutralizing IL1β, IL6 or both in medium. (Right): cell aggregation structure trends were quantified for each indicated groups.
Stem Cells and Development

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Figure 5. NF-κB dependent cell aggregation structure formation was associated with angiogenic
chemotaxis related genes. (A-D): Relative mRNA expressions of SELP, CCL23, CXCL2 and CXCL3 respectively in HUVECs after co-culture induction within 24 h. (E-H): Effects of PTDC (25 uM) treatment and neutralizing IL1β, IL6 or both on mRNA expression of SELP, CCL23, CXCL2 and CXCL3 in co-culture respectively. (I): Effects of selective and cooperative RNAi of SELP, CCL23, CXCL2 and CXCL3 on the formation of cell aggregation structure. “+” stands for RNAi of indicated genes; “-” for non-specific scramble siRNA control. ≥3 independent experimental fields were randomly selected for quantification, and data were represented as mean±SD. *p<0.05 and **p<0.01 vs. control group.
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Figure S6:

A. SRF

B. MYOC

C. CNN1

D. SM22α

Figure 6. HMSC was endowed with early smooth muscle differentiation trends upon co-culture.
(A, B): Myogenic and osteoblastic differentiation markers were represented as in the heatmaps; heatmaps were ordered via the fold of induction in log2 scale. Master regulatory factors in myogenesis and osteogenesis process were indicated by narrow. (C): Relative mRNA expressions of SRF, MYOCD, CNN1 and SM22a in HMSC after co-culture within 24 h. (D): TGF-β1 and TGF-β3 might regulate myogenic differentiation via mediating MYOCD through RhoA/ROCK pathway. Relative mRNA expressions of MYOCD with different treatments were indicated. (E): Schematic of reciprocal mechanisms in HMSC-HUVEC contact communication mediated pre-vascularization.

**Video S1:** Cell aggregation structure gradually formed within 24 h co-culture. GFP labeled HMSC and non-labeled HUVEC were merged in the video. The video acquisition was started at ~8 h after seeding. Acquisition timing and scale bar were indicated in the video.

**Figure S1:** (A) The innate angiogenic potential of HUVEC used in experiments were tested via Matrigel™ tube formation assay. HUVEC formed the tube structures within 10h to 14h on Matrigel™. (B) Indirect co-culture using Transwell™ or hMSC conditioned medium (CM) did not induce the formation of cell aggregation structures. Representative images for 24h, 48h and 96h of HUVEC culture in CM and Transwell™ were indicated. In Transwell™ experiment, hMSC were cultured in the inserts and HUVEC were cultured in the plate bottom.

**Figure S2:** Bioinformatics analysis pipeline for RNA-seq results.

**Figure S3:** PTDC effectively blocked nucleus translocation of p65 (RelA) in co-HUVEC. Western Blot of p65 nucleus translocation in co-HUVECs after treatment of indicated dose of PTDC. Mono-HUVEC group was used as a blank control group. GAPDH was used as a loading control for each group.

**Figure S4:** Effects of culture models on IL1β (A) and IL6 (B) mRNA2A expression induction.CM: Conditioned medium, HMSC cultured in HUVEC conditioned medium.

**Figure S5:** Potential NF-kB regulatory feedback mechanism in HMSCs on the expression and secretion of IL1β and IL6. Effects of neutralizing of IL1β, IL6 and NF-kB blocking (PTDC) on the mRNA expression level of IL1β (A) and IL6 (B). Effects of NF-kB blocking (PTDC) on the secretion of IL1β (C) and IL6 (D) in culture medium.

**Figure S6:** Effects of culture models on SRF (A), MYOCD (B), CNN1 (C), SM22a (D) mRNA
expression induction.

**Figure S7:** TGF-β1 and TGF-β3 were induced to expression in HMSC and HUVEC upon co-culture. Relative mRNA expressions of TGF-β1 and TGF-β3 in HMSC (A, B) and HUVEC (C, D) after co-culture within 24 h.

**Table S1:** Statistics for RNA-seq raw reads mapping to human reference transcript.

**Table S2:** Complete gene expression profiles for total mapped reads between 12 h co-HMSC and mono-HMSC.

**Table S3:** Complete gene expression profiles for total mapped reads between 24 h co-HMSCs and mono-HMSCs.

**Table S4:** Complete gene expression profiles for total mapped reads between 12h co-HUVEC and mono-HUVECs.

**Table S5:** Complete gene expression profiles for total mapped reads between 24 h co-HUVECs and mono-HUVECs.

**Table S6:** Filtered differential gene expression profiles between 12h co-HMSC and mono-HMSC. (Log2 (Fold change)>1, P <0.01)

**Table S7:** Filtered differential gene expression profiles between 24h co-HMSC and mono-HMSC. (Log2 (Fold change)>1, P <0.01)

**Table S8:** Filtered differential gene expression profiles between 12h co-HUVEC and mono-HUVEC. (Log2 (Fold change)>1, P <0.01)

**Table S9:** Filtered differential gene expression profiles between 24-h co-HUVECs and mono-HUVECs. (Log2 (Fold change)>1, P <0.01)

**Table S10:** Differential expression profiles for angiogenesis-related genes in HMSCs within 12 h and 24 h co-culture.

**Table S11:** Complete GO analysis for differentially-expressed genes in HMSCs within 12 h and 24 h co-culture.

**Table S12:** Complete GO analysis for differentially-expressed genes in HUVECs within 12 h and 24 h co-culture.
Table S13: Differential expression profiles for NF-kB target genes in HUVECs within 12 h and 24 h co-culture.

Table S14: Differential expression profiles for NF-kB target genes in HMSCs within 12 h and 24 h co-culture.

Table S15: Differential expression profiles for myogenesis markers in HMSCs within 12 h and 24 h co-culture.

Table S16: Differential expression profiles for osteogenesis markers in HMSCs within 12 h and 24 h co-culture.

Table S17: qPCR primer sequences used in this article.
Disclosure Statement

All authors declared no conflicts of interest.
Transcriptional profiling reveals cross-talk between mesenchymal stem cells and endothelial cells promoting pre-vascularization by reciprocal mechanisms. (doi: 10.1089/scd.2014.0330)

Figure S7:

![Bar charts showing mRNA relative expression of TGF-β1 and TGF-β3 in HMSC and HUVEC over time (control, 6h, 12h, 24h).](image_url)
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