Gene Regulation:

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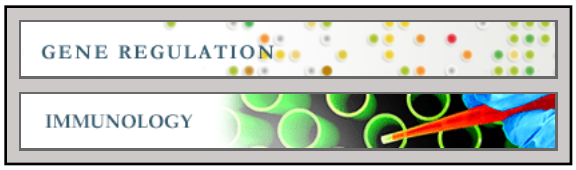

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Epigallocatechin Gallate Targeting of Membrane Type 1 Matrix Metalloproteinase-mediated Src and Janus Kinase/Signal Transducers and Activators of Transcription 3 Signaling Inhibits Transcription of Colony-stimulating Factors 2 and 3 in Mesenchymal Stromal Cells*

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Background: CSF-2 and CSF-3 confer proangiogenic and immunomodulatory properties to mesenchymal stromal cells (MSCs).
Results: Transcriptional regulation of CSF-2 and CSF-3 in concanavalin A-activated MSCs requires MT1-MMP signaling and is inhibited by EGCG.
Conclusion: The chemopreventive properties of diet-derived EGCG alter MT1-MMP-mediated intracellular signaling.
Significance: Pharmacological targeting of MSCs proangiogenic functions may prevent their contribution to tumor development.

Epigallocatechin gallate (EGCG), a major form of tea catechins, possesses immunomodulatory and antiangiogenic effects, both of which contribute to its chemopreventive properties. In this study, we evaluated the impact of EGCG treatment on the expression of colony-stimulating factors (CSF) secreted from human bone marrow-derived mesenchymal stromal cells (MSCs), all of which also contribute to the immunomodulatory and angiogenic properties of these cells. MSCs were activated with concanavalin A (ConA), a Toll-like receptor (TLR)-2 and TLR-6 agonist as well as a membrane type 1 matrix metalloproteinase (MT1-MMP) inducer, which increased granulocyte macrophage-CSF (GM-CSF, CSF-2), granulocyte CSF (G-CSF, CSF-3), and MT1-MMP gene expression. EGCG antagonized the ConA-induced CSF-2 and CSF-3 gene expression, and this process required an MT1-MMP-mediated sequential activation of the Src and JAK/STAT pathways. Gene silencing of MT1-MMP expression further demonstrated its requirement in the phosphorylation of Src and STAT3, whereas overexpression of a nonphosphorylatable MT1-MMP mutant (Y573F) abrogated CSF-2 and CSF-3 transcriptional increases. Given that MSCs are recruited within vascularizing tumors and are believed to contribute to tumor angiogenesis, possibly through secretion of CSF-2 and CSF-3, our study suggests that diet-derived polyphenols such as EGCG may exert chemopreventive action through pharmacological targeting of the MT1-MMP intracellular signaling.

Increasing evidence supports trophic activities for mesenchymal stromal cells (MSCs),[2] which employ their immunomodulatory functions and paracrine contribution toward the formation of vasculature *in vivo* (1, 2). In fact, proangiogenic properties have been documented for MSCs isolated from murine tissues, including bone marrow, white adipose tissue, skeletal muscle, and myocardium (3), whereas recent advances in our understanding of the MSC paracrine contribution to tumor-derived angiogenesis also imply that systemically infused MSCs must respond to serum-derived cues that direct their ultimate biodistribution within hypoxic tumors (4–6). The impact of secreted MSC-derived growth factors or cytokines responsible for paracrine regulation of angiogenesis remains, however, not fully understood.

All MSCs secrete multiple proangiogenic factors in culture, including members of the vascular endothelial growth factor (VEGF) and angiopoietin families (7). More recently, the angiogenic activity of classical hematopoietic cytokines was found to affect certain endothelial cell functions, and hematopoietic factors have been clearly demonstrated to influence angiogenesis (8). Furthermore, specific receptors for granulocyte colony-stimulating factor (CSF-2, G-CSF) and for granulocyte macrophage colony-stimulating factor (CSF-3, GM-CSF) have been detected on the surface of endothelial cells (9, 10). Accordingly, subnanomolar concentrations of CSF-2 and of CSF-3 have been shown to induce the proliferation of endothelial cells derived from human vessels (9, 11). Currently nothing is known about CSF-2 and CSF-3 expression and transcriptional regulation in MSCs.

Recently, we highlighted functional cross-talk between the membrane type 1 MMP (MT1-MMP) and JAK/STAT-medi-

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[2] The abbreviations used are: MSC, mesenchymal stromal cell; ConA, concanavalin A; CSF, colony-stimulating factor; EGCG, epigallocatechin gallate; MT1-MMP, membrane type 1 matrix metalloproteinase; qPCR, quantitative PCR; TLR, toll-like receptor.

ated signaling in concanavalin A (ConA)-activated MSCs that could regulate the expression of the inflammation biomarker cyclooxygenase-2 (12). Interestingly, aside from its canonical role in extracellular matrix proteolysis, MT1-MMP is also involved in transducing crucial intracellular signaling that may control several processes related to MSC mobilization and cell survival (13–15). In this study, we demonstrate that, as a consequence of ConA activation, sequential Src kinase and JAK/STAT3 signaling is required to up-regulate CSF-2 and CSF-3 transcription and that this necessitates the contribution of MT1-MMP transduction through the crucial involvement of a phosphorylatable Tyr^{573} residue located within its 20-amino acid cytoplasmic domain. Finally, we demonstrate that the anti-angiogenic, green tea-derived polyphenol (−)-epigallocatechin gallate (EGCG) is able to abrogate both ConA- and MT1-MMP-induced CSF-2 and CSF-3 transcription, which effects add to the EGCG pleiotropic chemopreventive properties.

EXPERIMENTAL PROCEDURES

Materials—Sodium dodecyl sulfate (SDS), EGCG, and bovine serum albumin (BSA) were purchased from Sigma. Cell culture media were obtained from Invitrogen. Electrophoresis reagents were purchased from Bio-Rad. The enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences. Microbicinchoninic acid protein assay reagents were from Pierce. The JAK family tyrosine kinase inhibitor tofacitinib (CP-690550) was from Cederlane (Burlington, ON), and AG490 was from Calbiochem. The anti-STAT3 (79D7), anti-phospho-STAT3 (Tyr^{705}), anti-Src, and anti-phospho-Src polyclonal antibodies were from Cell Signaling Technology. The polyclonal antibody against the MT1-MMP catalytic domain was from Millipore.

Cell Cultures—Human MSCs, obtained from marrow biopsies of volunteers undergoing hip replacement, were isolated by Ficoll gradient and plastic adherence and expanded in αMEM (Invitrogen) with 16.5% inactivated fetal bovine serum (iFBS) (Hyclone Laboratories). Serum starvation is classically performed by culturing the cells in high αMEM-containing 2 mM L-glutamine and 100 units/ml penicillin/streptomycin from which iFBS was omitted. Cell death was quantified using the NucleoCounter device from ChemoMetec (Mandel Scientific Company, Guelph, ON).

Immunoblotting Procedures—MSCs were lysed in a buffer containing 1 mM NaF and Na_3VO_4 (14), and proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes, and immunoreactive material was visualized by enhanced chemiluminescence as described previously (16).

Total RNA Isolation, cDNA Synthesis, and Real-time Quantitative RT-PCR—Total RNA was extracted from MSC monolayers using TRIzol reagent (Invitrogen). For cDNA synthesis, 1 μg of total RNA was reverse-transcribed into cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). cDNA was stored at −80 °C prior to PCR. Gene expression was quantified by real-time qPCR using iQ SYBR Green Supermix (Bio-Rad). DNA amplification was carried out using an Icycler iQ5 (Bio-Rad), 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: MT1-MMP (Hs_MMP14_1_SG, QT00001533), β-actin (Hs_ACTB_2_SG, QT01680476), GAPDH (Hs_GAPDH_1_SG, QT00079247), PPIA (Hs_PPIA_4_SG, QT01866137), STAT3 (Hs_STAT3_1_SG, QT00068754), CSF-1 (Hs_CSF1_1_SG, QT00035224), CSF-2 (Hs_CSF2_1_SG, QT00000896), CSF-3 (Hs_CSF3_1_SG, QT00001414), TLR-2 (Hs_TLR2_1_SG, QT00236131), and TLR-6 (Hs_TLR6_1_SG, QT00216272). The relative quantities of target gene mRNA against an internal control, β-actin/GAPDH/PPIA RNA, were measured by following a ΔCT method employing an amplification plot (fluorescence signal *versus* cycle number). The difference (ΔCT) 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), and the relative quantified value was expressed as 2-ΔCT. Semiquantitative 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—MSCs were transiently transfected with 20 nM siRNA against Src kinase (Src) (human Hs_src_10 Flexitube siRNA, SI02664151), MT1-MMP (human Hs_MMP14_6 HP siRNA, SI03648841), TLR-2 (human Hs_TLR2_1 Flexitube siRNA, SI00050015), TLR-6 (human Hs_TLR6_2 siRNA, SI00084168), or scrambled sequences (AllStar Negative Control siRNA, 1027281) using Lipofectamine 2000 transfection reagent (Invitrogen). Small interfering RNA and mismatch siRNA were synthesized by Qiagen and annealed to form duplexes. MSCs were transiently transfected with cDNA constructs encoding full-length MT1-MMP (15) or a nonphosphorylatable MT1-MMP mutant Y573F (17).

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 <0.05 were considered significant, and an asterisk identifies such significance in the figures.

RESULTS

ConA Transcriptional Regulation of CSF-1, CSF-2, and CSF-3 Gene Expression—We first assessed whether the TLR-2, TLR-6, CSF-1, CSF-2, and CSF-3 transcripts were present in MSCs. Total RNA was extracted from untreated MSCs, and cDNA was synthesized as described under "Experimental Procedures." qPCR was then performed, and end of cycle products loaded onto an agarose gel showed that single amplicons with the expected sizes were produced (Fig. 1*A*). When serum-starved MSCs were treated with various ConA concentrations for 18 h, we found that CSF-1 gene expression was only moderately induced, whereas those of CSF-2 and CSF-3 were significantly up-regulated with CSF-2 reaching a transient peak at 3 μg/ml ConA and CSF-3 reaching a maximal stimulation at 30 μg/ml ConA (Fig. 1*B*). In agreement with previously reported data (18), a slight decrease in MSC viability was also monitored at that maximal ConA concentration (Fig. 1*B*, *dashed lines*). Unless otherwise stated, all subsequent experiments were

therefore performed at either 3 μg/ml ConA for CSF-2 or 30 μg/ml ConA for CSF-1 and CSF-3.

TLR-2 and TLR-6 Gene Silencing Abrogates ConA-induced CSF-2 and CSF-3 Transcriptional Regulation—Given that MSCs express Toll-like receptors (TLRs) and that ConA is a TLR-2/6 agonist, we next assessed their respective impact as intermediates in ConA-mediated CSF transcriptional regulation. Efficient TLR-2 and TLR-6 gene silencing was achieved (Fig. 2*A*), and abrogated the ability of ConA to trigger CSF-2 and CSF-3 expression (Fig. 2*B*). Our data therefore suggest that either TLR-2 or TLR-6 independently from each other possesses the ability to transduce ConA effects.

EGCG Inhibits ConA-induced CSF-2, CSF-3, and MT1-MMP Gene Expression—Green tea polyphenol EGCG was next tested to evaluate its capacity to alter ConA-induced CSF transcription. MSCs were serum-starved and treated with ConA, and then total RNA was isolated. Gene expression levels were assessed by qPCR, and we observed that CSF-2 and CSF-3 (Fig. 3, *left panel*), as well as MT1-MMP (Fig. 3, *right panel*), were strongly down-regulated by EGCG with calculated IC_{50} ranging from 1.9 to 2.3 μM for CSF-2 and CSF-3. Moderate decreases in CSF-1 (~42%) and in STAT3 (~21%) gene expressions were observed in ConA-treated cells. EGCG alone up to 30 μM did

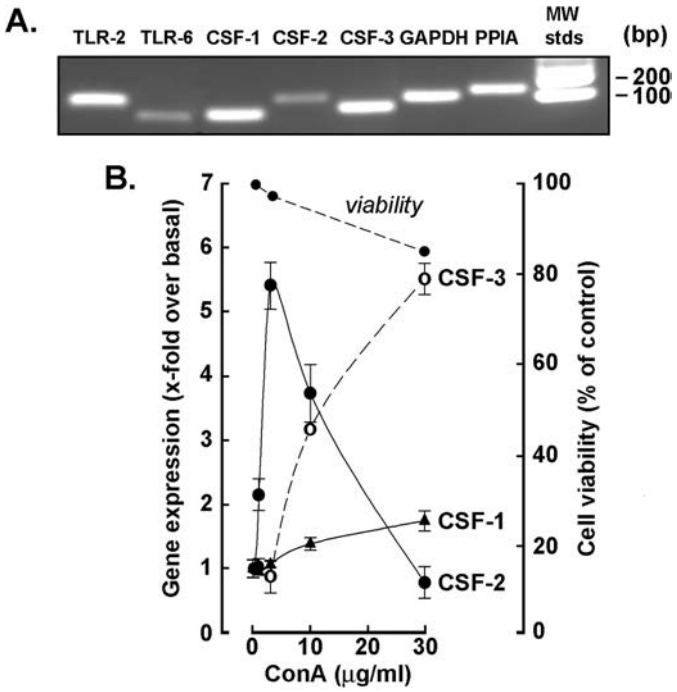

FIGURE 1. **ConA transcriptional regulation of CSF-1, CSF-2, and CSF-3 gene expression.** Subconfluent MSCs were serum-starved and treated with various concentrations of ConA for 24 h. Total RNA was extracted, and quantitative RT-PCR was used to assess gene expression. *A*, representative agarose gel of the corresponding single product amplicons. *B*, representative qPCR profile, of three independent experiments, for TLR-2, TLR-6, CSF-1, CSF-2, and CFS-3 genes. Data represent mean values ± S.D. (*error bars*) from triplicates. Typical cell viability readout was assessed as described under "Experimental Procedures" and is represented by *dashed lines*.

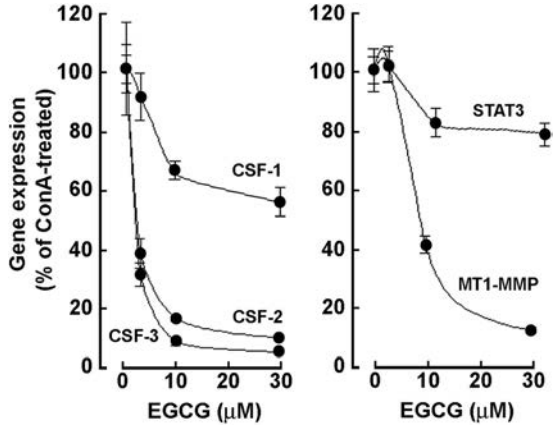

FIGURE 3. **EGCG inhibits ConA-induced CSF-2, CSF-3, and MT1-MMP gene expression.** Subconfluent MSCs were serum-starved and treated with ConA at either 3 μg/ml (CSF-2) or 30 μg/ml (CSF-1, CSF-3) for 24 h in the presence of increasing concentrations of EGCG (vehicle is 70% EtOH). Total RNA was extracted, and qRT-PCR was used to assess gene expression of CSF-1, CSF-2, CSF-3 (*A*) and MT1-MMP, STAT3 (*B*). A representative qPCR profile, from three independent experiments, is shown for the corresponding genes. Data represent mean values ± S.D. (*error bars*) from triplicates.

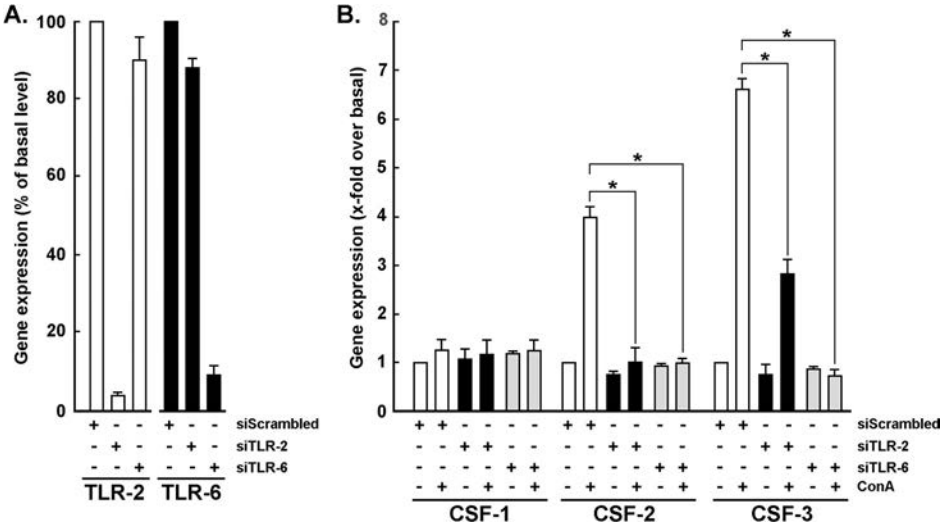

FIGURE 2. **TLR-2 and TLR-6 gene silencing abrogates ConA-induced CSF-2 and CSF-3 transcriptional regulation.** MSCs were transiently transfected with scrambled sequences (*siScrambled*), TLR-2 siRNA (*siTLR-2*), or TLR-6 siRNA (*siTLR-6*) as described under "Experimental Procedures." Total RNA was extracted, and qRT-PCR was used to assess TLR-2 and TLR-6 gene silencing (*A*) as well as CSF-1, CSF-2, and CSF-3 gene expression (*B*) upon treatment with 3 μg/ml (CSF-2) or 30 μg/ml (CSF-1, CSF-3) of ConA for 24 h. *Error bars*, S.D.; *, $p < 0.05$.

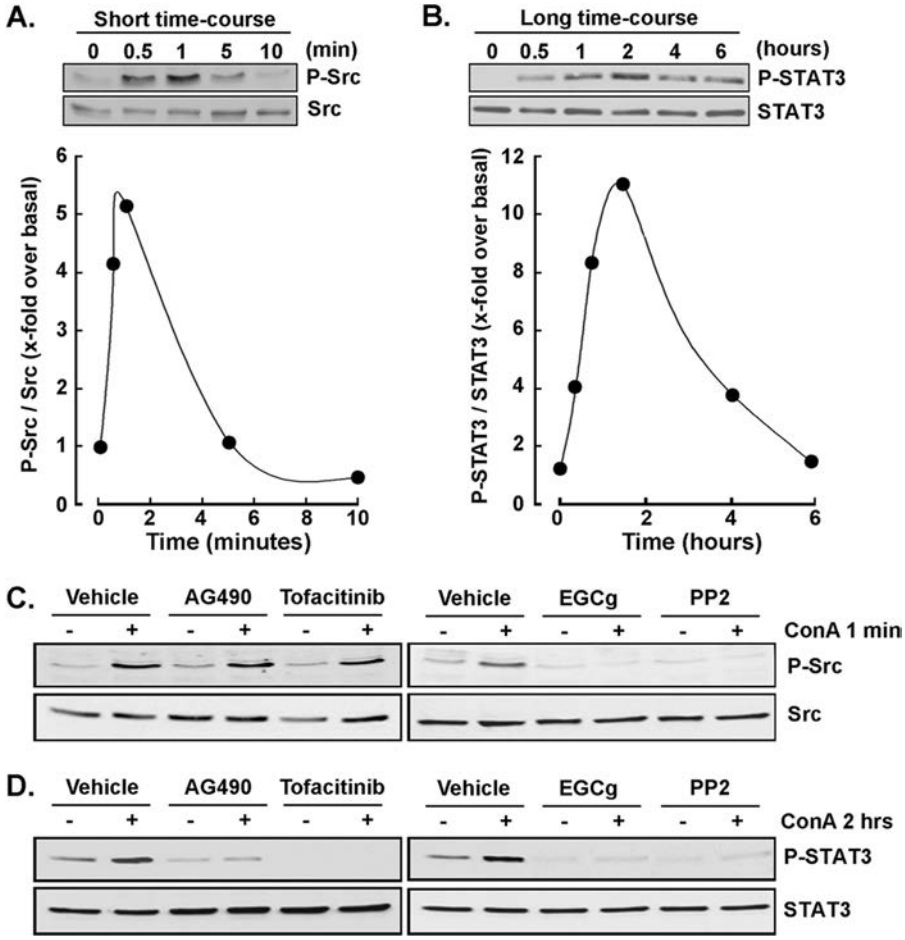

FIGURE 4. **EGCG inhibits ConA-induced short term Src phosphorylation and long term STAT3 phosphorylation.** Subconfluent MSCs were serum-starved and treated with 30 μg/ml ConA for the times indicated. Cell lysates were isolated, and Western blotting and immunodetection were performed using anti-phospho-Src and anti-Src (*A*) or with anti-STAT3 and anti-phospho-STAT3 antibodies (*B*). A representative scanning densitometry assessment was performed to show the respective extent of Src phosphorylation and of STAT3 phosphorylation. Preincubation of MSCs for 30 min was performed with vehicle, 30 μM AG490, 30 μM tofacitinib, 30 μM EGCG, or 10 μM PP2, followed by a treatment with 30 μg/ml ConA for 1 min (*C*) or 2 h (*D*). Cell lysates were isolated, and Western blotting and immunodetection were performed using the indicated antibodies. Vehicle for AG490 and tofacitinib was H_2O. Vehicle for EGCG and PP2 was 70% EtOH.

not affect MSC viability, whereas a slight 5–8% additional effect was found to decrease cell viability in the presence of ConA (data not shown).

ConA-induced Sequential Phosphorylation of Src and STAT3 Is Inhibited by EGCG—Given that ConA is a TLR-2/6 agonist, we next sought to evaluate the Src and JAK/STAT3 signaling pathways. MSCs were serum-starved and treated with ConA, and then cell lysates were isolated for Western blotting and immunodetection. We found that Src phosphorylation was rapidly and transiently induced within the 1st min of treatment (Fig. 4*A*), whereas STAT3 phosphorylation only peaked at 2 h of treatment (Fig. 4*B*). When MSCs were preincubated with EGCG to compare its effects with pharmacological inhibitors of Src (PP2) or JAK (AG490, tofacitinib), we found that EGCG effectively inhibited short term Src phosphorylation (Fig. 4*C*, *right panel*), as well as long term STAT3 phosphorylation (Fig. 4*D*, *right panel*). Short term phosphorylation of Src by ConA was not affected by AG490 or tofacitinib (Fig. 4*C*, *left panel*), whereas that of STAT3 was inhibited by PP2 preincubation (Fig. 4*D*, *left panel*), suggesting that a sequential signaling cascade occurs following ConA stimulation.

MT1-MMP and Src Are, Respectively, Required for Src and STAT3 Phosphorylation by ConA—Given that MT1-MMP was recently shown to regulate STAT3 phosphorylation (12) and that this also requires Src kinase activity, we decided to assess the potential relationship between MT1-MMP-mediated signaling and Src kinase activity in ConA action. Src (Fig. 5*A*) and MT1-MMP (Fig. 5*B*) protein expression in MSCs was significantly silenced using specific siRNA strategies. When MSCs were then treated with ConA, both Src phosphorylation (Fig. 5*A*, 1-min treatment) and STAT3 phosphorylation (Fig. 5*B*, 2-h treatment) were abrogated. This suggests that ConA-mediated phosphorylation of Src and of STAT3 requires an initial signaling cascade triggered by MT1-MMP.

MT1-MMP and Src Kinase Activity Are Required in ConA-induced CSF-2 and CSF-3 Transcription—A gene silencing strategy similar to that above was applied to assess whether ConA required either MT1-MMP or Src to trigger CSF gene expression. We found that MT1-MMP and Src gene silencing abrogated ConA-induced CSF-2 and CSF-3, but not CSF-1, transcription (Fig. 6). Interestingly, PP2 antagonized ConA-induced CSF-2 and CSF-3, confirming the requirement for Src kinase activity.

MT1-MMP Overexpression Triggers Src and STAT3 Phosphorylation Which Leads to Increased CSF-2 and CSF-3 Transcription—To better ascertain the importance of intracellular signaling originating from the cytoplasmic domain of MT1-MMP, recombinant forms of either WT-MT1-MMP or a mutant MT1-MMP, in which the crucial Tyr^{573} residue was mutated (Y73F), were overexpressed in MSCs. Both recombinant forms were efficiently overexpressed (Fig. 7, *A* and *B*). Only the WT-MT1-MMP signaled Src and STAT3 phosphorylation (Fig. 7*A*) and led to increased transcription of both the CSF-2 and CSF-3 genes (Fig. 7*C*). Mutation of Tyr^{573} within the MT1-MMP cytoplasmic domain prevented it from triggering Src or STAT3 phosphorylation or increasing the extent of CSF-2 and CSF-3 transcription. This demonstrates that increased MT1-MMP expression signals CSF-2 and CSF-3 transcriptional regulation.

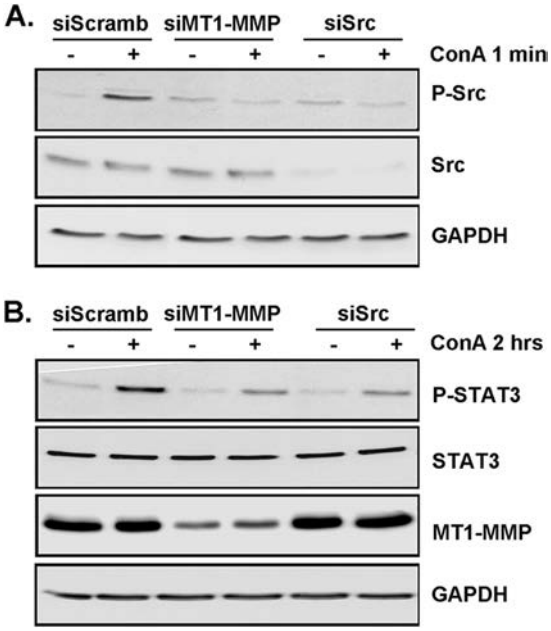

FIGURE 5. **MT1-MMP and Src gene silencing abrogates ConA-induced Src and STAT3 phosphorylation.** MSCs were transiently transfected with scrambled sequences (*siScramb*), Src siRNA (*siSrc*), or MT1-MMP siRNA (*siMT1-MMP*) as described under "Experimental Procedures." MSCs were then serum-starved and treated with 30 μg/ml ConA for either 1 min to monitor Src phosphorylation (*A*) or 2 h to monitor STAT3 phosphorylation (*B*). Cell lysates were isolated, and Western blotting and immunodetection were performed using anti-phospho-Src and anti-Src (*A*) or with anti-STAT3, anti-phospho-STAT3 antibodies (*B*).

EGCG Inhibits MT1-MMP-mediated Src and STAT3 Phosphorylation—In light of its ability to inhibit ConA-mediated Src and STAT3 phosphorylation and given the fact that MT1-MMP silencing abrogated ConA action, we next assessed the ability of EGCG to target MT1-MMP-mediated signaling functions. MT1-MMP was overexpressed in MSCs, and then cells were subjected to 30 μM EGCG treatment. We found that EGCG effectively diminished WT-MT1-MMP-mediated Src and STAT3 phosphorylation (Fig. 8*A*) and caused decreased CSF-2 and CSF-3 transcription (Fig. 8*B*), as was similarly observed in ConA-treated cells.

DISCUSSION

Since its discovery, MT1-MMP has been extensively investigated and is now among the best characterized MMPs. MT1-MMP has been implicated in various physiological and pathological processes such as wound healing, bone development, angiogenesis, inflammation, cancer invasion, and metastasis (19), all of which also involve the recruitment of circulating bone marrow-derived MSCs (20). Although MT1-MMP functions primarily immediately outside the cell surface, where it modifies the environment of the cell (21), it is now suggested that alteration of signaling driven from the extracellular matrix adhesion molecules or in response to chemokines can be transduced by MT1-MMP, thereby influencing cellular functions (22). In our study, we demonstrate the essential requirement for MT1-MMP in transducing intracellular signaling that regulates CSF-2 and CSF-3 transcription. Given its capacity to interact with extracellular components that impact intracellular functions, a plausible mechanism may, upon extracellular complex-

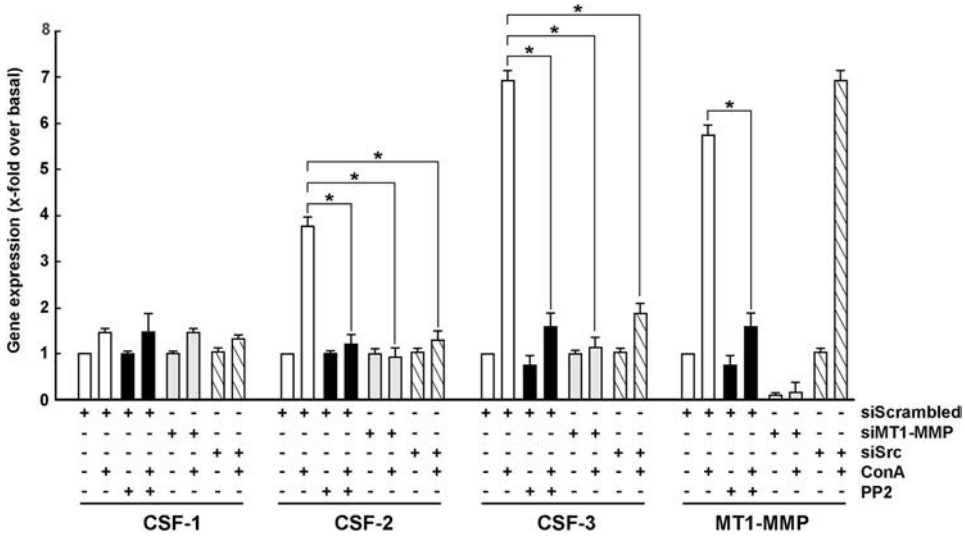

FIGURE 6. **MT1-MMP and Src gene silencing abrogates ConA-induced CSF-2 and CSF-3 transcriptional regulation.** MSCs were transiently transfected with scrambled sequences (*siScrambled*), Src siRNA (*siSrc*), or MT1-MMP siRNA (*siMT1*) as described under "Experimental Procedures." Total RNA was extracted, and qRT-PCR was used to assess CSF-1, CSF-2, CSF-3, and MT1-MMP gene expression upon treatment with 3 μg/ml (CSF-2) or 30 μg/ml (CSF-1, CSF-3) ConA for 24 h. Where indicated, cells were pretreated for 30 min with 10 μM PP2 prior to ConA treatment. *Error bars*, S.D.; *, $p < 0.05$.

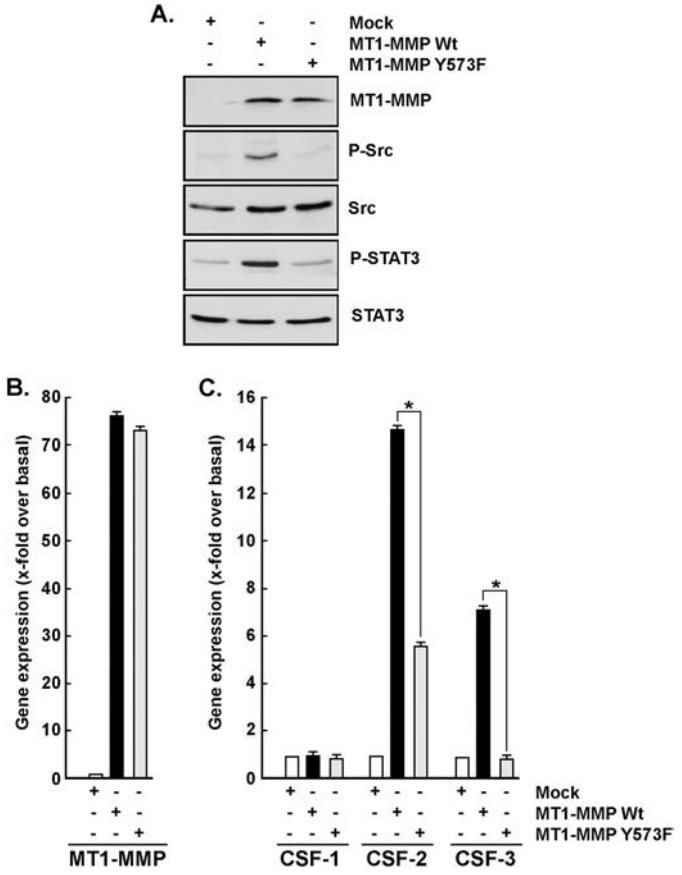

FIGURE 7. **MT1-MMP overexpression triggers CSF-2 and CSF-3 gene expression and requires phosphorylation of Tyr^{573} in its intracellular domain.** Subconfluent MSCs were transiently transfected with cDNA plasmids encoding WT full-length MT1-MMP or MT1-MMP mutant Y573F, as described under "Experimental Procedures." *A*, cell lysates were isolated, and Western blotting and immunodetection were performed using anti-phospho-Src, anti-Src, anti-STAT3, anti-phospho-STAT3, or anti-MT1-MMP antibodies. *B* and *C*, total RNA was also extracted from the lysates, and qRT-PCR was used to assess gene expression of MT1-MMP (*B*) or CSF-1, CSF-2, and CSF-3 (*C*). A representative qPCR profile, of three independent experiments, is shown for the corresponding genes. Data represent mean values ± S.D. (*error bars*) from triplicates. *, $p < 0.05$.

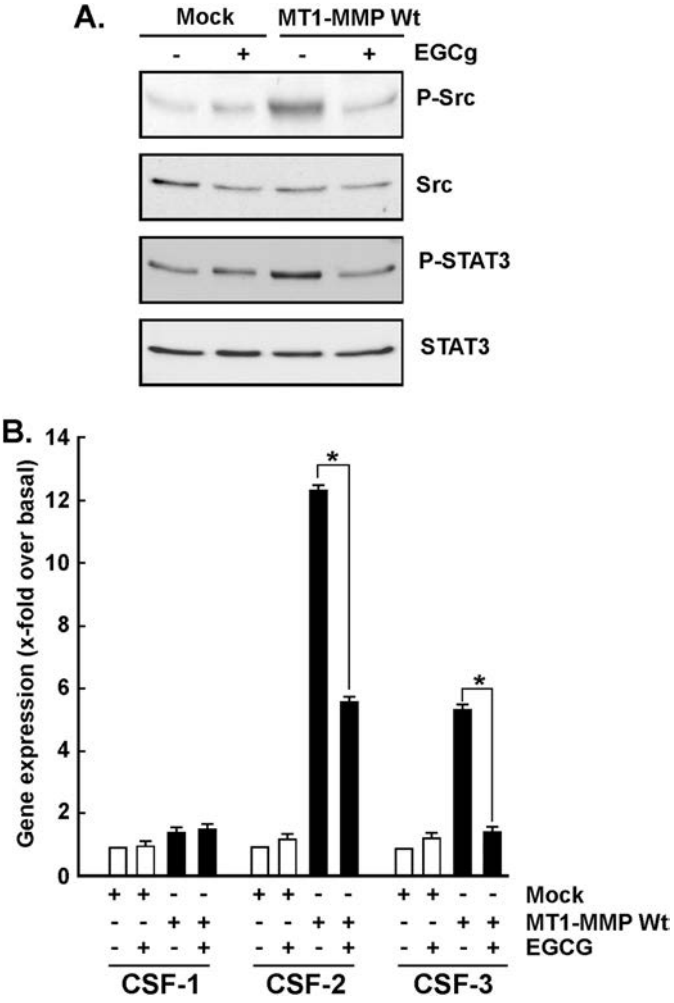

FIGURE 8. **EGCG inhibits MT1-MMP-induced Src and STAT3 phosphorylation and inhibits CSF-2 and CSF-3 gene expression.** Subconfluent MSCs were transiently transfected with a cDNA plasmid encoding the WT full-length MT1-MMP as described under "Experimental Procedures." *A*, cells were then treated with 30 μM of EGCG for 18 h, lysates were isolated, and Western blotting and immunodetection were performed using anti-phospho-Src, anti-Src, anti-STAT3 or anti-phospho-STAT3. *B*, total RNA was extracted from the lysates, and qRT-PCR was used to assess gene expression of CSF-1, CSF-2, and CSF-3. A representative qPCR profile of three independent experiments, is shown for the corresponding genes. Data represent mean values ± S.D. (*error bars*) from triplicates. *, $p < 0.05$.

ation of MMP-2/TIMP-2/MT1-MMP, trigger a potential conformational change in MT1-MMP structure that would impact on its ability to relay intracellular signals. To validate such an audacious hypothesis, one would need a complex structure-to-function study with MT1-MMP deletion mutants within its catalytic, hemopexin, and hinge domains all believed to recognize, anchor, and catalyze pro-MMP to MMP activation.

MT1-MMP intracellular function was also reported to impact on transcriptional regulation of other tumorigenic growth factors, such as VEGF-A (23). In fact, among the numerous circulating chemotactic mediators, chemokines play key roles in hematopoietic stem cell trafficking (24) and in endothelial progenitor cells homing to tumors. As such, high levels of serum VEGF and CSF were correlated with endothelial progenitor cell recruitment within tumor microvessels where they were found to contribute to tumor vasculogenesis (25). Furthermore, our findings that CSF-3 is induced through MT1-MMP signaling in MSCs add to its list of key roles in myeloid cell recruitment because it affects vasculogenesis through recruitment of endothelial progenitor cells (26). These studies, along with the fact that CSF-3 accelerates reendothelialization processes after vascular injury (26), suggest that CSF-3 plays several roles not only through effects on neutrophils but also through effects on other bone marrow-derived cells.

Our current study also sheds light on the emerging importance of TLR modulators such as the naturally occurring plant lectins. These lectins are used by the pharmaceutical industry as part of reliable *in vitro* cell functional assays for studying biological systems ranging from mitogenicity to proinflammatory cytokine production (27, 28). Given the use of the lectin ConA, a potent TLR-2 and TLR-6 agonist, in MSC activation, our study further supports the known cross-talk that exists between TLR and cancer (29), angiogenesis (30), and inflammation (31). Interestingly, ConA is well documented as inducing MT1-MMP, the expression of which has also been documented in all the above regulated processes, similar to TLR involvement (32, 33). Our study shows that EGCG inhibits both ConA- and

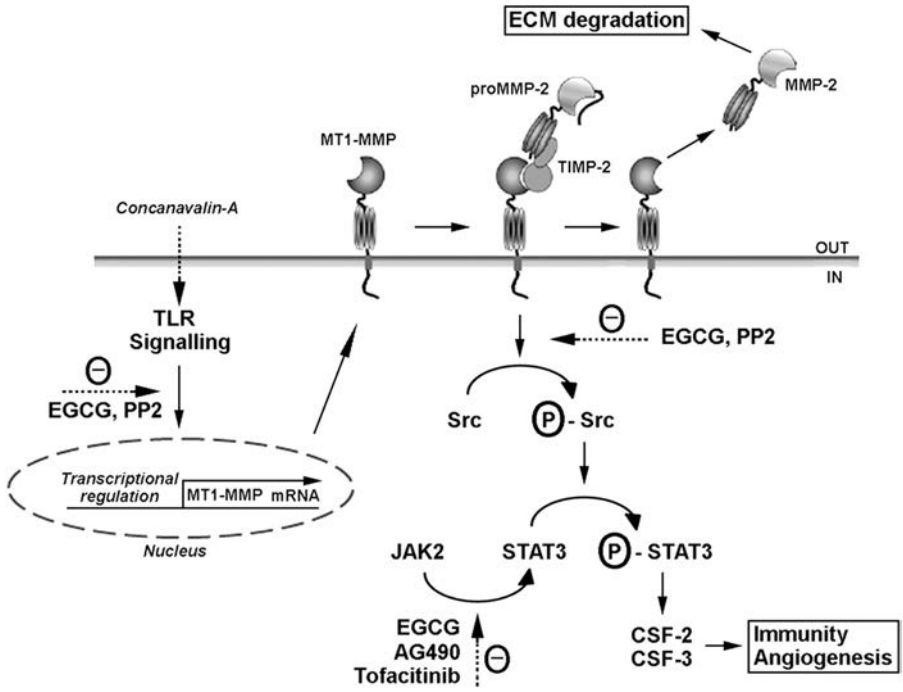

FIGURE 9. **Scheme summarizing the effects of EGCG against the molecular mechanisms involved in ConA- and MT1-MMP-mediated regulation of CSF-2 and CSF-3 transcription.** In this study, we show that ConA triggers MT1-MMP, CSF-2, and CSF-3 transcription, possibly through TLR-mediated signaling. Increased MT1-MMP gene/protein expression triggers sequential Src and STAT3 phosphorylation, which requires the involvement of a crucial phosphorylatable Tyr^{573} located within the MT1-MMP intracellular domain. Pharmacological inhibitors of the Src kinase (PP2) and JAK/STAT pathways (AG490, tofacitinib), as well as the green tea-derived EGCG, efficiently inhibit both ConA- and MT1-MMP-dependent signaling. *TIMP-2*, tissue inhibitor of matrix metalloproteinases-2.

MT1-MMP-mediated signaling that leads to increased CSF-2 and CSF-3 gene expression. In support of this, EGCG was already described as inhibiting TLR-2 and TLR-4 signaling (34, 35) as well as the signaling that leads to diminished MT1-MMP gene expression (36). We further show that EGCG inhibits a ConA- and MT1-MMP-mediated signaling cascade that leads to Src and STAT3 phosphorylation (Fig. 9). Such Src phosphorylation was previously reported in MCF-7 breast cancer cells where it regulated the transcription of VEGF-A (23). Besides its impact on cancer progression and angiogenesis, MT1-MMP-mediated transcriptional regulation of genes seems to also be among the functions that impact on the expression of biomarkers in autophagy (37) as well as in neuroinflammation (38).

In the current study, we also confirmed a role for the Tyr^{573} residue located within the intracellular domain of MT1-MMP because the Y573F mutation disabled the capacity of MT1-MMP to trigger Src phosphorylation and subsequent CSF-2 and CSF-3 transcription. Interestingly, MT1-MMP-mediated phosphorylation of Src is also believed to trigger a positive feedback loop resulting in increased MT1-MMP phosphorylation which may also regulate MT1-MMP ubiquitination at Lys^{581} and lead to increased cell migration (39). Src-mediated phosphorylation was, in fact, shown to occur within the Tyr^{573} of the intracellular domain of MT1-MMP where it was suggested to affect endothelial cell migration (40). Impaired phosphorylation of Tyr^{573} was shown to reduce tumor growth in mice (18), but further transcription studies were not performed. In our study, we demonstrate that impaired Tyr^{573} phosphorylation abrogates the ability of MT1-MMP to regulate CSF-2 and CSF-3 gene transcription which may, in part, explain the previously reported low tumor-associated angiogenesis.

Given previous moderate-to-no success targeting of soluble secreted MMPs, membrane-bound MMPs have recently become critical pharmacological targets in angiogenesis-related disease treatment (33). Accordingly, preclinical proof of principle rationale was recently provided for the design and development of novel and selective MT1-MMP inhibitors that specifically target its hemopexin domain (42), whereas recent molecular profiling has identified actinonin, originally classified as an aminopeptidase N/CD13 pharmacological inhibitor, with MT1-MMP targeting functions (43). Finally, impact on lymphangiogenesis with selective targeting of MT1-MMP was also achieved through the use of specific monoclonal antibodies (44), whereas AG3340, a potent small molecule MT1-MMP antagonist, was required for the design of novel therapies for type 1 diabetes (45).

In conclusion, primary vascularizing and metastatic tumors are thought to attract MSCs in their microenvironment where they become tumor-associated fibroblasts, affect tumor cell survival and angiogenesis, and have an immunomodulatory function (4, 46). Provided that those IC_{50} values of EGCG we report in this study (Fig. 3) closely approximate the reported plasmatic concentrations of ~1 μM (41, 47), with optimal pharmacological effects achieved at 30 μM, our study puts emphasis on potential strategies aimed at targeting MSC proangiogenic and immunomodulatory functions, thereby preventing their contribution to tumor development. Given that MSCs contribute to tumor angiogenesis, possibly through secretion of CSF-2

and CSF-3, we provide the first molecular evidence that one such strategy makes use of the chemopreventive properties of diet-derived polyphenols such as EGCG that may act through pharmacological targeting of the MT1-MMP intracellular signaling.

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