invitrogen

Two data sets. One step. Zero doubt.

Confidently confirm your cell profiles with a new flow cytometer that delivers flow cytometry and imaging data simultaneously. Now, you can acquire dual data quickly and easily. The new Invitrogen™ Attune™ CytPix™ Flow Cytometer delivers both brightfield images and flow cytometry data sets simultaneously, so you can confirm cellular characteristics and sample quality confidently, without changing your protocols.

Enhance analysis and confidence at **thermofisher.com/cytpix**

ThermoFisher SCIENTIFIC

STEM CELLS®
Tissue-Specific Stem Cells

Hepatocyte Growth Factor Effects on Mesenchymal Stem Cells: Proliferation, Migration, and Differentiation

GIANCARLO FORTE,[a] MARILENA MINIERI,[a] PAOLO COSSA,[a] DANIELE ANTENUCCI,[a] MARILENA SALA,[b] VIOLA GNOCCHI,[b] ROBERTA FIACCAVENTO,[a] FELICIA CAROTENUTO,[a] PAOLO DE VITO,[c] PATRIZIA MORENA BALDINI,[c] MARIA PRAT,[b] PAOLO DI NARDO[a]

[a]Molecular and Cellular Cardiology Lab, Department of Internal Medicine, University of Rome "Tor Vergata," Italy. [b]Department of Medical Sciences, University "A. Avogadro" of Piemonte Orientale, Novara, Italy. [c]Department of Biology, University of Rome "Tor Vergata," Italy

Key Words. Met receptor • Mesenchymal stem cells • Hepatocyte growth factor • p38 • Akt

ABSTRACT

Hepatocyte growth factor (HGF), a pleiotropic cytokine of mesenchymal origin promoting migration, proliferation, and survival in a wide spectrum of cells, can also modulate different biological responses in stem cells, but the mechanisms involved are not completely understood so far. In this context, we show that short-term exposure of mesenchymal stem cells (MSCs) to HGF can induce the activation of its cognate Met receptor and the downstream effectors ERK1/2, p38MAPK, and PI3K/Akt, while long-term exposure to HGF resulted in cytoskeletal rearrangement, cell migration, and marked inhibition of proliferation through the arrest in the G_1-S checkpoint. When added to MSCs, the K252A tyrosine kinase inhibitor prevented HGF-induced responses. HGF's effect on MSC proliferation was reversed by p38 inhibitor SB203580, while the effects on cell migration were abrogated by PI3K inhibitor Wortmannin, suggesting that HGF acts through different pathways to determine its complex effects on MSCs. Prolonged treatment with HGF induced the expression of cardiac-specific markers (GATA-4, MEF2C, TEF1, desmin, α-MHC, β-MHC, and nestin) with the concomitant loss of the stem cell markers nucleostemin, c-kit, and CD105. STEM CELLS *2006;24: 23–33*

INTRODUCTION

Bone marrow mesenchymal stem cells (MSCs) display a great transdifferentiation potential in adult organisms, being able to differentiate in cell lineages different from those of the original tissue [1]. So far, unidentified mechanisms and factors may disclose such a plasticity that could be exploited by the organism to regulate cell turnover, substitute injured cells, and repair tissue architecture. In this context, a major challenge is represented by the identification of growth factors that selectively promote MSC proliferation, migration, and differentiation, opening great prospects for future stem cell–based tissue engineering [1, 2].

Hepatocyte growth factor (HGF) is a pleiotropic cytokine of mesenchymal origin, promoting motility, proliferation, inva-

Correspondence: Paolo Di Nardo, M.D., Dipartimento di Medicina Interna, Università di Roma "Tor Vergata," Via Montpellier, 1, 00133 Roma, Italy. Telephone: +39-06-72594215; Fax: +39-06-2024130 or +39-06-72594263; e-mail: dinardo@med.uniroma2.it
Received August 3, 2004; accepted for publication June 20, 2005; first published online in STEM CELLS *EXPRESS* August 11, 2005.
 1066-5099/2006/$12.00/0 doi: 10.1634/stemcells.2004-0176

sion, morphogenesis, and survival of a wide spectrum of cells, namely epithelial and endothelial cells [3, 4]. The coordinated integration of these processes plays a pivotal role in organ formation during embryogenesis and tissue homeostasis in adults. HGF directs tubulogenesis during kidney [5] and mammary gland [6] development and angiogenesis [7] and is a chemoattractant for motor neuron axon [8] and myoblast precursors [9, 10] and a survival factor for hepatocytes [11] and placenta [12]. In adults, HGF is involved in kidney and liver regeneration [13]; it displays in vivo cytoprotective activity in different cell types of injured organs [14]. Inappropriate activation of the HGF signaling pathway results in tumorigenesis and metastatic spreading of tumor cells [15]. Biological responses elicited by HGF are mediated by its high affinity receptor, the tyrosine kinase encoded by the *met* proto-oncogene [16, 17]. The Met receptor is a disulfide-linked heterodimeric glycoprotein, in which the transmembrane β-chain contains the kinase domain and a unique multifunctional docking site [18], which upon tyrosine phosphorylation, recruits different signal transducers and adaptors, such as Gab-1, SHC, and GRB2 [3, 4, 19]. The latter two couple the Met receptor with the ras-mitogen-activated protein kinase (ras-ERK1/2 MAPK) pathway, while Gab-1 binds PI3-kinase efficiently.

Although biochemical and biological responses induced by HGF on primary cultured cells or established cell lines have been extensively studied, little information is presently available on undifferentiated cells. In human embryonic stem cells, HGF induced differentiation into the three embryonic germ layers [20]. In the presence of Matrigel and FGF-4, HGF induced multipotent adult progenitor cells from bone marrow to differentiate into functional hepatocytes in vitro, but its presence seemed to be dispensable [21]. Hemopoietic stem cells purified from bone marrow and primed with HGF in vitro were also found to differentiate into a hepatocyte lineage [22, 23]. Moreover, it was recently reported that human MSCs produce HGF and express a functional Met receptor [24]. Finally, Duan and colleagues [25] demonstrated that the overexpression of HGF by MSCs engrafted into ischemic myocardium can improve their incorporation in the organ, reducing infarct size, improving heart functions, and inducing angiogenesis.

The present study was undertaken to investigate (a) the biochemical pathways involved in HGF activity on mouse MSCs isolated from bone marrow in terms of proliferation and migration, and (b) the possible effects on cell differentiation.

Materials and Methods

Cell Culture

MSCs were obtained from 6-week-old female C3H/He mice femurs according to Friedenstein's protocol [26]. Ice-cold phosphate-buffered saline (PBS) was flushed into the bone shaft, the pellet was resuspended in 5 ml of 5 mM EDTA for 30 seconds, and then an equal volume of 1.8% NaCl was added (hypotonic lysis). The Lin^{pos} fraction was eliminated by a magnetic cell-sorting protocol (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com). Briefly, cells obtained from bone marrow were incubated with a cocktail of Biotin-conjugated monoclonal antibodies (CD5, CD45R [B220], CD11b, antiLy-6G [Gr-1], 7-4, and Ter-119) and then separated by anti-Biotin microbeads-conjugated secondary antibody. Aliquots of the two separated cell subpopulations (Lin^{pos} and Lin^{neg}) were then stained with anti-Biotin phycoerythrin-conjugated secondary antibody and analyzed with fluorescence-activated cell sorting (FACS). The Lin^{neg} fraction was resuspended in complete Iscove's modified Dulbecco's medium (IMDM; Cambrex Bio Science, Verviers, Belgium, http://www.cambrex.com) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cell concentration was adjusted at 0.5×10^6 per cm^2. After 4–6 days, an adherent population of MSCs appeared. Medium was replenished, floating cells were removed after 7 and 12 days, and the final adherent cell population was used for the experiments. Serum was omitted from the culture medium in the final passage.

RNA Extraction, Reverse Transcription, and Semiquantitative and Quantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted by Trizol Reagent (GIBCO BRL, Gaithersburg, MD, http://www.gibcobrl.com).

Reverse transcription (RT) was carried out with 2 μg of RNA for each sample using RT M-MLV (Invitrogen Corporation, Carlsbad, CA, http://www.invitrogen.com) in the presence of random hexamers. Semiquantitative analysis of RNA expression was carried out by RT–polymerase chain reaction (PCR) by comparing the control transcript (GAPDH [glyceraldehyde-3-phosphate dehydrogenase]) and the transcript of interest when their amplification was in the exponential phase. The primers used are reported in Table 1. PCR products were size-fractionated in 2% agarose gel electrophoresis.

For real-time RT-PCR, each reaction was performed in a final volume of 20 microl of Universal Master Mix without Amperase Uracyl N-Glycosilase 2X (UMM no UNG, Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com), murine HGF, Met primers, probe 20X (Mm0115182 ml and Mm00434924 ml assays; Applied Biosystems), and 0.5 microl of template cDNA. As active references, 18S RNA (mammalian 18S PDAR; Applied Biosystems) was used. In each experiment, single samples were amplified three times and each experiment was a triplicate. The system used was the 700 Sequence Detection System (Applied Biosystems). In inhibition

Table 1. Primers used in the study

Primer	Forward sequence	Reverse sequence
HGF	5′-GGCCTTCGTTTTTGATAAGTC-3′	5′-TTCATGGGGGATCATGGAAT-3′
c-MET	5′-TTCTTGAATGCTACACCCCA-3′	5′-TCACACCCACTTCATGCACAT-3′
NST	5′-GGGAAAAGCAGTGTCATTA-3′	5′-GGGATGGCAATAGTAACC-3′
α-MHC	5′-GGAAGAGTGAGCGGCCATCAAGG-3′	5′CTGCTGGAGAGGTTATTCCTGG-3′
β-MHC	5′-GCCAACACCAACCTGTCCAAGTTC-3′	5′-TGCAAAGGCTCCAGGTCTGAGGGC-3′
MEF2C	5′-AGCAAGAATACGATGCCATC-3′	5′-GAAGGGGTGGTGGTACGGTC-3′
TEF1	5′-AAGCGTCAAGCCCTTTGTG-3′	5′-AAAGGAGCACACTTTGGTGG-3′
MLC 2a	5′-CAGACCTGAAGGAGACCT-3′	5′-GTCAGCGTAAACAGTTGC-3′
MLC 2v	5′-GCCAAGAAGCGGATAGAAGG-3′	5′-CTGTGGTTCAGGGCTCAGTC-3′
ANP	5′-TTGGCTTCCAGGCCATAATTG-3′	5′AAGAGGGCAGATCTATCGGA-3′
Desmin	5′-TCTACGAGGAGGAGATGCGC-3′	5′-GGACCTGCTGTTCCTGAAGC-3′
GAPDH	5′-CAAGATGGTGAAGGTCGGTGTG-3′	5′-GGGGTAAGCAGTTGTGTCAGGAT-3′

Abbreviations: ANP, atrial natriuretic peptide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; MEF2C, myogenic enhancer factor 2C; MHC, myosin heavy chain; MLC 2a/v, myosin light chain 2 atrial/ventricular; NST, nucleostemin; TEF1, transcriptional enhancer factor 1.

experiments, cells were concomitantly incubated with K252a tyrosine kinase inhibitor (EMD Biosciences, Merck KGaA, Darmstadt, Germany, http://www.emdbiosciences.com).

Stimulation, Immunoprecipitation, and Western Blot Analysis

Quiescent MSCs were incubated for 15 minutes at 37°C in the absence, or presence, of 20 ng/ml of human recombinant HGF (ReliaTech, Braunscwheig, Germany, http://www.reliatech.de), washed twice with cold PBS, and lysed in radio-immunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 0.25% sodium deoxycholate, and 2 mM ortho-vanadate) and a cocktail of protease inhibitors (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com). The HGF dose was chosen after preliminary dose-response experiments. Cell lysates were centrifuged at 13,000 rpm at 4°C for 15 minutes and incubated 2 hours with a mixture of DO-24 and DN-30 monoclonal antibodies and rabbit anti-mouse antibodies coupled to Sepharose–protein A (Amersham Pharmacia Biotech, Amersham, U.K., http://www1.amershambiosciences.com), as described by Prat et al. [27]. Immunoprecipitates were washed with ice-cold RIPA buffer, solubilized in reducing Laemmli buffer, and resolved on SDS-PAGE, followed by transfer onto polyvinylidene difluoride (PVDF) filters. Filters were blocked with methanol for 10 minutes at 20°C and probed with the appropriate antibodies (antiphosphotyrosine [UBI, Lake Placid, NY] or anti-Met SP-260 [Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com]) diluted in Tris-buffered saline–5% bovine serum albumin for 2 hours at 22°C.

MSCs were also treated with 20 ng/ml HGF for 10, 30, and 60 minutes and, after washing with cold PBS, lysed in 100 μl of reducing Laemmli sample buffer; extracts were clarified and protein content quantified by Bradford method (Amresco, Inc., Solon, OH, http://www.amresco-inc.com). Thirty micrograms of the clarified extracts was run in 12.5% SDS-PAGE followed by transfer to a PVDF membrane. Western analysis was carried out using the following primary antibodies: monoclonal antibodies (mAbs) against phosphorylated-ERK1/2 MAPK (Cell Signaling Technology, Inc., Beverly, MA, http://www.cellsignal.com), mAbs against phosphorylated-Akt, phosphorylated-p38, p38 (Sigma-Aldrich, Milan, Italy), polyclonal Abs against Akt1/2, ERK1/2, Met, αHGF (Santa Cruz Biotechnology), polyclonal antibodies (pAbs) against $p27^{kip1}$, $p21^{waf1}$ (Lab Vision Corporation, Fremont, CA, http://www.labvision.com), and mAb against pRB (BD Biosciences Pharmingen, San Jose, CA, http://www.bdbiosciences.com/pharmingen). After extensive washing, immunocomplexes were detected with horseradish peroxidase–conjugated appropriate secondary antibodies followed by enhanced chemiluminescence reaction (ECL™; Amersham).

Proliferation Assay

For cell growth assay, 2.5×10^3 cells were seeded in 96-well microplates, grown for 12 hours in 10% FCS and then starved in 2% FCS for 24 hours. Cells were then switched to 2% serum supplemented, or not supplemented, with 20 ng of HGF per ml, or to 10% serum. Fresh medium plus and minus HGF was replenished. Cells were also incubated in 10% FCS, in the presence of 20 ng HGF per ml. Cells were pulsed with 1 μCi per ml [^{3}H]-thymidine and incubated for 3 hours. After trypsin treatment, cells were harvested by centrifugation and treated with 5% trichloroacetic acid (TCA) at 4°C for 30 minutes. The TCA-insoluble fraction was resuspended in 0.1% SDS in 200 mM NaOH, and the samples, after addition of 7 ml Optifluor (Packard Instruments, Downers Grove, IL, www.packardbioscience.com), were counted for radioactivity by a liquid scintillation counter (Tricarb 2180 TR, Packard Instruments). As indicated in some experiments, prior to stimulation, cells were preincubated for 1 hour with specific inhibitors. Statistical analysis of the data was carried out using the Student's *t*-test.

FACS Analysis

To stain isolated nuclei, cells were incubated with 25 μg/ml of propidium iodide (PI; Sigma-Aldrich, Milan, Italy) in a solution containing 2% Triton X-100. In parallel experiments, MSCs were doubly stained with PI and fluorescein isothiocyanate (FITC)–labeled Annexin V (Sigma-Aldrich, Milan, Italy). After 30 minutes of incubation, cells were washed with ice-cold PBS and analyzed in a FACScalibur flow cytometer (BD Biosciences Pharmingen).

Wound Healing Assay

For wound healing assay, 5×10^5 cells were grown in a 15-mm culture dish and allowed to reach confluence and further incubated in medium containing 2% FCS for 18 hours. The monolayers were then wounded with a plastic pipette as described [28]. After wounding, cells were washed with PBS and incubated for 24 hours in medium containing 2% FCS, with or without HGF (20 ng/ml), in the presence, or absence, of 30 nM of Met tyrosine kinase inhibitor K252a (Calbiochem-Novabiochem Intl.) added immediately before stimulation with HGF, fixed with 11% glutaraldehyde and stained with hematoxylin and eosin. Images of cell samples were taken with a digital camera.

Transwell Migration Assay

The assay for chemotaxis was performed in Transwell chambers (Corning Costar Italia, Concorezzo, Italy). Briefly, 200 μl of medium containing MSCs was seeded on the upper side of a porous polycarbonate membrane (pore size: 8 μm). Five hundred microliters of medium containing, or not containing, HGF in the presence of 2% FCS was added to the lower compartment. The plates were incubated at 37°C in 5% CO_2 for 48 hours. In inhibition experiments, cells were preincubated with K252A inhibitor for 1 hour. At the end of incubation, the cells at the upper side of the filter were mechanically removed. Cells that had migrated to the lower side of the filter were fixed for 30 minutes in 11% glutaraldehyde and stained with hematoxylin and eosin. Five to ten random fields were counted for each filter.

Immunofluorescence

MSCs were seeded on chamber slides (Nalge Nunc International, Rochester, NY, USA, http://www.nalgenunc.com) and treated, or not treated, with 20 ng/ml HGF for 24, 48, 72, and 96 hours. Cells were washed in PBS, fixed in paraformaldehyde 4% in PBS containing $CaCl_2$ for 30 minutes at 4°C and permeabilized with 0.1% Triton X-100. F-actin was labeled with Tetra-rhodamine– conjugated Phalloydine in methanol 50% for 30 minutes. Cells were stained with antibodies for c-kit, CD105, GATA-4 (Santa Cruz Biotechnology), nestin (Chemicon International, Temecula, CA, http://www.chemicon.com), and α–myosin heavy chain (monoclonal antibody MF-20 [29]). As secondary antibodies, FITC-conjugated mouse anti-goat and goat anti-mouse (Vector Laboratories, Ltd., Peterborough, England, http://www.vectorlabs.com/uk) were used. Nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole). Incubation with FITC-labeled secondary antibody in the absence of specific primary antibody was used to exclude the occurrence of unspecific signals.

RESULTS

Phenotype of the Cells Purified from Bone Marrow

Cells were negatively selected by magnetic cell sorting on the basis of the expression of Lin surface molecules and positively selected for their ability to adhere to plastic substrate. Before plating on plastic dishes, aliquots of the Lin^{neg} and Lin^{pos} fractions were analyzed by FACS to assess the preparation quality and the Lin^{neg} fraction was found to be 98% pure (data not shown). The cells resulting after plating were c-kit^{pos}, $CD105^{pos}$, nucleostemin^{pos}, α–smooth muscle actin^{low}, nestin^{neg}, Sca-1^{neg}, $CD34^{neg}$, and $CD45^{neg}$. They did not express any muscle-specific marker.

MSCs Express Functional HGF and Met Receptor, Both of Which Can Be Upregulated

Recently, Neuss and colleagues demonstrated that human MSCs express HGF and its receptor c-Met [24]. To assess the expression of HGF and c-Met in mouse MSCs from bone marrow, PCR was performed on cDNA obtained from RNA extracts of purified MSCs. MSCs were found to express low levels of mRNAs for HGF and Met, both of which were upregulated upon 48 hours of treatment with HGF (20 ng/ml) (Fig. 1A). In parallel, PCR was performed also on non–reverse-transcribed RNA samples, to exclude the possibility of positive results due to contaminating genomic DNA. Real time RT-PCR performed on cells stimulated with HGF confirmed the upregulation of both HGF and its receptor, Met. Simultaneous treatment with 30 nM tyrosine kinase inhibitor K252a [30] nearly completely abolished the expression of HGF and its receptor (Fig. 1B). Untreated MSCs released in their supernatants low levels of HGF, detectable and quantified in a scatter assay in 32 scatter U/ml, equivalent to 7 ng/ml, when assayed in ELISA (enzyme-linked immunosorbent assay) [31]. The receptor was functional, as it could be phosphorylated upon stimulation with 100 ng/ml of HGF for 10 minutes (Fig. 1B). These data suggest that the exogenously added HGF could trigger the activation of both its own receptor and the ligand. MLP29 oval cells [32] were used as positive controls for Met expression.

HGF Activates ERK1/2, p38 MAP Kinases, and Akt

Because HGF activates downstream effectors, such as ERK1/2, p38 MAPKs, and PI3K in several cell types [18, 33], we

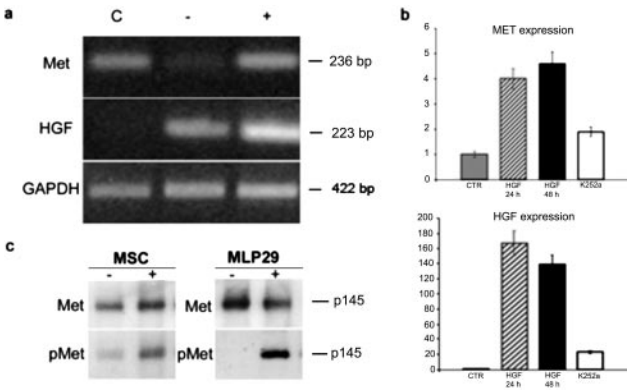

Figure 1. Hepatocyte growth factor (HGF) and Met expression in mouse mesenchymal stem cells (MSCs). **(A):** Reverse transcription–polymerase chain reaction (RT-PCR) analysis of RNA isolated from quiescent MSCs either untreated (−) or treated (+) with HGF (20 ng/ml) for 48 hours. The Met (top panel) and the HGF (middle panel) major transcripts are detectable in untreated MSCs and upregulated by HGF treatment of 48 hours. RNA from MLP-29 was used as control (**C**). To verify the quality of RNA, RT-PCR was carried out in parallel with a probe specific for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (bottom panel). **(B):** Quantitative PCR using HGF and Met-specific probes was performed on cDNA obtained from RNA extracted from MSCs treated, or not treated, with HGF in the presence, or absence, of tyrosine kinase inhibitor K252a. **(C):** Western immunoblotting analysis of Met expression (top panel) and HGF-induced activation (bottom panel) in MSCs. Detergent extracts from MSCs, as well as from cells expressing a physiological level of the Met receptor (MLP-29, positive control), were immunoprecipitated with a mixture of DO-24 and DN-30 Met antibodies, separated by SDS-PAGE, transferred onto membrane, and probed with SP-260 Met antibodies (Met) and antiphosphotyrosine antibodies (P-Met). Similar amounts of proteins were used as detected in Western blot with anti-Met antibodies (top panel). All experiments are representative of three replicates.

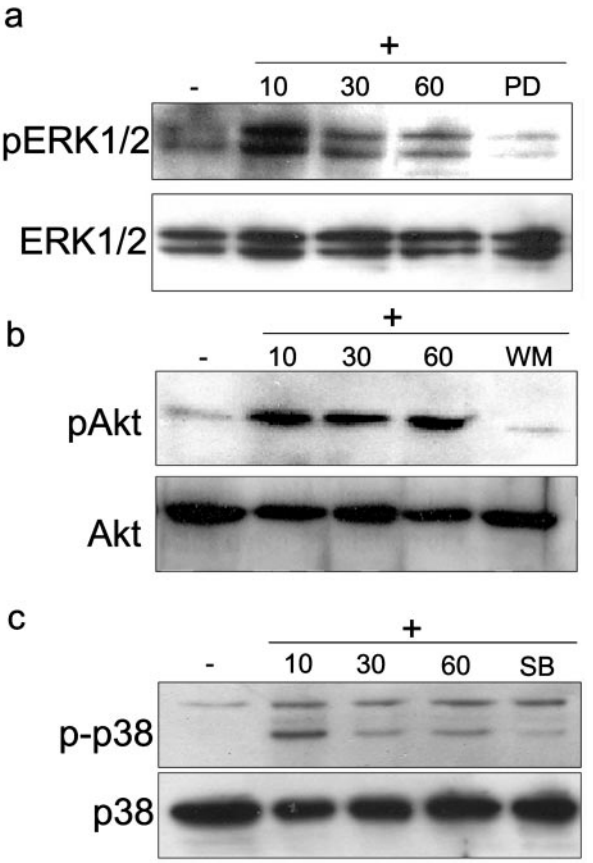

Figure 2. Hepatocyte growth factor (HGF) activates ERK1/2, p38 MAPKs, and Akt. **(A):** Quiescent mesenchymal stem cells were either left unstimulated (−) or stimulated with HGF at 20 ng/ml for the indicated times. Total cell lysates were resolved in SDS-PAGE, Western blotted, and immunoprobed with antibodies against the active phosphorylated forms of ERK1/2, p38, and Akt (pERK1/2, pp38, and pAkt) or against the total proteins (ERK1/2, p38, Akt). Protein phosphorylation induced at 10 minutes of stimulation was inhibited if cells were pretreated for 1 hour with specific inhibitors: PD98059 (30 μM PD), SB230580 (30 μM SB), and Wortmannin (100 nM WM), respectively. All experiments are representative of three replicates.

investigated the potential involvement of these effectors in HGF-stimulated MSCs. Indeed, phosphorylation of ERK 1/2 and p38 MAPKs was time-dependent, peaking within 10 minutes (Figs. 2A, 2C), whereas Akt phosphorylation was prolonged for at least 1 hour (Fig. 2B). These responses were completely abolished by 1 hour of pretreatment with 30 μM PD98059, 30 μM SB230580, and 100 nM Wortmannin, specific inhibitors of ERK1/2, p38, and PI3K, respectively. Finally, to strengthen the idea that these responses were specifically dependent on HGF-induced Met-mediated activation, preliminary experiments infecting MSCs with a lentiviral vector [34] carrying a small interfering RNA (siRNA) specific for Met (sequence 5′ ACUCUAGAUGCUCAGACUU 3′), previously reported to downregulate Met expression and HGF-dependent effects in other cell types [35], were performed. Indeed, in these cells, a significant reduction of ERK 1/2 phosphorylation was observed (Taulli et al., personal communication).

HGF Inhibits MSC Proliferation

HGF is a pleiotropic cytokine promoting multiple biological effects, namely mitogenesis, motogenesis, survival, and morphogenesis in a cell type–specific fashion [4]. Experiments were thus carried out to investigate the effect of HGF on MSC proliferation. Cells were plated at low density in IMDM plus 10% FCS for 24 hours. They were then starved in low FCS–containing medium (2%) for an additional 24 hours, treated or not treated for 1 hour with specific inhibitors for ERK 1/2, p38 MAPKs, and PI3K, and finally switched to 2% FCS supplemented or not supplemented with 20 ng HGF/ml, or to 10% FCS (control). Cells were replenished with respective fresh medium after 24 hours, and cell proliferation was monitored by [^{3}H]-thymidine uptake in a time-course experiment. The results re-

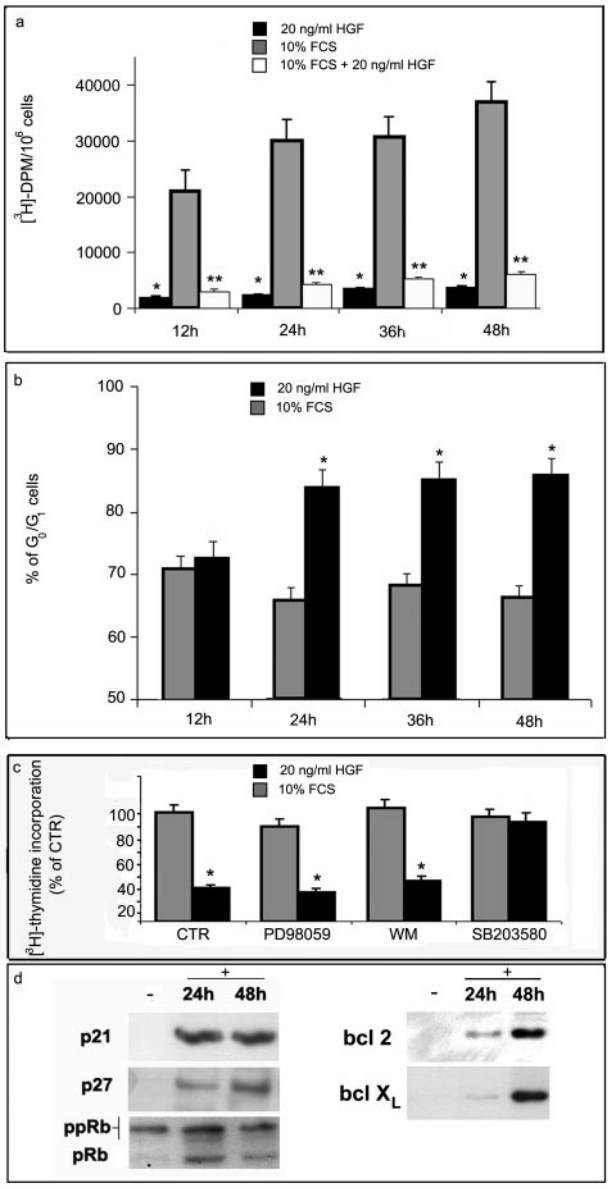

Figure 3. Effects of hepatocyte growth factor (HGF) on mouse mesenchymal stem cell (MSC) proliferation. **(A):** Cells (2.5×10^3) were seeded in 96-well microplates, grown for 12 hours in 10% fetal calf serum (FCS), and then starved in 2% serum for 24 hours. Cells were switched to 2% serum supplemented with HGF 20 ng/ml (black), or to 10% serum (grey), or to 10% serum containing HGF 20 ng/ml (white), and incubated for the indicated times, with change of medium every second day. Cells were pulsed with 1 μCi/ml [^{3}H]-thymidine, incubated for 3 hours, and counted for radioactivity by a liquid scintillation counter (Tricarb 2180 TR). **(B):** Cells prepared as described in Materials and Methods were stained with 25 μg/ml of propidium iodide in a solution containing 2% Triton X-100. The number of cells in G_0/G_1 phase was analyzed in a FACScalibur flow cytometer. **(C):** MSCs were treated, or not treated, with HGF after 1 hour of pretreatment with the indicated inhibitors: PD98059, Wortmannin, and SB203580. Cells were pulsed with 1 μCi/ml [^{3}H]-thymidine, incubated for 3 hours, and counted for radioactivity by a liquid scintillation counter (Tricarb 2180 TR). Each value represents the mean of six replicates $\pm$ SEM. Samples treated with SB203580 were not significantly different from the untreated control samples, indicating that this drug reverted the inhibition exerted by HGF. **(D):** Western blot analysis of p21^{waf1}, p27^{kip}, pRb (left panel) and of bcl-2 and bcl-X_L (right panel) expression in MSCs either untreated or treated with HGF for the indicated times. Bars represent the mean of four independent experiments $\pm$ SEM. *, $p <$ .001, compared with MSCs treated with 10% FCS. **, $p <$ .001, compared with MSCs treated with 10% FCS. All the experiments are representative of three replicates, unless otherwise stated.

ported in Figure 3A show that HGF did not induce a proliferative response in MSCs, whereas cells treated with 10% FCS grew significantly. The finding that HGF can inhibit the proliferation of distinct cell types [36] prompted us to investigate whether this was also the case for MSCs. Indeed, when quiescent MSCs were cultured in medium containing 20 ng/ml of HGF, cell proliferation induced by 10% FCS was nearly abolished (Fig. 3A). FACS analysis performed on stem cells treated, or not treated, with 20 ng/ml HGF for 12, 24, 36, and 48 hours, and stained with PI shows that HGF induces a significant increase in the G_0/G_1 ratio with respect to control untreated MSCs (Fig. 3B). In a parallel experiment, stem cells were doubly stained with PI and Annexin V to rule out the possibility that HGF treatment could induce cell death by apoptosis or necrosis. Indeed, no increase in the apoptotic or in the necrotic fractions was detectable in MSCs after HGF treatment (data not shown), and, actually, HGF displayed a faint, but reproducible, antiapoptotic activity. We also investigated which signaling pathway was responsible for the HGF-dependent inhibition of FCS-driven cell proliferation, by using specific inhibitors. As shown in Figure 3C, the block in cell proliferation, which was evident within 9 hours, was completely reverted when MSCs were pretreated with the p38 inhibitor SB203580, whereas no effects were observed using PD98059 and Wortmannin, inhibitors of ERK 1/2 MAPK and PI3K, respectively. HGF induced the expression of p21^{waf1} and p27^{kip}, both of which are recognized as universal cell cycle progression inhibitors. Consistently, the HGF-driven accumulation of the hypophosphorylated

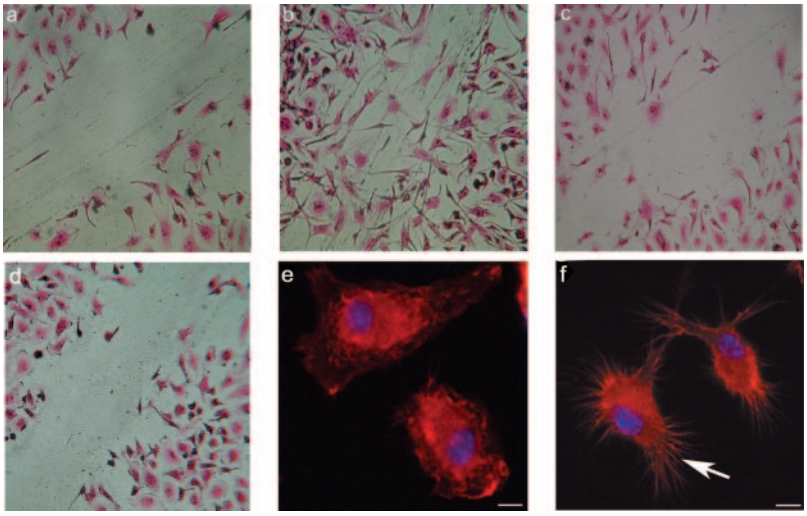

Figure 4. Hepatocyte growth factor (HGF) induces a motogenic response in mouse mesenchymal stem cells (MSCs). A confluent monolayer of MSCs grown in 5% fetal calf serum (FCS) and made quiescent by a 12-hour 2% FCS treatment was 'wounded' with a pipette tip and incubated for 24 hours in the absence **(A)** or presence of 20 ng/ml HGF **(B)** or pretreated with PI3K inhibitor Wortmannin **(C)** or tyrosine kinase inhibitor K252a **(D)** and then exposed to 20 ng/ml HGF. At the end of the treatment, cells were fixed with 2.5% glutaraldehyde and stained with hematoxilin and eosin. MSCs were also plated on coverslip, and when they reached 60% confluence, they were starved in low serum for 12 hours, untreated **(E)** or treated with HGF 20 ng/ml **(F)** for 24 hours, fixed with paraformaldehyde, permeabilized with Triton X-100, and stained for polymerized actin with tetramethylrhodamine isothiocyanate (TRITC)–conjugated Phalloydine. Arrow indicates microspikes protruding from cell surface. Bar = 7.5 μm.

forms of Rb, typical of quiescent cells [37], was detected. Finally, HGF treatment induced the expression of bcl-2 and bcl-X_L proteins, which is consistent with the antiapoptotic activity displayed by HGF on MSCs (Fig. 3D).

HGF Activates MSC Migration

HGF is identical to scatter factor, a molecule that was independently identified for its ability to induce motility in epithelial cells [38]. The chemoattractant ability of HGF on human MSCs was recently demonstrated [24]. A conventional scatter assay, in which cell colonies are dispersed, could not be performed, because MSCs are already basally spindle-shaped. A wound healing assay, which is generally considered a simple and reliable test for evaluation of cell motility, was thus carried out. Confluent cell monolayers were cultured in low FCS for 12 hours, wounded, and finally cultured in 2% FCS for 24 hours in the absence or presence of HGF. In the absence of HGF, MSCs displayed barely detectable healing after 24 hours, which is indicative of very low basal motility (Fig. 4A). Cells responded to HGF treatment by filling the wound within 24 hours (Fig. 4B), in a dose-response way (not shown), and this effect was completely abolished by adding 30 nM kinase inhibitor K252a or 100 nM Wortmannin 1 hour before HGF stimulation (Figs. 4C, 4D). This evidence suggested that PI3K/Akt pathway is involved in the scattering effect of HGF. At the molecular level, HGF-dependent migration was accompanied by cytoskeleton remodeling. Indeed, MSC treated with 20 ng/ml HGF for 24 hours, fixed, permeabilized, and decorated with Rhodamine-labeled Phalloydine displayed typical F actin–rich microspikes (Figs. 4D, 4E), which are peculiar for migrating cells [39]. HGF was also tested in a directional transwell migration assay. In the absence of ligand, MSCs showed a limited ability to cross the filter, whereas, in the presence of HGF (20 ng/ml) in the lower compartment, the number of cells crossing the filter was significantly increased (Fig. 5). This effect was completely inhibited by concomitant incubation with Wortmannin or the tyrosine kinase inhibitor K252a at concentrations mentioned above.

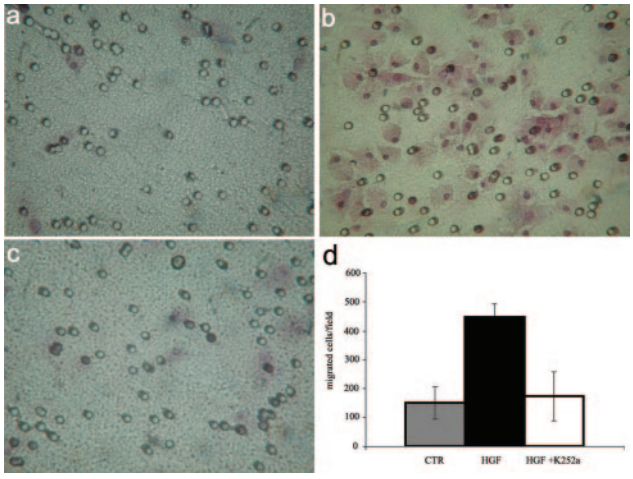

Figure 5. Hepatocyte growth factor (HGF) acts as chemoattractant for mouse mesenchymal stem cells. Cells were untreated **(A)** or treated with 20 ng/ml HGF **(B)** or pretreated with tyrosine kinase inhibitor K252a before adding HGF **(C)** in a transwell directional migration assay. At the end of incubation, the cells at the upper side of the filter were mechanically removed. Cells that had migrated to the lower side of the filter were fixed for 30 minutes in 11% glutaraldehyde and stained with hematoxylin and eosin. Five to ten random fields were counted for each filter **(D)**.

HGF Prompts MSC Differentiation Toward Cardiac Lineage

HGF is a potent differentiating factor for human embryonic stem cells and rat bone marrow mesenchymal cells [20, 22]. To test this potential effect on mouse MSCs, cells were cultured for several days in the presence of HGF (20 ng/ml) and the outcoming phenotype was thus examined by determining the expression of some muscle-specific genes using RT-PCR. RNA was isolated from cells treated for different periods of time.

After day 2 of treatment, MSCs lost stem cell markers, like nucleostemin, and started to express mRNAs for the muscle-specific transcription factors MEF2C (myocyte enhancer factor 2C) and TEF1 (transcriptional enhancer factor 1) (Fig. 6). Furthermore, at the same time, they expressed transcripts for desmin, α-MHC (α–myosin heavy chain), and β-MHC, while they did not express other muscular markers, such as MLC 2a (myosin light chain 2 atrial), MLC 2v (myosin light chain 2 ventricular), or ANP (atrial natriuretic peptide) (data not shown). Interestingly, α-MHC transcript was detected in a higher amount than β-MHC transcript. HL-1 cardiac cell line [40] was used as a positive control for myogenic differentiation of MSCs. Consistently with the onset of a differentiative program, immunofluorescence analysis (Fig. 7) showed that MSCs were no longer reactive for the stem cell markers c-kit (panels A, B) and CD105 (panels C, D) and they showed a positive staining for GATA-4 (panels M, N), a crucial transcription factor operating during the early phases of cardiac development, the intermediate filament protein nestin (panels E, F), and α-MHC, a component of the cardiac contractile system (panels G, H).

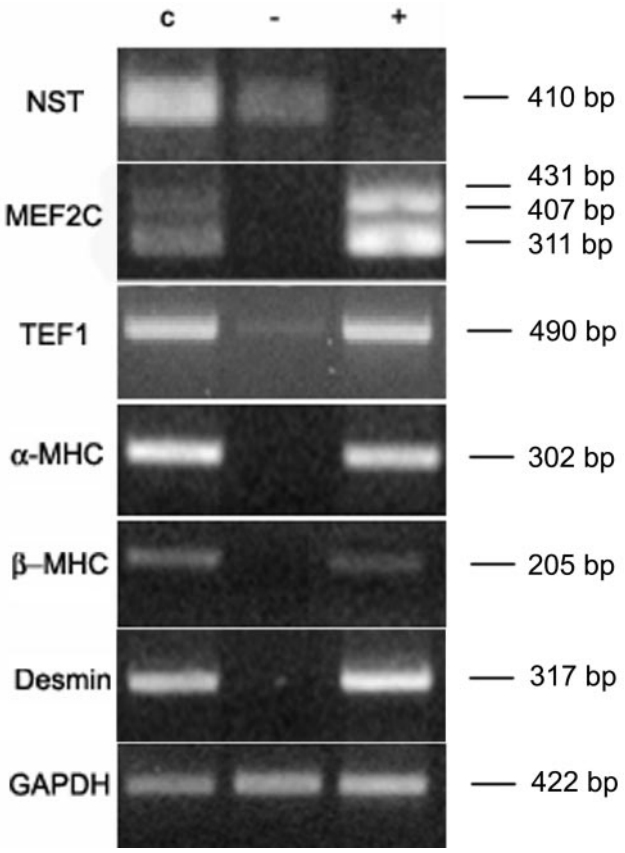

Figure 6. Hepatocyte growth factor (HGF) induces the expression of myocyte-specific transcription factors and structural genes in mesenchymal stem cells. Cells were untreated (−) or treated with 20 ng/ml of HGF (+) for 48 hours. Total RNA was isolated and after DNase I treatment, reverse transcription–polymerase chain reaction was performed with primers specific for nucleostemin (NST), myocyte enhancer factor 2C (MEF2C), transcriptional enhancer factor 1 (TEF1), α–myosin heavy chain (α-MHC), β–myosin heavy chain (β-MHC), desmin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described in Materials and Methods. HL-1 cells were used as positive controls. All experiments are representative of three replicates.

DISCUSSION

In this study, we investigated the effects of HGF on murine MSCs and found that HGF can induce early biochemical effects, such as receptor tyrosine phosphorylation and upregulation, activation of the major signaling pathways, as well as delayed biological responses, namely block of proliferation, cytoskeletal rearrangement, cell migration, and expression of cardiac-specific markers with concomitant loss of stem cell markers. We also show in some experiments that these effects were dependent on HGF stimulation, because they could be inhibited by the natural alkaloid K252a, which was shown to strongly impair the oncogenic properties of Met [30] or in MSCs infected with a lentiviral vector [34] carrying an siRNA specific for Met [35]. Finally, we present evidence that cell migration is PI3K-dependent, whereas inhibition of cell proliferation is p38-dependent.

Adult stem cells are attracting increasing attention because of their potential use in both developmental biology and medical applications, such as tissue and organ repair [1, 2]. It is now widely accepted that they are present in nearly all tissues, where they are probably already committed to a particular lineage, dictated by the microenvironment. In this context, bone marrow is the source of adult stem cells with greater differentiation potential [1], because it can give origin not only to hemopoietic and endothelial stem cells, but also to precursors of other tissues of mesenchymal origin, such as adipose tissue, bone, and cartilage [2], as well as to cells that can transdifferentiate in epithelial components, such as hepatocytes [21–23]. The fate of these stem cells is determined by microenvironment and growth/differentiation/mobilization factors, which can recruit them in different organs and situations, such as after injury.

HGF is a pleiotropic cytokine displaying mitogenic, motogenic, morphogenetic, and antiapoptotic activities in a cell type–specific manner [3], the main target cells being epithelial

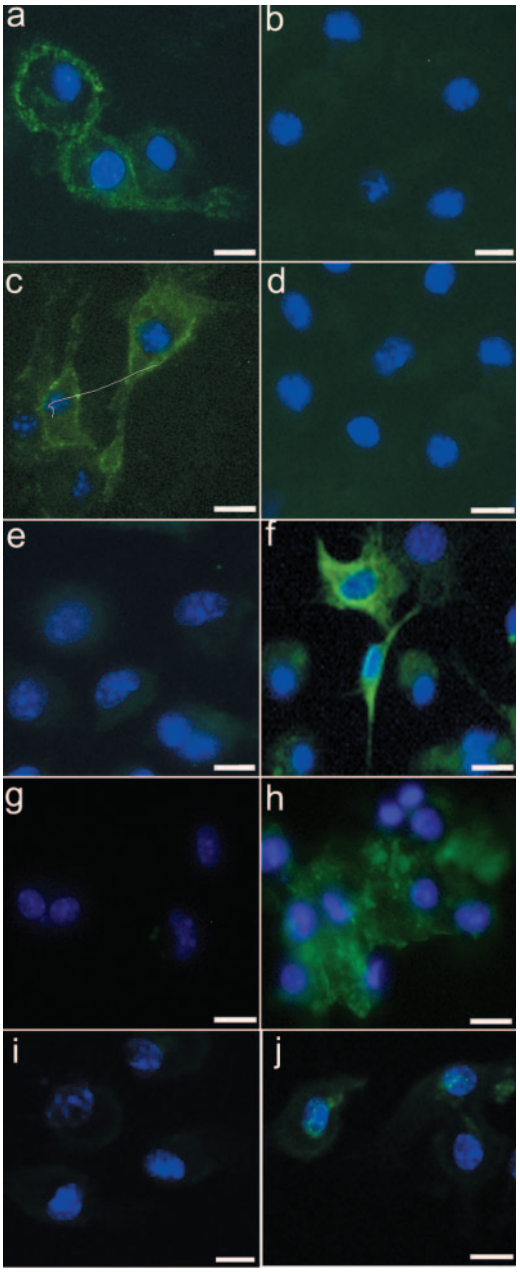

Figure 7. Hepatocyte growth factor (HGF) induces differentiation of mesenchymal stem cells. Cells were plated on coverslip, and when they reached 60% confluence, they were untreated **(A, C, E, G, I)** or treated with 20 ng/ml of HGF **(B, D, F, H, J)** for 96 hours. They were then fixed with paraformaldehyde, permeabilized with Triton X-100, and stained with antibodies for c-kit **(A, B)**, CD105 (TGFβrIII) **(C, D)**, nestin **(E, F)**, MHC (myosin heavy chain) **(G, H)**, and GATA-4 **(I, J)**, followed by the appropriate FITC (fluorescein isothiocyanate)–labeled secondary antibodies. DAPI (4′,6′-diamidino-2-phenylindole) counterstaining was used to visualize nuclei. Bar = 12.5 μm. In control experiments, in which the primary antibody was omitted, no fluorescent signal was detected. All experiments are representative of three independent replicates.

and endothelial cells, which predominantly express the cognate receptor. Indeed, HGF promotes hepatocytes, keratinocytes, renal tubule cell and endothelial cell proliferation, dissociation of epithelial cell colonies, cell motility, and invasion through extracellular matrix. The coordinated orchestration of these processes plays a major role in organogenesis, such as tubulogenesis and branching morphogenesis, and in tissue and organ homeostasis in adults, such as liver and kidney regeneration [14]. In vivo, HGF and Met receptor have been reported to play an important role in the early cardiac development [41], when they are transiently expressed. Recently, Duan and colleagues [25] showed that HGF is able to enhance MSC engraftment in injured heart. In the adult, upon heart injury, HGF plasma levels are rapidly and markedly elevated with cardioprotective significance. Indeed, exogenous HGF has a potent cardioprotective role in experimentally induced myocardial infarction, preventing cardiomyocyte apoptosis, inducing significant angiogenesis, and improving impaired heart functions [7, 42, 43]. These findings show that HGF can induce biological responses in cells other than epithelial.

More recently, a few studies have shown that HGF can induce biological responses also in stem cells [20, 22, 24].

Our results show that mouse MSCs coexpress functional forms of Met receptor as well as of its ligand. Yet, the low levels of HGF found in the culture medium of control samples are not sufficient to activate its own receptor on MSCs; the receptor, however, maintains the biochemical and biological responsiveness to exogenously added ligand. In fact, both the receptor and the ligand can be upregulated upon long-term HGF treatment. Indeed, it was already reported that Met receptor behaves as a delayed early gene, the expression of which in epithelial cells could be upregulated by treatment with serum, phorbol esters, and HGF itself [44]. After full ligand-dependent tyrosine phosphorylation, the Met receptor expressed on MSCs was able within a few minutes to activate the ras-ERK1/2 and p38 MAPKs as well as the PI3K/Akt pathways, which are the main transduction pathways activated in other HGF biologically responsive cells [3, 4, 19]. Depending on cell type, these pathways were reported to variably contribute to the different biological responses elicited by HGF. The ras-ERK1/2 MAPK pathway was shown to be mostly associated with HGF mitogenic and morphogenic effect, whereas the PI3K/Akt pathway seems to be mainly related to motogenic and antiapoptotic effects [4, 19, 45].

In our system, HGF activated cell migration in a PI3K-dependent way, because the effect of wound healing could be blocked by the PI3K pharmacological inhibitor Wortmannin.

Surprisingly, HGF inhibited cell proliferation by blocking cells in the G_0-G_1 phase. This response was accompanied by the induction of $p21^{waf1}$ and $p27^{kip}$ proteins, which are known as universal cell cycle progression inhibitors, acting through their binding to cyclins-CDK complexes and PCNA [46, 47] and by

the accumulation of the hypophosphorylated forms of pRb. Although HGF is generally recognized as a proliferation factor, it was already reported to inhibit the proliferation of certain cell types [36] as well as human MSCs [24]. No significant apoptosis was detected in the conditions used, and actually the low basal level of apoptosis was even decreased upon treatment with HGF. Consistent with this finding, bcl-2 and bcl-X_L proteins were induced by HGF. The arrest of cell proliferation was dependent on p38 MAPK, because it was abrogated by treatment with its specific inhibitor, SB203580. P38 has been shown to exert a similar role also in FGF-dependent chondrocyte proliferation [45]. Moreover, p38 MAPK pathway has been already implicated in chondrocyte and skeletal muscle differentiation [48–50].

In our model, upon 48 hours of HGF treatment, MSCs started to express mRNAs for MEF2C and TEF1, two transcription factors, and desmin, which are typically detectable during the first stages of muscle differentiation. Moreover, MSCs expressed transcripts for contractile proteins, such as α-MHC and β-MHC. In this transition toward myogenic differentiation, MSCs lost the expression of markers typically associated with the stem cell phenotype, such as CD105 (TGFβRIII), c-kit, and nucleostemin; remarkably, after 7 days of exposure to HGF, MSCs expressed nestin, a poorly organized form of MHC, and the transcription factor GATA-4, which is crucially involved in early phases of cardiac development. By contrast, these cells failed to express other muscle markers, such as MLCs or ANP, suggesting that further differentiative steps probably require additional factors. Other treatments were also reported to drive bone marrow cells toward the cardiomyocyte phenotype: for example, 5-azacytidine was able to induce morphologic, biochemical, and functional cardiomyocytes [51, 52], although the molecular events involved have yet to be identified. PDGF was reported to enhance the generation of bone marrow cell–derived cardiomyocytes in rat hearts [53], but these cells did not integrate with resident myocytes, again suggesting that additional factors are required for their full functionality.

In conclusion, our findings suggest that HGF could be one of the factors involved in the mobilization and commitment of MSCs toward a cardiomyocyte phenotype even if more detailed experiments will be necessary to clarify whether these processes occur in vivo as well.

ACKNOWLEDGMENTS

The authors are grateful to Isabella Screpanti (University of Rome ‘La Sapienza’), Andrea Graziani (Università del Piemonte Orientale ‘A. Avogadro’) for helpful discussion throughout these studies and for critically reading the manuscript. The authors thank also Riccardo Taulli (Università di Torino) for kindly providing the lentivirus-siRNA for Met and Syntech srl, Roma, Italy for its technical assistance. This study was supported by grants from Ministero Istruzione, Università e Ricerca (FIRB 2001 and PRIN 2003), ‘Compagnia di S. Paolo’, Torino, University of Piemonte Orientale ‘A. Avogadro’, Novara, and Regione Piemonte (Ricerca Scientifica Applicata 2004-CIPE), Italy.

DISCLOSURES

The authors indicate no potential conflicts of interest.

REFERENCES

1 Herzog EL, Chai L, Krause DS. Plasticity of marrow-derived stem cells. Blood 2003;102:3483–3493.

2 Bianco P, Riminucci M, Gronthos S et al. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 2001;19:180–192.

3 Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. Nat Rev Cancer 2002;2:289–300.

4 Birchmeier C, Birchmeier W, Gherardi E et al. Met, metastasis, motility and more. Nat Rev Mol Cell Biol 2003;4:915–925.

5 Santos OF, Barros EJ, Yang XM et al. Involvement of hepatocyte growth factor in kidney development. Dev Biol 1994;163:525–529.

6 Yang Y, Spitzer E, Meyer D et al. Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. J Cell Biol 1995;131:215–226.

7 Bussolino F, Di Renzo MF, Ziche M et al. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J Cell Biol 1992;119:629–641.

8 Ebens A, Brose K, Leonardo ED et al. Hepatocyte growth factor/scatter factor is an axonal chemoattractant and a neurotrophic factor for spinal motor neurons. Neuron 1996;17:1157–1172.

9 Bladt F, Riethmacher D, Isenmann S et al. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature 1995;376:768–771.

10 Maina F, Casagranda F, Audero E et al. Uncoupling of Grb2 from the Met receptor in vivo reveals complex roles in muscle development. Cell 1996;87:531–542.

11 Schmidt C, Bladt F, Goedecke S et al. Scatter factor/hepatocyte growth factor is essential for liver development. Nature 1995;373:699–702.

12 Uehara Y, Minowa O, Mori C et al. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. Nature 1995;373:702–705.

13 Matsumoto K, Nakamura T. Hepatocyte growth factor renotropic role and potential therapeutics for renal diseases. Kidney Int 2001;59:2023–2038.

14 Michalopoulos GK, DeFrances MC. Liver regeneration. Science 1997;276:60–66.

15 Danilkovitch-Miagkova A, Zbar B. Dysregulation of Met receptor tyrosine kinase activity in invasive tumors. J Clin Invest 2002;109:863–867.

16 Bottaro DP, Rubin JS, Faletto DL et al. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science 1991;251:802–804.

17 Naldini L, Weidner KM, Vigna E et al. Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. EMBO J 1991;10:2867–2878.

18 Ponzetto C, Bardelli A, Zhen Z et al. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell 1994;77:261–271.

19 Furge KA, Zhang YW, Vande Woude GF. Met receptor tyrosine kinase: enhanced signaling through adapter proteins. Oncogene 2000;19:5582–5589.

20 Schuldiner M, Yanuka O, Itskovitz-Eldor J et al. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2000;97:11307–11312.

21 Schwartz RE, Reyes M, Koodie L et al. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest 2002;109:1291–1302.

22 Oh SH, Miyazaki M, Kouchi H et al. Hepatocyte growth factor induces differentiation of adult rat bone marrow cells into a hepatocyte lineage in vitro. Biochem Biophys Res Commun 2000;279:500–504.

23 Okumoto K, Saito T, Hattori E et al. Differentiation of bone marrow cells into cells that express liver-specific genes in vitro: implication of the Notch signals in differentiation. Biochem Biophys Res Commun 2003;304:691–695.

24 Neuss S, Becher E, Woltje M et al. Functional expression of HGF and HGF receptor/c-met in adult human mesenchymal stem cells suggests a role in cell mobilization, tissue repair and wound healing. Stem Cells 2004;22:405–414.

25 Duan HF, Wu CT, Wu DL et al. Treatment of myocardial ischemia with bone marrow-derived mesenchymal stem cells overexpressing hepatocyte growth factor. Mol Ther 2003;8:467–474.

26 Friedenstein AJ, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hemopoietic organs. Exp Hematol 1976:4;267–274.

27 Prat M, Crepaldi T, Gandino L et al. C-terminal truncated forms of Met, the hepatocyte growth factor receptor. Mol Cell Biol 1991;11:5954–5962.

28 Montaldo F, Maffé A, Morini M et al. Expression of functional tyrosine kinases on immortalized Kaposi's sarcoma cells. J Cell Physiol 2000;184:246–254.

29 Condorelli G, Borello U, De Angelis L et al. Cardiomyocytes induce endothelial cells to trans-differentiate into cardiac muscle: implications for myocardium regeneration. Proc Natl Acad Sci U S A 2001;98:10733–10738.

30 Morotti A, Mila S, Accornero P et al. K252a inhibits the oncogenic properties of Met, the HGF receptor. Oncogene 2002;21:4885–4893.

31 Grano M, Galimi F, Zambonin G et al. Hepatocyte growth factor is a coupling factor for osteoclasts and osteoblasts in vitro. Proc Natl Acad Sci U S A 1996;93:7644–7648.

32 Medico E, Mongiovi AM, Huff J et al. The tyrosine kinase receptors Ron and Sea control 'scattering' and morphogenesis of liver progenitor cells in vitro. Mol Biol Cell 1996;7:495–504.

33 Muller M, Morotti A, Ponzetto C. Activation of NF-kappaB is essential for hepatocyte growth factor-mediated proliferation and tubulogenesis. Mol Cell Biol 2002;22:1060–1072.

34 Taulli R, Accornero P, Follenzi A et al. RNAi technology and lentiviral delivery as a powerful tool to suppress Tpr-Met-mediated tumorigenesis. Cancer Gene Ther 2005;12:456–463.

35 Pennacchietti S, Michieli P, Galluzzo M et al. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell 2003;3:347–361.

36 Shiota G, Rhoads DB, Wang TC et al. Hepatocyte growth factor inhibits growth of hepatocellular carcinoma cells. Proc Natl Acad Sci U S A 1992;89:373–377.

37 Niculescu AB 3rd, Chen X, Smeets M et al. Effects of p21(Cip1/Waf1) at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. Mol Cell Biol 1998;18:629–643.

38 Stoker M, Gherardi E, Perryman M et al. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature 1987;327:239–242.

39 Taher TE, Derksen PW, de Boer OJ et al. Hepatocyte growth factor triggers signaling cascades mediating vascular smooth muscle cell migration. Biochem Biophys Res Commun 2002;18:80–86.

40 Claycomb WC, Lanson NA Jr, Stallworth BS et al. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci U S A 1998;17:2979–2984.

41 Rappolee DA, Lyer A, Patel Y. Hepatocyte growth factor and its receptor are expressed in cardiac myocytes during early cardiogenesis. Circ Res 1996;78:1028–1036.

42 Aoki M, Morishita R, Taniyama Y et al. Angiogenesis induced by hepatocyte growth factor in non-infarcted myocardium and infarcted myocardium: up-regulation of essential transcription factor for angiogenesis, ets. Gene Ther 2000;7:417–427.

43 Jayasankar V, Woo YJ, Bish LT et al. Gene transfer of hepatocyte growth factor attenuates postinfarction heart failure. Circulation 2003;9:230–236.

44 Boccaccio C, Gaudino G, Gambarotta G et al. Hepatocyte growth factor (HGF) receptor expression is inducible and is part of the delayed-early response to HGF. J Biol Chem 1994;269:12846–12851.

45 Giordano S, Maffe A, Williams TA et al. Different point mutations in the met oncogene elicit distinct biological properties. FASEB J 2000;14:399–406.

46 Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1999;13:1501–1512.

47 Coqueret O. New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? Trends Cell Biol 2003;13:65–70.

48 Raucci A, Laplantine E, Mansukhani A et al. Activation of the ERK1/2 and p38 mitogen-activated protein kinase pathways mediates fibroblast growth factor-induced growth arrest of chondrocytes. J Biol Chem 2004;279:1747–1756.

49 Stanton LA, Sabari S, Sampaio AV et al. p38 MAP kinase signalling is required for hypertrophic chondrocyte differentiation. Biochem J 2004;378:53–62.

50 Baeza-Raja B, Munoz-Canoves P. p38 MAPK-induced nuclear factor-kappaB activity is required for skeletal muscle differentiation: role of interleukin-6. Mol Biol Cell 2004;15:2013–2026.

51 Makino S, Fukuda K, Miyoshi S et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 1999;103:697–705.

52 Grassi G, Maccaroni P, Meyer R et al. Inhibitors of DNA methylation and histone deacetylation activate cytomegalovirus promoter-controlled reporter gene expression in human glioblastoma cell line U87. Carcinogenesis 2003;24:1625–1635.

53 Xaymardan M, Tang L, Zagreda L et al. Platelet-derived growth factor-AB promotes the generation of adult bone marrow-derived cardiac myocytes. Circ Res 2004;94:E39–E45.