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ORIGINAL ARTICLE

Cavin-2 Functions as a Suppressive Regulator in TNF-induced Mesenchymal Stromal Cell Inflammation and Angiogenic Phenotypes

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Tumour necrosis factor (TNF)-α activation of mesenchymal stromal cells (MSC) enhances their tumour-suppressive properties and tumour-homing ability. The molecular actors involved are unknown. We found that TNF induced MSC migration and tubulogenesis which correlated with a dose-dependent increase in *Cavin-1* and *Cavin-3* transcript levels. TNF triggered cyclooxygenase (COX)-2 expression, whereas specific siRNA-mediated gene silencing of *Cavin-2* resulted in an amplified COX-2 expression, tubulogenesis, and migratory response partially due to a rapid and sustained increase in NF-κB phosphorylation status. Our results highlight a suppressive role for the caveolar component Cavin-2 in the angiogenic and inflammatory regulation of TNF-activated MSC.

Keywords: Mesenchymal stromal cells, Cavin, TNF, Cancer, Angiogenesis, Inflammation

Introduction

Mesenchymal stromal cells (MSC), most commonly isolated from the bone marrow (1, 2), are a population of pluripotent adult stem cells that have been shown to be recruited within experimental vascularizing tumours (3-5). This incorporation of MSC within a pro-inflammatory tumour architecture, combined with intrinsic immunomodulatory mechanisms, implies that they must also respond to tumour-derived growth factor or cytokine cues (5, 6). The homing of MSC to tumours is therefore believed to be among the earliest phenomena involving MSC-cancer interactions, as was recently reported in a mouse model where injected human MSC preferentially migrated towards implanted human melanoma tumours (3). Subsequent studies have further confirmed MSC homing to tumours and to sites of metastasis (7), and cotransplantation of MSC with melanoma cells in mice was found to enhance tumour engraftment and growth (8). More recently, MSC were reported to migrate to the tumour parenchyma and to differentiate into pericytes, inducing tumour vasculogenesis and promoting tumour recurrence (9). MSC were also found to promote tumour growth both *in vitro* and *in vivo*, suggesting that tumour promotion may be partially attributable to enhanced angiogenesis (10), and through interactions with endothelial cells (11). Although some molecular partners have been identified, most of the signal transducing contributors and the subcellular compartments involved still remain to be determined.

Multiple cytokine-mediated signaling cascades are compartimentalized within caveolae, which represent specialized cholesterol- and sphingolipid-enriched plasma mem-

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brane invaginations, and which, in cancer cells, regulate oncogenic functions (12). Among these functions, roles have been described for caveolae in mechanosensing, vesicular trafficking, and shuttling of molecules through the cell (13, 14). Caveolae have also been ascribed functions in many physiological events (15, 16), and in pathological settings such as cancer progression and metastasis (17). Cancer cell invasion, which involves several cellular processes including disruption of cell adhesion, degradation of the extracellular matrix (ECM) and regulation of cell invasion through blood and lymph vessels, has also been shown, in part, to require caveolae (18). Furthermore, the clustering of signaling molecules within these cholesterol-rich membrane microdomains has already revealed a role for Caveolin-1 in osteogenic differentiation of MSC (19, 20). It is, however, unknown whether alternate caveolar components are involved in the regulation of the angiogenic and inflammation phenotypes of MSC.

Coat proteins called Cavins have recently been suggested not only to work together with Caveolins to regulate the formation of caveolae, but also to have the potential to transmit signals dynamically from caveolae in response to angiogenic or inflammatory cues (21-23). Of the four Cavin proteins, PTRF/Cavin-1 has been shown to mediate reduced production of the pro-inflammatory biomarker MMP-9, independent of caveolar formation, and has been reported to decrease metastatic PC3 prostate cancer cell migration (24). Interestingly, MMP-9 transcriptional regulation is already considered to be triggered by cytokines, such as tumour necrosis factor (TNF)-α, through transcription factors including SP-1, AP-1 and NF-κB (25). Additionally, several studies have pointed out dual roles for other Cavin proteins (26), including Cavin-2 (SDPR, for serum deprivation protein response), Cavin-3 (SRBC, as an Sdr-related gene product that binds to c-kinase), and Cavin-4 (MURC, for muscle restricted coiled-coil protein). Most notably, a decrease in Cavin-2 expression is known to lead to decreased caveolar formation and numbers, as it co-localizes at the plasma membrane along with Caveolin-1 and Cavin-1 (27). Cavin-3 was first identified as a substrate of PKC delta and is located in a tumour suppressor region at chromosome 11p15.5, which is lost in breast, prostate and pancreatic cancer cells (28). Cavin-4 expression is restricted to smooth muscle, such as skeletal and cardiac myocytes (29).

In line with recent evidence which indicated an elevated inflammatory response in Caveolin-1-deficient mice (30), one may envision that caveolae, in fact, exert some repressive functions upon inflammatory cues. In addition, Caveolin-1 KO mice exhibit upregulated transcriptional control of inflammatory cytokines including IL-6 and TNF-α (30). Whether any of the Cavin proteins contribute to such repressive control and could thus impact the TNF-mediated inflammatory or angiogenic phenotype of MSC is unknown. In the current study, we examined which of the Cavin family members could alter the TNF-mediated response in MSC.

Materials and Methods

Materials

Tumour necrosis factor (TNF)-α was from EMD Millipore (Gibbstown, NJ), sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The enhanced chemiluminescence (ECL) reagents were from Perkin Elmer (Waltham, MA). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). The polyclonal antibodies against IκB and phosphorylated IκB were from Cell Signaling (Danvers, MA). The polyclonal anti-Cavin-1 antibody (ABE1953) was from EMD Millipore (Etobicoke, ON), whereas the anti-Cavin-2 and anti-Cavin-3 antibodies were from ABNOVA (Walnut, CA). The monoclonal antibody against GAPDH was from Advanced Immunochemical Inc. (Long Beach, CA). The anti-COX-2 antibody (610203) was from BD Biosciences (San Jose, CA). Horseradish peroxidase-conjugated donkey anti-rabbit and anti-mouse IgG secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). All other reagents were from Sigma-Aldrich Canada.

Cell cultures

Human MSC were obtained from marrow biopsies of volunteers undergoing hip replacement and were isolated by Ficoll gradient. Cells were plated in high glucose Dulbecco's modified Eagle's medium (DMEM; GibcoBRL) supplemented with 10% inactivated fetal bovine serum (iFBS) (Hyclone Laboratories, Logan, UT) and 100 units/ml Penicillin/Streptomycin. After 5～7 days of incubation in a humidified incubator at 37°C with 5% CO_2, the nonadherent hematopoietic cells were discarded. Adherent MSC were further grown on non-coated culture dishes and maintained over 14 passages. Analysis by flow cytometry performed at passage 14 revealed that MSC expressed CD44, yet were negative for CD45, CD31, KDR/flk1 (VEGF-R2), flt-4 (VEGF-R3), and Tie2 (angiopoietin receptor) (data not shown). Serum starvation was performed by culturing the cells in high αMEM, 2 mM

L-glutamine, and 100 units/ml Penicillin/Streptomycin from which the inactivated fetal bovine serum was omitted.

Immunoblotting procedures

Following treatment or transfection, MSC were washed with PBS and lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1% Triton) in the presence of phosphatase and protease inhibitors on ice for 30 minutes. Cell debris was pelleted by centrifugation for 10 min at high speed. Protein concentration was quantified using a micro bicinchoninic acid protein assay kit (Thermo Fisher Scientific Inc). Proteins from control and treated cells were separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes, which were then blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with primary antibodies directed against COX-2 (1/10,000), IκB, phosphorylated IκB (1/1,000), or GAPDH (1/1,500). Washing was then performed in TBST, followed by a 1 hour incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/10,000) or anti-mouse IgG (1/5,000) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by Western Lightning Enhanced Chemiluminescence Pro reagents (Perkin Elmer).

Real-time cell migration assay

Cell migration assay experiments were carried out using the Real-Time Cell Analyzer (RTCA) Dual-Plate (DP) Instrument of the xCELLigence system (Roche Diagnostics). MSC were trypsinized and 20,000 cells/well were seeded onto CIM-Plates 16 (Roche Diagnostics). These plates are similar to conventional Transwells (8 μm pore size) but with gold electrode arrays on the bottom side of the membrane to provide a real-time measurement of cell migration. Prior to cell seeding, the underside of the wells from the upper chamber was coated with 25 μL of 0.15% gelatin in PBS and incubated for 1 hour at 37°C. TNF was dissolved in serum-free culture medium and added to the upper wells, while the lower chamber was filled with 10% Fetal Calf Serum medium to act as a chemoattractant. Cell migration was monitored for 8 hours. The impedance values were measured by the RTCA DP Instrument software and were expressed in arbitrary units as the Relative Cell Migration Index. Each experiment was performed in triplicate.

Total RNA isolation, cDNA synthesis and real-time quantitative RT-PCR

Total RNA was extracted from MSC monolayers using TRIzol reagent (Life Technologies, Gaithersburg, MD). For cDNA synthesis, 1 μg of total RNA was reverse-transcribed into cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was stored at −80°C prior to PCR. Gene expression was quantified by real-time quantitative PCR using iQ SYBR Green Supermix (BIO-RAD, Hercules, CA). DNA amplification was carried out using an Icycler iQ5 (BIO-RAD, Hercules, CA) and product detection was performed by measuring binding of the fluorescent dye SYBR Green I to double-stranded DNA. The following primer sets were provided by QIAGEN (Valencia, CA): Cavin-1 (Hs_CAV1_1_SG, QT00001533), Cavin-2 (Hs_CAV2_1_SG, QT00068754), Cavin-3 (Hs_CAV3_1_SG, QT00001414), β-Actin (Hs_ACTB_2_SG, QT01680476), GAPDH (Hs_GAPDH_1_SG, QT00079247), PPIA (Hs_PPIA_4_SG, QT01866137). The relative quantities of target gene mRNA against an internal control, β-Actin/GAPDH/PPIA RNA, were measured by following a ΔC_T method employing an amplification plot (fluorescence signal *vs.* cycle number). The difference (ΔC_T) between the mean values in the triplicate samples of target gene and those of β-Actin/GAPDH/PPIA RNA were calculated by iQ5 Optical System Software version 2.0 (BIO-RAD, Hercules, CA) and the relative quantified value (RQV) was expressed as $2^{-\Delta C_T}$. Semi-quantitative PCR was performed to examine amplification products and amplicons resolved on 1.8% agarose gels containing 1 μg/ml ethidium bromide.

Transfection method and RNA interference

Subconfluent (30～50%) MSC were transiently transfected for 24 hours in serum-free media and with 20 nM siRNA against Cavin-1 (human Hs_CAV1_7 FlexiTube siRNA, SI02662338), Cavin-2 (human Hs_CAV2_6 HP siRNA, SI03648841), Cavin-3 (HS_CAV3_5 FlexiTube siRNA, SI02223221), or scrambled sequences (AllStar Negative Control siRNA, 1027281) using Lipofectamine 2000 transfection reagent (Invitrogen, CA). Small interfering RNA and mismatch siRNA were synthesized by QIAGEN and annealed to form duplexes.

Capillary-like structure formation assay

Induction of tubulogenesis was performed using Matrigel. Matrigel was thawed on ice to prevent premature polymerization; aliquots of 50 μl were plated into individual wells of 96-well tissue culture plates (Costar) and allowed to polymerize at 37°C for at least 60 minutes. Cells were re-

moved from confluent cultures by treatment with 0.05% trypsin, 0.53 mM EDTA. The cells were washed in serum-containing medium then resuspended to 10^6 cells/ml. Each culture well received 100-μl of the cell suspension with or without 100 μM TNF. Each control or TNF treatment was assayed in duplicate, and all experiments were performed at least three times. For quantitation of tube formation, the total length of the tubes formed in a unit area was digitized and measured using ECLIPSE software. For each test, five randomly chosen areas were measured and averaged.

Statistical data analysis

Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t*-test. Probability values of less than 0.05 were considered significant and an asterisk (*) identifies such significance in the figures.

Results

TNF triggers a global MSC angiogenic phenotype by inducing cell migration and tubulogenesis

We first assessed the effect of TNF treatment on MSC's angiogenic phenotype using both a real-time cell migration assay as well as measuring the cells ability to generate a three-dimentional capillary-like structure formation (tubulogenesis) on Matrigel. We found that the cell migration response to TNF was increased in a dose-dependent manner (Fig. 1A). When data were compared after 8 hours, cell migration tended to plateau between 30～100 μM TNF (Fig. 1B) and it was decided to maintain the TNF at 100 μM for all subsequent experiments in order to correlate the molecular signatures associated with an optimal TNF-mediated cellular effect. We have previously observed that MSC possess intrinsic angiogenic properties since they can generate capillary-like structures *in vitro*, and that this process was regulated at the molecular level in part through a specific membrane-bound MMP, namely membrane-type (MT)1-MMP and by sphingosine-1-phosphate, which are both involved in tumour angiogenesis (31, 32). Increasing quantities of MSC were therefore seeded onto Matrigel and tubulogenesis was assessed within 6 hours as described in the Methods section. We found that significant tubulogenesis was observed when 40,000～80,000 cells were seeded (Fig. 1C), reached a plateau at 100,000 cells, and that TNF augmented this process (Fig. 1D). The potential interrelationship between the MSC angiogenic phenotype and Cavin expression was next assessed.

TNF triggers Cavin-1 and Cavin-3 transcription, while Cavin-2 transcripts are decreased

Cavin gene and Cavin protein expression profiling was next performed using respectively total RNA or total cell lysates isolated from control and TNF-treated MSC. We found that each of the *Cavin-1*, *Cavin-2*, and *Cavin-3* transcripts were expressed in MSC because single specific amplicons were generated upon RT-qPCR and visualized on an agarose gel (Fig. 2A). *Cavin-1* and *Cavin-3* transcript levels were dose-dependently increased in response to TNF, whereas that of *Cavin-2* decreased upon TNF treatment (Fig. 2B). Cavin-1, -2, and -3 protein levels were assessed by Western blotting (Fig. 2C) and confirmed TNF-mediated increases in Cavin-1 and -3, whereas Cavin-2 (Fig. 2C, arrow) levels decreased (Fig. 2D). Altogether, our data confirm that TNF triggers an angiogenic phenotype in MSC by increasing cell migration and tubulogenesis (Fig. 1), and that it is able to differentially modulate *Cavin* expression in MSC. We next sought to investigate the potential impact of Cavins on the TNF response.

Silencing of Cavin-2, but not Cavin-1 or -3, potentiates TNF-mediated COX-2 expression

MSC were next transiently transfected with siRNA in order to silence *Cavin-1*, *-2*, or *-3* gene expression. Gene silencing specificity was validated by qRT-PCR, and found to effectively reduce gene expression between 75～90% (Fig. 3A). When cyclooxygenase (COX)-2, a pro-inflammatory biomarker known to be induced by TNF, expression was assessed, we found that neither *Cavin-1* nor *Cavin-3* gene silencing affected TNF-induced COX-2 expression (Fig. 3B). In contrast, silencing *Cavin-2* was found to potentiate TNF-mediated COX-2 induction (Fig. 3B). Our observations suggest that Cavin-2 exerts a pivotal role in TNF-mediated signalling in MSC as its expression represses basal TNF signalling.

Silencing of Cavin-2, but not Cavin-1 or -3, potentiates TNF-mediated MSC migration and tubulogenesis

When MSC migration was asessed, none of the three *Cavin* gene silencings altered basal migration (Fig. 4A, open circles). TNF-mediated MSC migration was induced in siScrambled, siCavin-1 and in siCavin-3 transfected cells, although to a lesser extent than in untransfected cells, due to transfection conditions, whereas it was potentiated in the MSC where *Cavin-2* gene expression was reduced (Fig. 4A, closed circles). Given that TNF is able to increase MSC migration and that Cavin-2 regulates this

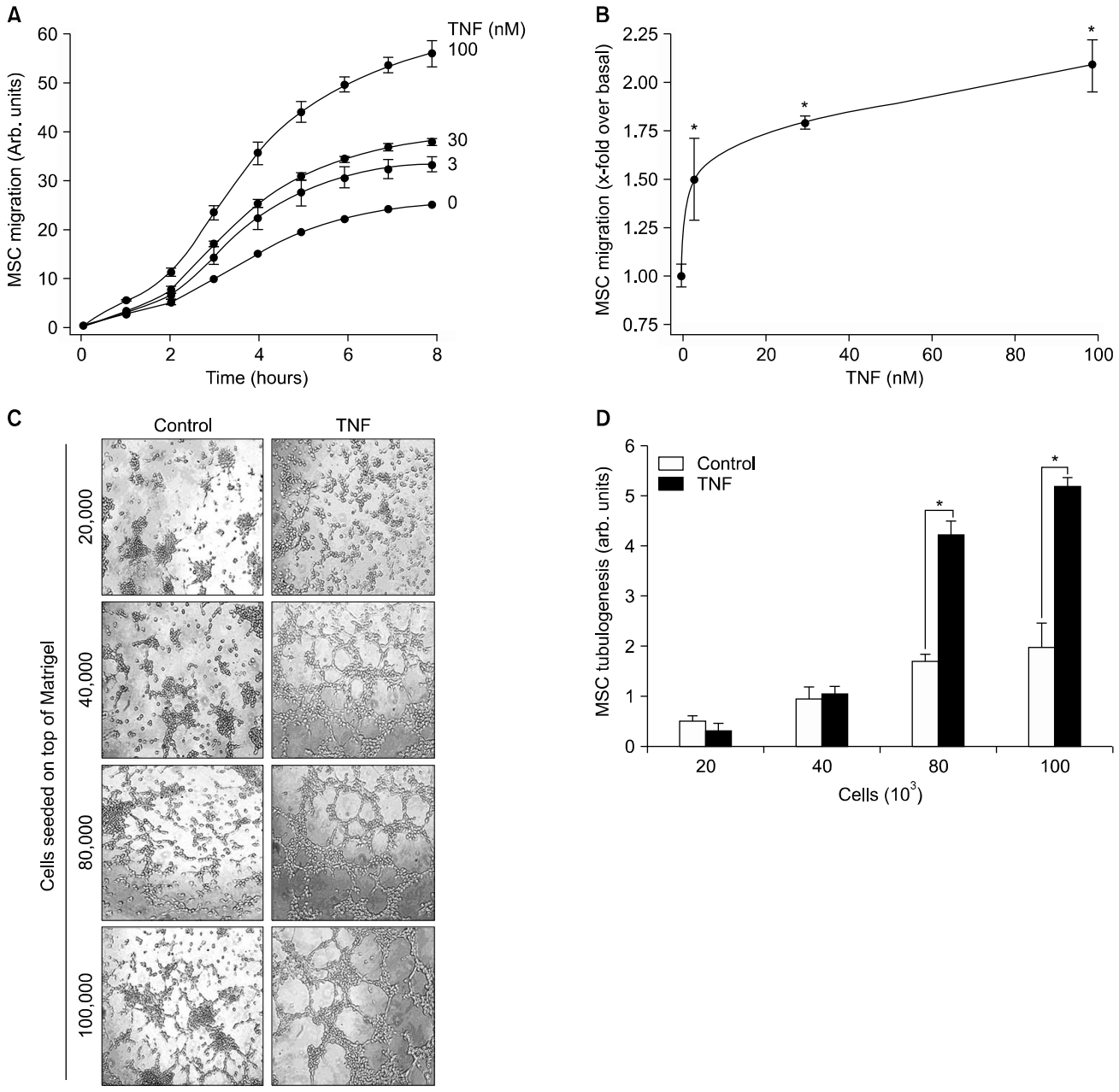

Fig. 1. TNF triggers a global MSC angiogenic phenotype by inducing cell migration and tubulogenesis. (A) MSC were harvested and the rate of cell migration monitored in real-time in response to various TNF concentrations using the xCELLigence system. Representative plots from two experiments measuring impedance responses are shown for each condition. (B) Plot of cell migration at time=8 hours is shown in response to each TNF concentration used. (C) Various quantities of MSC were seeded on top of Matrigel and tubulogenesis was allowed to proceed for 6 hours as described in the Methods section. (D) Quantification of the 3D-capillary-like structures was performed as described in the Methods section for control (open box) and 100 nM TNF-treated cells (closed box). Data is from a representative experiment, and each point is the mean of 5 randomly chosen areas.

response, we next examined whether tubulogenesis was also altered. When *Cavin-2* gene expression was silenced and tubulogenesis measured at a sub-saturating cell density (40,000 cells), we found that TNF, similar to its effect on cell migration and COX-2 expression, showed greater amplification of MSC tube formation as compared to control (siScrambled) cells (Fig. 4B).

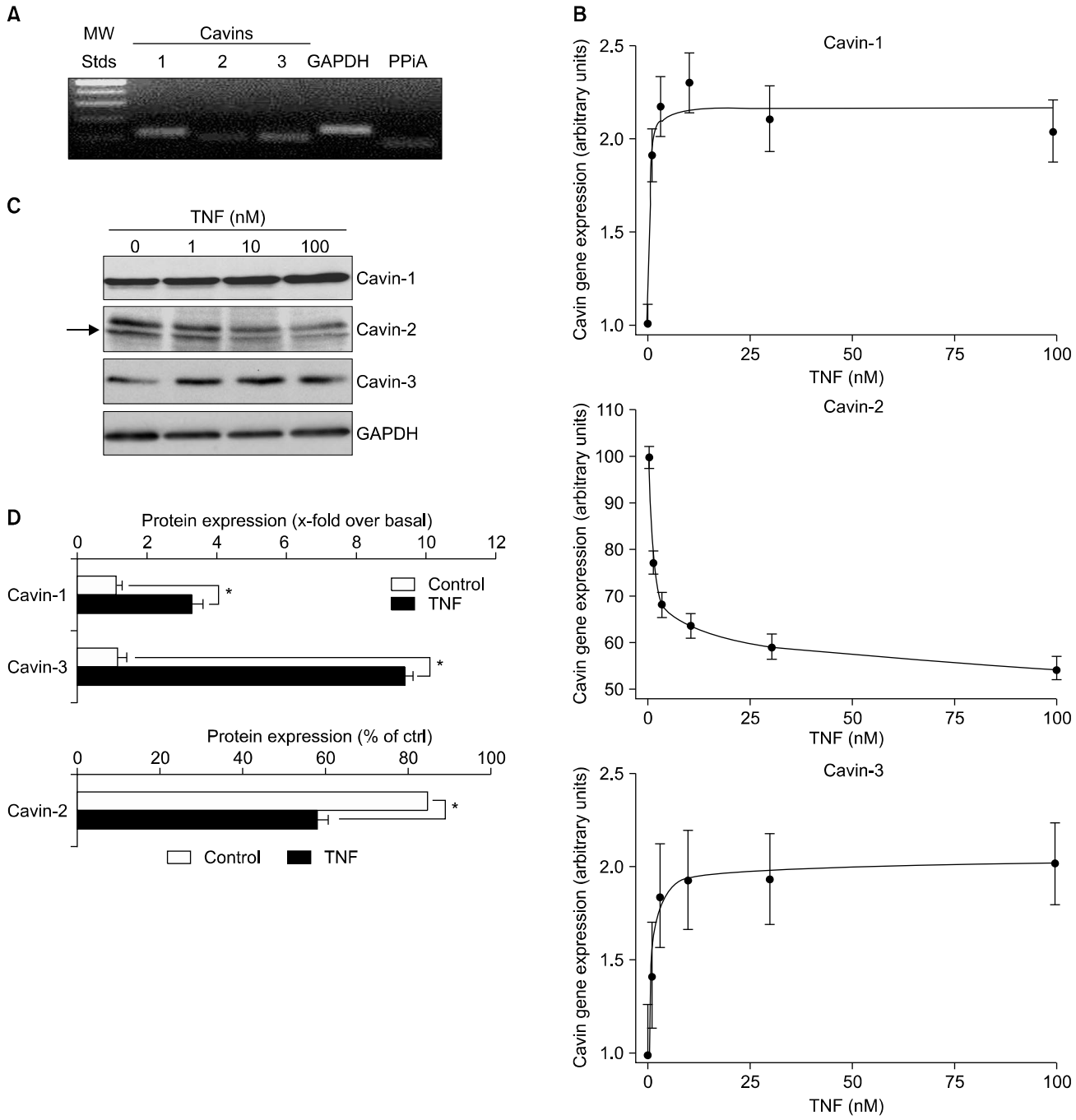

Fig. 2. TNF triggers *Cavin-1* and *Cavin-3* transcription, while *Cavin-2* transcripts are decreased. Total RNA was extracted from MSC and RT-qPCR performed as described in the Methods section. (A) Amplicons of Cavins were migrated on agarose gels and each showed a single product. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PPIA, peptidyl prolylisomerase A. (B) *Cavin-1*, *Cavin-2*, and *Cavin-3* gene expression and (C) protein expression were assessed in serum-starved MSC following 24 hours treatment with the indicated TNF concentrations. (D) Protein levels were assessed by scanning densitometry in vehicle (white bars) and 100 nM TNF-treated (black bars) MSC.

TNF-mediated NF-κB signaling occurs rapidely and is sustained upon Cavin-2 gene silencing

In order to investigate the possible signal transducing mechanisms responsible for increased TNF-induced MSC migration, tubulogenesis and COX-2 expression upon *Cavin-2* silencing, we decided to examine the phosphorylation status of IκB and its subsequent proteasomal-mediated degradation. MSC were transiently-transfected with

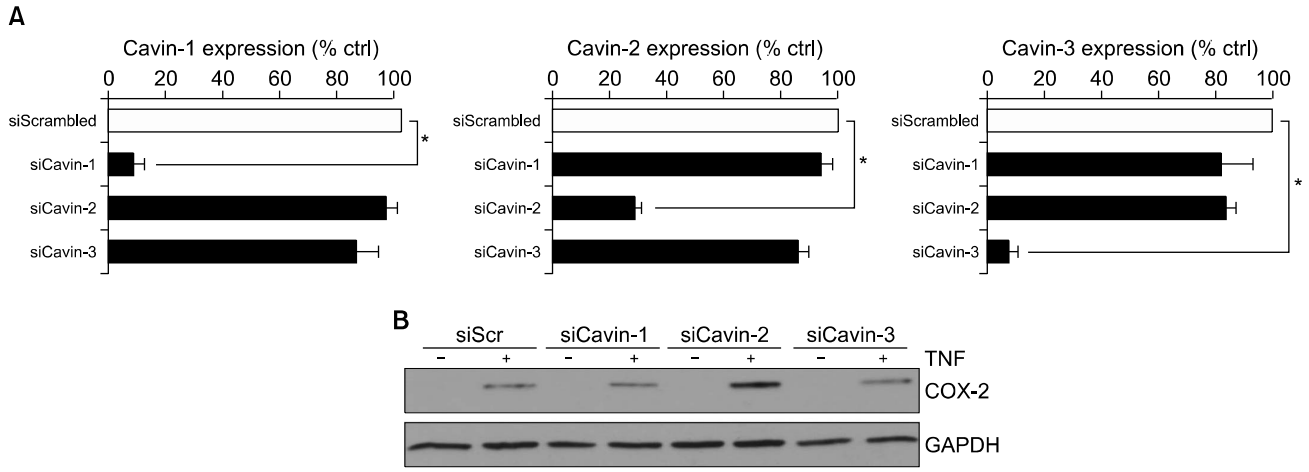

Fig. 3. Silencing of *Cavin-2*, but not *Cavin-1* or *-3*, potentiates TNF-mediated COX-2 expression. MSC were transiantly transfected with specific siRNA against each of the *Cavin-1*, *-2*, or *-3* genes as described in the Methods section. (A) Total RNA was extracted and *Cavin* gene silencing efficacy and specificity were assessed by qRT-PCR. (B) MSC were treated with 100 nM TNF for 24 hours and COX-2 expression was immunodetected in lysates as described in the Methods section. GAPDH expression served as a loading control.

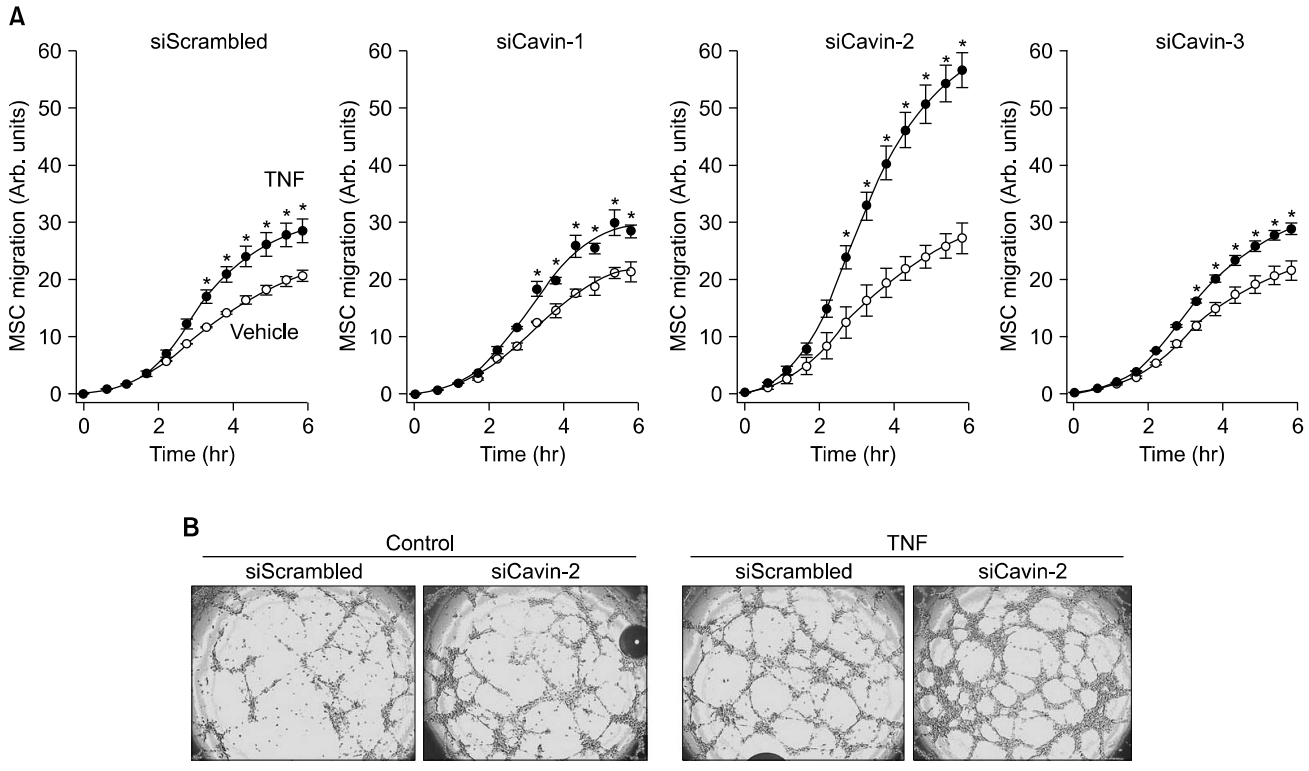

Fig. 4. Silencing of *Cavin-2*, but not *Cavin-1* or *-3*, potentiates TNF-mediated MSC migration and tubulogenesis. MSC were transiently transfected with specific siRNA against each of the *Cavin-1*, *-2*, or *-3* genes as described in the Methods section. (A) Transfected-MSC were harvested and cell migration in response to vehicle (open circles) or to 100 nM TNF (closed circles) was assessed as described in the Methods section. (B) Transient gene silencing was performed in control (siScrambled) and in siCavin-2-transfected MSC as described in the Methods section. Cells were then left to recuperate, trypsinized and 40,000 cells were seeded on top of Matrigel. A tubulogenesis assay was performed as described in the Methods section and images were taken at 6 hours.

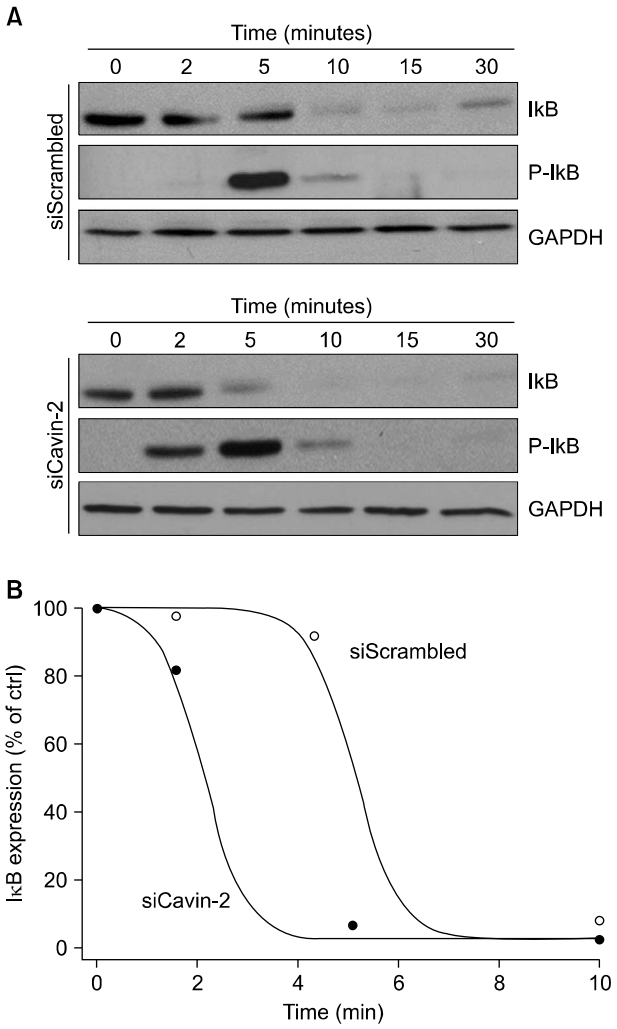

Fig. 5. TNF-mediated NF-κB signaling is sustained upon *Cavin-2* gene silencing. MSC were transiently transfected with a scrambled siRNA sequence, or with a specific siRNA against Cavin-2 (siCavin-2). Cells were then treated with 100 μM TNF for the indicated time and (A) IκB, phosphorylated IκB, and GAPDH expression were immunodetected in lysates, as described in the Methods section, and (B) quantified by scanning densitometry.

siScrambled or siCavin-2, then treated with TNF and lysates were harvested at the indicated times. We found that TNF effectively triggered IκB phosphorylation at 5 minutes, and that this subsequently led to IκB degradation as expected (Fig. 5A, upper panels). In contrast, *Cavin-2* silencing resulted in a more rapid (<5 min) and sustained IκB phosphorylation (Fig. 5A, lower panels) which, in turn, also led to a rapid decrease in IκB (Fig. 5B). This data suggests that increased translocation to the nucleus of the freed p50 and p60 NF-κB subunits may lead to sustained and/or increased COX-2 transcription and protein expression, as well as to increased MSC migration.

Discussion

Assessing the *in vitro* migration and tubulogenesis of MSC generally requires strategies to prime the cells, such as in response to serum-derived growth factors or to specific cytokines (33). Given the lack of *in vitro* inflammatory-like microenvironment components or extracellular growth factor-mediated cues, treatment with TNF-α was used here to mimic and trigger a pro-inflammatory response. While TNF signal transducing events are, in part, believed to take place through caveolar components (34), neither the contribution of Cavins nor the balance in their stimulatory/repressive functions in inflammation and angiogenesis had yet been assessed in MSC.

Our current study specifically provides new insight into the inflammatory-suppressive functions of Cavin-2. While *Cavin-1* and *Cavin-3* expression are dose-dependently induced by TNF, their individual silencing did not alter TNF-induced MSC migration nor COX-2 expression. In agreement with this, Cavin-1 and Cavin-3 expression were also induced by TNF in airway smooth muscle cells (35). In contrast, we found that only endogenous Cavin-2 expression is decreased upon TNF treatment, while its silencing led to amplified TNF-mediated MSC migration, tubulogenesis, and COX-2 expression. Silencing of *Cavin-2* expression was, in fact, shown to alter the NF-κB pathway, partially explaining the TNF-α effects. Collectively, we conclude that Cavin-2 exerts a suppressive role against the pro-inflammatory action of TNF since its silencing leads to increased TNF effects. Cavin-2 affects the formation of large caveolae, and accumulating evidence suggests that it is present in many cellular types; however, its clinical relevance remains debated. Whether Cavin-2 alters MSC tumour-homing ability *in vivo* remains unexplored, but opens up the possibility of investigating and possibly optimizing genetically-modified MSC-mediated targeted delivery of anti-cancer genes to tumours. Whether any regulation is exerted by Caveolin-1 on the repressive functions of Cavin-2 also remains to be investigated. Future studies will aim at defining whether Cavin-2 functions are caveolae-dependent or independent.

Cavin-2 was recently suggested to regulate Caveolin-1 expression, leading to slow oral squamous cell carcinoma (OSCC) proliferation by inactivation of the extracellular regulated kinase (ERK) pathway (36). Cavin-2 may therefore be a possible key regulator of OSCC progression via a Cavin-2/Caveolin-1/ERK pathway and a potential therapeutic target for developing new treatments for OSCCs. Further investigations on the roles of Cavin-2 will be required in inflammatory and angiogenic settings. Metabolic

effects may also be expected upon modulation of Cavin-2 expression. Indeed, hypoxia was recently shown to inhibit Cavin-2 expression in adipocytes and to alter caveolar formation (37). This led to the inhibition of insulin signaling and the establishment of insulin resistance. Interestingly, downregulation of Cavin-2 upon hypoxia (37) correlates with increased hypoxic MSC endogenous tubulogenesis (31). The combined hypoxic and TNF-mediated inflammatory cues, which both led to decreased Cavin-2 expression, again reinforces the fact that Cavin-2 exerts a repressive control upon the angiogenic responses of a given cell model and within conditions approximating those found within the solid tumour microenvironment. While Cavin-1 expression is thought to be induced by TNF in MSC (our study), its regulation by hypoxia has yet to be assessed. On the other hand, Cavin-2 expression was found decreased upon hypoxia in 3T3 L1 adipocytes, that of Cavin-1 was also found decreased (37). How Cavin-1 is regulated in MSC upon hypoxia, and how this controls 3D-tubulogenesis is currently unknown. Although speculative, one can only safely envision at this point of our knowledge specific contributions of all Cavins in response to inflammatory and hypoxic cues which are expected to vary from a cell model to another. Further studies on each of Cavins' promoter sequences will be needed and will allow us to understand their transcriptional control.

In the current study, Cavin-1 and Cavin-3 were upregulated in MSC upon TNF treatment, suggesting that this positively correlates with the increased angiogenic phenotype of MSC upon pro-inflammatory stimulation. Interestingly, *Cavin-3* transcript levels were also found to be induced by an alternate pro-inflammatory agent such as the phorbol ester PMA, and to regulate the expression of the biomarker MMP-9 in a human fibrosarcoma cell line (38). In that model, PMA was used to prime cell migration (39), and to increase secretion of MMP-9 (38), the secretion of which is generally correlated with inflammation, pro-angiogenic and metastatic processes (40). Such observations regarding the differential involvement of Cavins upon pro-inflammatory cues, whether these are triggered through receptor-mediated processes (TNF/TNFR) or through receptor-independent processes (PMA), suggest that pro-inflammatory and pro-angiogenic conditions can be effectively regulated by caveolar components. Through its capacity to repress TNF-mediated signaling, we have shown here the ability of Cavin-2 to regulate MSC migration and tubulogenesis. Given that MMP-9 was not inducible by TNF in MSC (not shown), the involvement of Cavins in MMP-9 regulation in that model remains to be established.

In summary, our study provides a better understanding of roles for the Cavin family members in regulating the angiogenic and inflammatory phenotype of MSC. Given the recognized contribution of MSC within (patho)physiological settings is currently of great interest towards either their therapeutic applications or as intermediates in solid tumour development, the dual roles of Caveolins and of Cavins must be better documented. Our study defines Cavin-2 as a putative suppressor regulator in TNF-mediated pro-inflammatory and pro-angiogenic conditions, and supports the recent evidence found in OSCC where its down-regulation was functionally and clinically linked to tumoural progression (36).

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Potential conflict of interest

The authors have no conflicting financial interest.

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