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Original article

# HMGB1-stimulated human primary cardiac fibroblasts exert a paracrine action on human and murine cardiac stem cells

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**Abstract**

High Mobility Box 1 Protein (HMGB1) is a cytokine released into the extracellular space by necrotic cells and activated macrophages in response to injury. We recently demonstrated that HMGB1 administration into the mouse heart during acute myocardial infarction induces cardiac tissue regeneration by activating resident cardiac c-kit$^+$ cells (CSCs) and significantly enhances left ventricular function. In the present study it was analyzed the hypothesis that human cardiac fibroblasts (cFbs) exposed to HMGB1 may exert a paracrine effect on mouse and human CSCs. Human cFbs expressed the HMGB1 receptor RAGE. Luminex technology and ELISA assays revealed that HMGB1 significantly enhanced VEGF, PlGF, Mip-1α, IFN-γ, GM-CSF, Il-10, Il-1β, Il-4, Il-1ra, Il-9 and TNF-α in cFbs cell culture medium. HMGB1-stimulated cFbs conditioned media induced CSC migration and proliferation. These effects were significantly higher to those obtained when HMGB1 was added directly to the culture medium. In conclusion, we provide evidence that HMGB1 may act in a paracrine manner stimulating growth factor, cytokine and chemokine release by cFbs which, in turn, modulate CSC function. Via this mechanism HMGB1 may contribute to cardiac tissue regeneration.


*Keywords:* Cardiac stem cells; Cytokines; Chemokines; Growth factors; Migration; Proliferation; Paracrine action

## 1. Introduction

The High Mobility Group Box 1 protein is a highly conserved non-histone DNA-binding protein involved in the regulation of gene expression [1]. In addition to its nuclear role, HMGB1 has been identified as a critical mediator of inflammation in response to injury. HMGB1 serum level strongly increases during sepsis [2], synovitis [3], arthritis [4] and ischemic injury [5,6]. Further, HMGB1 is released by necrotic cells and also by monocytes–macrophages upon exposure to proinflammatory cytokines [7]. Extracellular HMGB1 induces inflammatory cytokine production by monocytes–macrophages and neutrophils [2,8,9] and elicits endothelial cell proinflammatory responses by increasing ICAM and VCAM expression as well as Il-8 and MCP1 secretion [10,11]. These extracellular functions of HMGB1 are mediated by its binding to the Receptor for Advanced Glycation End products (RAGE) [12] as well as to the Toll Like Receptors 2, 4 and 9 (TLR2, TLR4, and TLR9) [13,14].

Recent studies indicate that HMGB1 can modulate some stem cell functions. Specifically, HMGB1 is a strong chemoattractant

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*in vitro* and *in vivo* for vessel-associated stem cells (mesoangioblasts) [15] and endothelial precursor cells (EPCs) [16]. In mice, HMGB1 administration to healthy skeletal muscle results in the recruitment of intra-arterial delivered mesoangioblasts [15] and EPC pre-stimulation with HMGB1 promotes their homing to ischemic tissues via an integrin-dependent EPC adhesion [16]. When delivered to skeletal and cardiac muscle following acute ischemia, HMGB1 enhances regeneration by activating endogenous stem cells [17,18]. We have previously shown that HMGB1 promotes *in vivo* CSC proliferation and differentiation into cardiomyocytes resulting in significant cardiac regeneration and preservation of cardiac function [17]. In the present study it was investigated whether HMGB1 affected c-kit$^+$ cardiac stem cell (CSC) function *in vitro*; specifically, it was addressed the direct effect of HMGB1 on these cells, as well as the effect of conditioned media from human cardiac fibroblasts (cFbs) pre-exposed to HMGB1. We reasoned that HMGB1 might increase growth factor and cytokine release by cardiac cells, which in turn modulate CSC functions. cFbs reside in the heart in their undifferentiated form. In pathologic conditions, including myocardial infarction, cFbs convert into myofibroblasts (MyoFbs), which exhibit morphologic features of both fibroblasts and smooth muscle cells and release growth factors important for granulation tissue and scar formation [19]. Growing experimental evidences propose an active role both for cFbs and MyoFbs in cardiac pathophysiology, including modulation of electrical properties and inflammatory response [20–22]. Recently, Urbanek et al. reported that physical interactions exist between cFbs and cardiac stem cells in the heart niches, suggesting that cFbs may represent nurse cells for CSC [23].

Our findings show that HMGB1 had a chemotactic effect on cFbs and enhanced growth factors, cytokine and chemokine release in the culture medium. Importantly, conditioned medium of HMGB1-treated cFbs stimulated CSC migration and proliferation.

## 2. Materials and methods

### 2.1. Reagents

Expression and purification of HMGB1 was performed by HMGBiotech (Milan, IT). Endotoxins were removed by passage through Detoxy-Gel columns (Pierce Biotechnology Inc., Rockford IL, USA). Recombinant HMGB1 was diluted in PBS and stored at −80 °C.

### 2.2. Isolation and culture of primary human cardiac fibroblasts

Human auricle fragments were obtained after signed informed consent from patients that underwent cardiac surgical intervention with extracorporeal circulation. Fragments were minced and incubated for 45 min at 37 °C with a PBS solution $Ca^{++}$ and $Mg^{++}$ free containing 1 mg/ml Collagenase II (Worthington, Biochemical Corporation, Lakewood, USA) and 0.2% Bovine Serum Albumin (BSA, Sigma-Aldrich, Milan, Italy). After digestion, the solution was passed trough nylon filter (70 μm, BDBiosciences, Milan, Italy) and centrifuged for 10 min at 1300 rpm. The pellet was suspended in culture medium and cells placed in an humidified incubator gassed with 5% $CO_2$ at 37 °C. After 2–3 h, adherent cells were mainly composed by fibroblasts [24] and non-adherent cells were removed. Cardiac fibroblasts (cFbs) were cultured either with complete Endothelial Growth Medium-2 (EGM-2, Cambrex, Milan, Italy) or Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Milan, Italy): F12 (Invitrogen) 1:1 containing 10% Fetal Bovin Serum (FBS, HyClone, Logan, USA), supplemented with 10.000 U/ml Penicillin, 10.000 μg/ml Streptomycin (Invitrogen) and 20 mM L-Glutamine (Sigma-Aldrich). Starvation media for cFbs and MyoFbs were Endothelial Basal Medium (EBM, Cambrex), which differently from EGM contained neither growth factors nor serum, and DMEM:F12 without serum, respectively. Normal cFbs and MyoFbs were used up to 12th passage.

### 2.3. Conditioned medium (CM) was obtained from untreated- and HMGB1-treated cFbs and MyoFbs

HMGB1 (0, 10, and 100 ng/ml) was added to the culture medium every 24 h. After 72 h, supernatants were collected and stored at −80 °C. CMs were used undiluted as culture medium for CSCs. The concentrated medium was used to culture auricle fragments.

### 2.4. Human auricle fragment processing

Human cardiac cells containing a fraction of c-kit$^+$ cells were obtained from auricle fragments cut in small pieces (1–2 $mm^3$) and plated at a density of 5–6 pieces in a 35 mm plate. These explants were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen), supplemented with 10% FBS, 10.000 U/ml Penicillin, 10.000 μg/ml Streptomycin, 20 mM L-Glutamine and 0.1 mM β-mercaptoethanol (Complete Explant Medium- CEM) [26]. After 2–3 weeks, when small bright round cells emerged from the explants, 10 ng/ml HMGB1 were added to the culture. After 4 days round bright cells were harvested and the percentage of c-kit$^+$ cells determined by FACS analysis.

### 2.5. Cytokine, chemokine and growth factor detection

Bio-Plex assay (Bio-Rad Laboratories, Milan, Italy), a bead-based multiplex immunoassay, was used to quantify cytokines chemokines and growth factors in the supernatants of untreated and HMGB1-treated cFbs and MyoFbs. For these experiments, a 27-plex assay was used (Bio-Rad) [25]. ELISA assays were performed to detect growth factors not included in the package (R&D Systems, Minneapolis, USA) according to manufacturer's instructions.

### 2.6. Cardiac murine cell isolation

Mouse CSCs were obtained from total heart of adult CD1 male mice and selected for the expression of c-kit antigen. Briefly, the heart was removed under anesthesia and perfused through a

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Langendorff system with: (a) washing solution to remove blood, composed by Minimum Essential Medium Eagle (MEM, Sigma-Aldrich) supplemented with 3 mM HEPES, 10 mM Taurine, 2 mM L-Glutamine, 10.000 U/ml Penicillin, 10.000 μg/ml Streptomycin (Invitrogen), at pH 7.4; (b) enzymatic solution obtained by adding 1 mg/ml Collagenase II (Worthington Biochemical Corporation, Lakewood, USA) and 0.1% BSA to the solution described in (a). After 20 min the heart was minced and suspended in 10 ml PBS containing 5% FBS. Centrifugation at 500 rpm for 2 min was used to separate and discard the cardiomyocyte fraction. The remaining cells were centrifuged in Lymphoprep gradient (Axis-Shield, Oslo, Norway) to obtain the mononuclear heart cell fraction. C-kit$^+$ cells were isolated from the mononuclear cell fraction by magnetic sorting (MACS, Miltenyi Biotech, Bergisch Gladbach, Germany) with phycoerythrin (PE)-conjugated anti-mouse CD117 (c-kit) antibody (clone 2B8, BD Pharmingen, San Diego, USA) and Miltenyi immunomagnetic beads against PE fluorochrome. Purity of sorted cells was determined by cytofluorimetry measurements.

All experimental procedures complied with the Guidelines of the Italian National Institutes of Health, with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee.

### 2.7. Western blot analysis

Adherent cFbs and MyoFbs were lysed with RIPA buffer (10 mM Tris–HCl at pH 7.4, 150 mM NaCl, 1% NP40, 1% Deoxycolic acid, 0.1% SDS and 10% Glycerol) containing proteinase inhibitor mixture (Boehringer Mannheim, Indianapolis, USA) and phosphatase inhibitors (10 mM sodium fluoride, 20 mM sodium vanadate). Intracellular proteins were extracted after 20 min rotation on ice and centrifugation at 14,000 rpm, 4 °C. Protein concentration was assessed using Bradford assay (Bio-Rad Laboratories). Total proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). After 1 h blocking in Tris Buffered Saline (TBS) containing 0.1% Tween 20, 5% skimmed milk, the membrane was incubated overnight at 4 °C with primary antibody (2 μg/ml mouse anti-human α-SMA, clone 1A4, Sigma-Aldrich; 1 μg/ml rabbit anti-RAGE, Sigma-Aldrich). After 30 min washing with the appropriate HRP-conjugated secondary antibody for 1 h, bound antibody was detected by enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

### 2.8. Migration assay

Chemotaxis was performed in microwell chemotaxis chambers (Neuroprobe, Cabin John, MD) using polycarbonate filters (Costar Scientific Corporation, Cambridge, USA). Briefly, filters (Costar Scientific Corporation, Cambridge, USA) with 8 μm pores were coated with 5 μg/ml porcine skin gelatin (Sigma-Aldrich) and used for cFb migration. CFbs starved overnight were harvested with trypsin, resuspended in EBM and placed in the upper chamber at a density of 80.000 cells/ml. EBM alone or supplemented with 5, 10, 100 or 200 ng/ml of HMGB1 was placed in the lower chamber; EGM-2 was used as positive control. After 8 h at 37 °C in 5% $CO_2$, cFbs migrated to the lower surface of the filter were stained with the Diff Quick kit (Dade Behring, Milan, Italy). Migrated cFbs were counted at 40× in 10 random fields per filter. Each experiment was performed in duplicate and repeated 4 times in independent experiments using cFbs obtained from 3 different patients.

To evaluate CSC migration, filters with 3 μm pores were used. For every experiment $2\times10^4$ cardiac kit$^+$ cells were resuspended in EBM, after magnetic sorting for c-kit antigen, and placed in the upper chambers. The lower chambers were filled with EBM alone, EBM supplemented with 10 ng/ml HMGB1, CM from untreated cFbs and 10 ng/ml HMGB1 treated cFbs. Cells that had migrated to the lower chamber after 4 h at 37 °C were collected by pipetting, viable cells were stained with Trypan Blue, allowed to settle on plastic dishes and counted at 40× in 5 random fields.

### 2.9. Immunofluorescence

CFbs and MyoFbs were fixed in PBS 4% paraformaldheyde and permeabilized in 0.1% Triton X-100. After blocking with PBS containing 1% BSA, cells were incubated overnight at 4 °C with the following primary antibodies: anti-α-smooth muscle actin (SMA) (clone 1A4, Sigma-Aldrich); anti-vimentin (clone VIM 13-2, DakoCytomation, Carpinteria, CA), anti-fibroblast surface protein, (clone 1B10, Sigma-Aldrich) followed by the incubation with the proper secondary antibody fluorescein isothiocyanate (FITC)-conjugated.

To evaluate DNA synthesis, CSCs were exposed to 30 μM 5-bromo-2′-deoxy-uridine (BrdU, Sigma-Aldrich) for 24 h, fixed in PBS containing 4% paraformaldheyde, permeabilized in 0.2% Triton X-100 and then incubated for 1 h in PBS/0.1% Triton X-100/5 mM $MgSO_4$ in the presence of mouse monoclonal anti-BrdU primary antibody (BD Bioscience, Belgium) and 100 U/ml DNAse. After 30 min incubation with secondary antibody cells were rinsed with PBS and nuclei stained by Hoescht 33342 (Sigma-Aldrich).

Cells were observed and photographed with an inverted Zeiss microscope equipped for epifluorescence.

### 2.10. Co-culture experiments

Total mononuclear cardiac murine cells were placed in the upper chamber of a Transwell permeable support (pore 0.4 μM, Corning Incorporated, Acton, USA), so that cFbs remained confined in the lower compartment. The Transwell system allowed the free diffusion of solutes, but prevented contact between the two cell populations.

### 2.11. Flow cytometry

Cardiac murine cells were incubated in PBS containing 5% FBS for 30 min on ice with Phycoerythrin-PE-conjugated

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monoclonal antibodies against c-kit (clone 2B8, BD Pharmingen) at 4 μg/mL and analyzed using FACScalibur Fluorescence Activated Cell Sorter (Becton-Dickinson, San Jose, CA). Isotype control was performed for each experiment (BD Pharmingen).

### 2.12. Endothelial differentiation assays

Endothelial differentiation assays were performed in glass chamber slides (Invitrogen, Frederick, MD) coated with fibronectin (FN, 20 μg/mL, Sigma-Aldrich). $10^5$ c-kit$^+$ cells were cultured for 6 days in CM from untreated and 10 ng/ml HMGB1-treated cFbs. and during the last 24 h, cells were incubated with 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labeled acetylated LDL (Ac-LDL-DiI 10 ng/ml, Biomedical Technologies, Inc, Stoughton, MA, USA) as an indicator of endothelial cells differentiation. After fixation with 4% paraformaldheyde, cells were stained with Hoechst 33258 nuclear dye and Ac-LDL-DiI$^+$ cells, counted.

### 2.13. Statistical analysis

Results are presented as mean ± standard error (SE). Statistical analysis was performed by paired Student's *t* test. A probability value of $p < 0.05$ was considered significant.

## 3. Results

### 3.1. Culture and characterization of human primary cardiac fibroblasts

Cells expressing vimentin and α-smooth muscle actin (α-SMA) and negative for desmin (a marker for smooth muscle cells), can be reliably identified as cFbs [27]. MyoFbs exhibit a more spreaded shape compared to cFbs and well defined stress fibers constituted by organized α-SMA.

Since cFbs plated in plastic dishes and cultured in 21% $O_2$, spontaneously differentiate into MyoFbs [27,28], we sought to identify culture conditions to keep cFbs in their undifferentiated

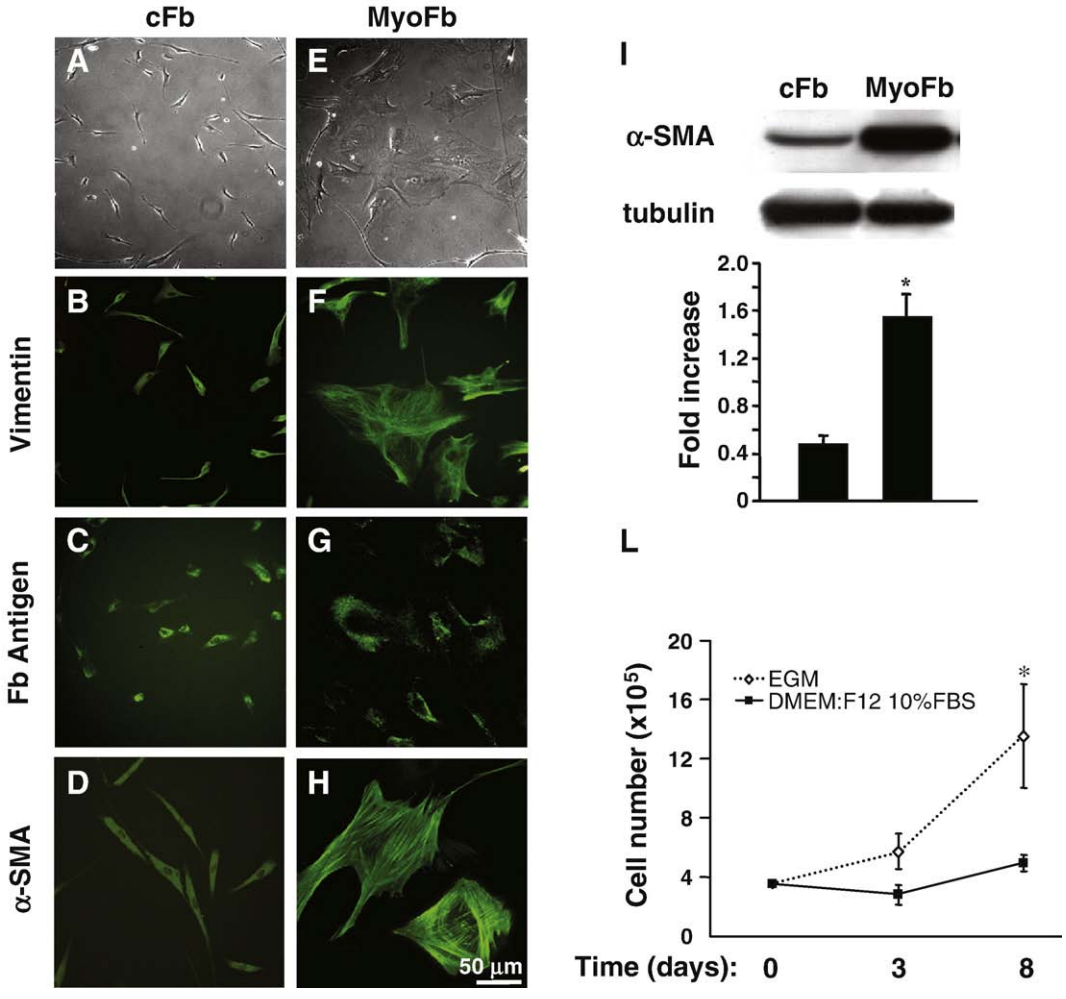


Fig. 1. Characterization of human cardiac fibroblasts. cFbs were cultured either in EGM-2 (A–D) or in DMEM:F12 1:1 containing 10% FBS (E–H). Contrast images (A, E) and immunofluorescence analysis to detect vimentin (B, F), fibroblast (Fb) antigen (C, G) and α-Smooth Muscle Actin (α-SMA) (D, H). (I) Western blot analysis of total extracts from cFbs cultured in EGM-2 and DMEM:F12 with 10% FBS, respectively to detect α-SMA. The same filter was probed with anti α-tubulin to show equal proteins loading. Lower panel: average results, normalized to α-tubulin, of α-SMA densitometric analyses of western blots ($n = 3$, $^*p < 0.05$). (L) CFbs and MyoFbs proliferation assay. Time-dependent changes in the number of cFbs and MyoFbs cultured in EGM and DMEM:F12 with 10% FBS, respectively ($n = 3$, $^*p < 0.05$).

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form. This represented an important issue since the regenerative effects induced by HMGB1 injection into the infarcted mouse heart was obtained 4 h after coronary ligation [17], a time when cFbs do not display a MyoFbs phenotype. It is noteworthy that MyoFbs number in the granulation tissue achieves its peak approximatively 7 days after infarction in human [29] and 4 days after infarction in mouse [30].

CFbs, isolated from human auricles and cultured either in EGM-2 (Figs. 1A–D) or DMEM:F12 with 10% FBS (Figs. 1E–H) expressed vimentin (Figs. 1B, F) and were negative for desmin. Moreover by immunoflourescence, cFbs expressed neither endothelial markers Flk-1 and Flt-1 nor cardiac markers, MEF-2C and Nkx2.5 (data not shown). Like human skin fibroblasts, EGM and DMEM-cultured cFbs were also positive for the specific human fibroblast surface protein (Figs. 1C, G) [31]. However, DMEM:F12-cultured cFbs exhibited a MyoFb phenotype since they appeared larger than EGM-cultured cFbs and expressed higher levels of α-SMA organized in stress fibers (Figs. 1D, H, I). Moreover, proliferation rate of DMEM:F12-cultured cFbs was statistically lower than EGM-cultured cFbs (Fig. 1L). EGM-cultured cFbs, retained the more undifferentiated phenotype at least up to 12th passage and acquired the MyoFb phenotype within 7 days (data not shown) of culture in DMEM:F12 containing 10% FBS. Therefore, EGM and DMEM culture conditions were used in the present study to grow cFbs and MyoFbs, respectively.

### 3.2. HMGB1 effects on cFbs functions

To evaluate whether HMGB1 modulated cFb cell functions, we first investigated the expression levels of HMGB1 receptor RAGE in both cFbs and MyoFbs. Western blot analysis revealed higher levels of RAGE in cFbs compared to MyoFbs (Fig. 2A). This result prompted us to evaluate whether HMGB1 modulated cFb invasion, proliferation and their differentiation into MyoFbs. Invasion assays were performed in a modified Boyden chamber assay in which cells migrate from the upper chamber

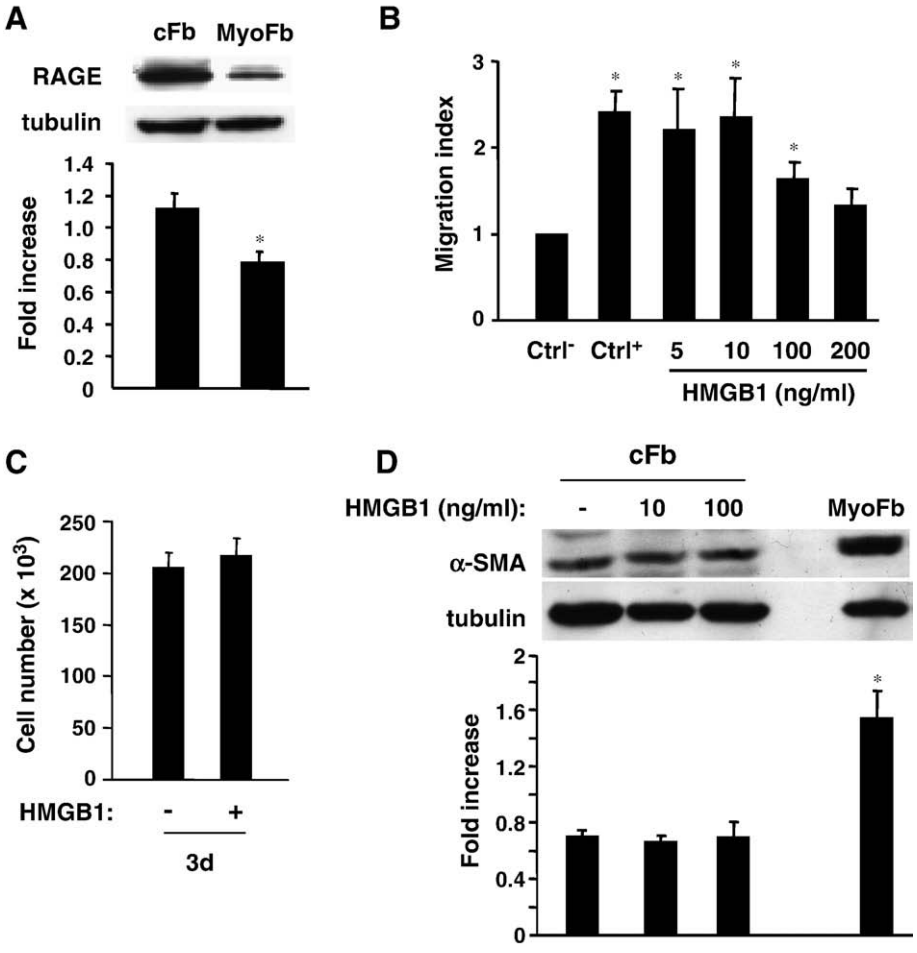


Fig. 2. Effect of HMGB1 on cFbs. (A) RAGE expression in cFbs and MyoFbs. Western blot analysis of total extracts from cFbs and MyoFbs. Filter was also probed with α-tubulin, to demonstrate equal loading. Lower panel: average results, normalized to α-tubulin, of RAGE densitometric analyses of western blots ($n=3$, $^*p<0.05$). (B) HMGB1 exerts a chemotactic effect on cFbs. EBM and EGM-2 were used as negative ($Ctrl^-$) and positive controls ($Ctrl^+$), respectively. HMGB1 was added in EBM medium at the indicated concentrations. The data are expressed as the fold increase in the number of migrated cells relative to the number of migrated cells in the absence of factor (migration index) and represent the mean ± SE of 4 independent experiments performed in duplicate ($^*p<0.05$ vs the negative control). (C) HMGB1 has no effect on cFb proliferation. cFbs ($2\times10^5$) were plated in EBM and either left untreated (−) or treated with 10 ng/ml HMGB1 (+) for 3 days. (D) HMGB1 has no effect on α-SMA expression. Western blot analysis of total extracts from EGM-2-cultured fibroblast untreated and treated for 3 days with the indicated concentrations of HMGB1. Lower panel: average results of α-SMA densitometric analyses normalized to α-tubulin, of western blots ($n=3$, $^*p<0.05$).

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through a gelatin-coated nucleopore filter to the lower chamber containing the chemotactic factor. HMGB1, added to the culture medium in the lower compartment, exhibited a dose-dependent chemotactic effect on cFbs. The chemotactic activity of both 5 and 10 ng/ml HMGB1 was comparable to that induced by EGM-2, while the higher dose of 200 ng/ml failed to enhance cell migration (Fig. 2B). In contrast, 10 ng/ml HMGB1 had no effect on cFb proliferation and cell number of HMGB1-treated cFbs was similar to control after 3 days in culture either (Fig. 2C).

To investigate whether HMGB1 modulated differentiation of cFbs into MyoFbs, cells were exposed to EGM either in the presence or absence of HMGB1. Western blot analysis of total extracts revealed no difference in α-SMA accumulation between untreated and HMGB1-treated cFbs. As expected, in the absence of treatment, α-SMA levels were higher in MyoFbs compared to cFbs (Fig. 2D).

### *3.3. HMGB1 stimulates growth factor, cytokine and chemokine release by cFbs*

To explore whether HMGB1 promoted growth factors and cytokines release by cFbs and MyoFbs, a multiplex bead-based immunoassay of 27 cytokines was performed.

Unstimulated cFbs in serum free medium (EBM) released basal amounts of VEGF, IFN-γ, MIP-1α, Il-9, Il-10, Il-4, Il-1ra, GM-CSF, Il-1β, TNF-α, PDGF-BB, Mip-1β, Il-13, Il-7, bFGF, Rantes, Eotaxin, and IP-10 (Table 1). MyoFbs also released cytokines in basal conditions, with a different secretion profile compared to cFbs (Table 1). Specifically, MyoFbs secreted significantly higher quantities of VEGF and Eotaxin, while GM-CSF, Il-1β, TNF-α, PDGF-BB, Mip-1β and Il-13 were undetectable in MyoFb-conditioned medium. HMGB1 administered to cultured cFbs at the concentration of 10 ng/ml, significantly increased levels of VEGF, IFN-γ, Mip-1α, Il-9, Il-10, Il-4, Il-1ra, GM-CSF, Il-1β, and TNF-α (Fig. 3 and Table 1). The HMGB1-mediated production of these factors was attenuated in the presence of a higher HMGB1 concentration (100 ng/ml). HMGB1 stimulation had a less prominent effect on MyoFbs: in the conditioned medium of 10 ng/ml HMGB1-treated MyoFbs only Il-9 and bFGF statistically increased (Table 1).

ELISA assay was performed to confirm findings obtained with Multiplex immunoassay and to detect growth factors of interest not included in the array package. By ELISA, VEGF levels were significantly higher in HMGB1-treated compared to untreated cFbs confirming the high degree of correlation between the two technologies (Fig. 4A). PlGF, IGF-1, HGF and SDF-1 levels were evaluated in the medium of untreated- and HMGB1-treated cFbs. CFbs secreted all these factors in basal conditions (IGF-1 = 81.28 ± 43.02 pg/$10^5$ cells/24 h, $n = 5$; HGF = 192.70 ± 87.21 pg/$10^5$ cells/24 h, $n = 4$; SDF-1 = 1977.83 ± 374.08 pg/$10^5$ cells/24 h, $n = 3$), however only PlGF secretion increased in response to both 10 and 100 ng/ml HMGB1 (Fig. 4B).

Taken together these results demonstrate that HMGB1 modulates cytokines, chemokines and growth factors release by cFbs. Moreover, HMGB1-induced factor release is attenuated in MyoFbs, a result consistent with the reduced HMGB1 receptor levels in these cells.

Table 1
Cytokines chemokines and growth factors released in the supernatant of untreated and HMGB1-treated cFbs and MyoFbs

| | Fibroblasts | | | Myofibroblasts | | |
|---|---|---|---|---|---|---|
| | pg/10^5 cells/24h | Fold increase vs 0 ng/ml HMGB1 | | pg/10^5 cells/24h | Fold increase vs 0 ng/ml HMGB1 | |
| Cytokine | 0 ng/ml HMGB1 | 10 ng/ml HMGB1 | 100 ng/ml HMGB1 | 0 ng/ml HMGB1 | 10 ng/ml HMGB1 | 100 ng/ml HMGB1 |
| VEGF | 255.01± 152.54 | 1.72±0.35 * | 1.28±0.32 | 939.46±249.06 § | 1.55±0.29 | 1.57±0.45 |
| IFN-γ | 23.69±10.48 | 1.81±0.33 * | 1.44±0.34 | 37.59±15.62 | 1.25±0.17 | 1.35±0.23 |
| MIP-1α | 1.12±0.40 | 1.46±0.12 * | 1.25±0.06 * | 1.87±0.74 | 1.22±0.16 | 1.93±0.68 |
| Il-9 | 5.55±1.55 | 1.49±0.20 * | 1.45±0.25 | 6.60±1.63 | 2.05±0.27 * | 1.92±0.77 |
| Il-10 | 3.29±2.18 | 3.72±1.63 * | 2.59±1.02 | 8.45±2.37 | 1.58±0.41 | 0.76±0.38 |
| Il-4 | 1.02±0.64 | 2.96±0.87 * | 2.71±0.89 * | 0.87±0.24 | 1.42±0.44 | 1.27±0.32 |
| Il-1ra | 5.85±2.75 | 2.82±1.09 * | 1.71±0.28 | 11.08±3.69 | 1.24±0.13 | 1.73±0.60 |
| GM-CSF | 2.63±1.43 | 2.92±0.87 * | 3.53±1.45 | OOR< | | |
| Il-1β | 0.30±0.21 | 2.10±0.66 * | 1.86±0.73 | OOR< | | |
| TNF-α | 1.67±0.96 | 1.51±0.21 * | 3.33±2.20 | OOR< | | |
| PDGF-BB | 0.12±0.07 | 1.24±0.14 | 1.33±0.17 | OOR< | | |
| MIP-1β | 0.09±0.04 | 2.46±0.96 | 3.69±2.43 | OOR< | | |
| Il-13 | 0.06±0.04 | 1.53±0.39 | 2.45±1.71 | OOR< | | |
| Il-7 | 2.76±1.91 | 1.59±0.59 | 1.42±0.53 | 0.57±0.21 | 1.05±0.24 | 2.29±1.47 |
| bFGF | 6.99±4.28 | 1.11±0.11 | 0.74±0.22 | 0.64±0.18 | 1.68±0.22 * | 1.15±1.07 |
| RANTES | 389.25±203.24 | 2.32±0.78 | 1.48±0.28 | 43.49±27.69 | 1.23±0.32 | 1.64±0.62 |
| Eotaxin | 122.72±73.37 | 2.13±0.54 | 1.31±0.39 | 633.42±92.08 § | 1.39±0.29 | 1.08±0.32 |
| IP-10 | 176.77±65.51 | 2.46±0.90 | 2.67±1.11 | 33.15±23.08 | 1.08±0.31 | 1.44±0.64 |

CFbs and MyoFbs were cultured in EGM-2 and in DMEM:F12 containing 10% FBS, respectively. Then cells were transferred in starvation medium (EBM for cFbs and DMEM for MyoFbs). HMGB1 was added at 10 and 100 ng/ml. Supernatants were collected after 3 days and analysed by Bio-plex assay. Data were expressed both in pg/ml normalized for cell number and as fold increase of HMGB1-treated vs untreated cells. Results were obtained from 4 independent experiments performed with cFbs and MyoFbs isolated from 4 different patients. *$p < 0.05$ vs untreated cFbs (−).

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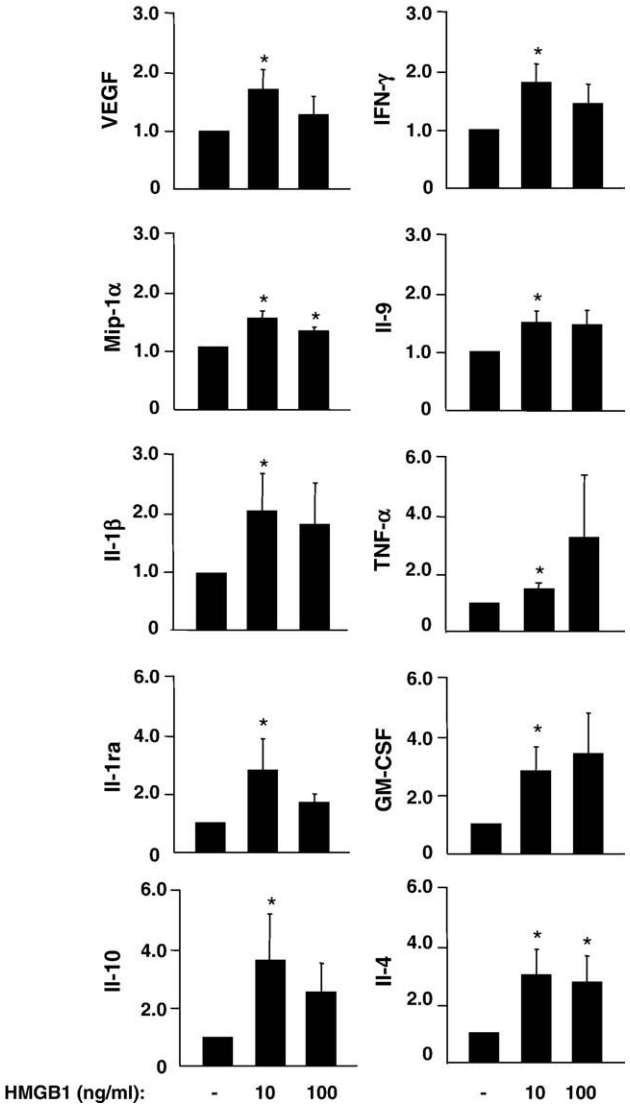


Fig. 3. HMGB1 enhances growth factors, cytokines and chemokines release from cFbs. CFbs were cultured in EGM-2 and then transferred in starvation medium (EBM). HMGB1 was added at 10 and 100 ng/ml. Supernatants were collected after 3 days and analysed by Bio-plex assay. Data were obtained from 4 independent experiments performed with cFbs isolated from 4 different patients and expressed as fold increase. *$p<0.05$ vs untreated cFbs (−).

### 3.4. Conditioned medium obtained from HMGB1-treated cFbs stimulates CSC migration and proliferation

To investigate whether HMGB1 modulated CSC functions in a paracrine manner, the conditioned medium (CM) obtained from untreated- and HMGB1-treated cFbs was used to stimulate CSC migration and proliferation. CSCs were isolated from murine hearts and selected for the expression of c-kit antigen [32]. Usually, it was possible to obtain from a single mouse heart from 2 to $5\times10^5$ c-kit$^+$ cells. FACS analysis revealed that c-kit$^+$ cells were negative for the hematopoietic marker CD45 [32] while about 20% of c-kit$^+$ cells expressed the endothelial precursor markers Flk-1 and Flt-1 (data not shown). Assays performed in the Boyden chamber, revealed that the CM from HMGB1-stimulated cFbs significantly increased CSC migration compared to CM of untreated cFbs (Fig. 5A). Importantly, the chemotactic effect of CM from HMGB1 stimulated cFbs was higher to that obtained when HMGB1 was directly added to the culture medium (Fig. 5A). The CM from unstimulated cFbs had a chemotactic activity to that of HMGB1 alone (Fig. 5A).

To determine whether CM from HMGB1-stimulated cFbs also induced CSC proliferation, murine c-kit$^+$ cells were plated at a density of $1.5\times10^5$ cells/ml and cultured in the presence of CM of untreated- and HMGB1-treated cFbs. After 48 h, the number of cells was significantly higher when CM was obtained from HMGB1-treated cFbs compared to CM from untreated cFbs ($16.8\pm2.2\times10^4$ vs $11.9\pm1.0\times10^4$ cells/ml) (Fig. 5B). At the same time point and in presence of HMGB1 added to the culture medium, CSCs were not detected (data not shown). BrdU incorporation experiments performed under these experimental conditions, revealed a significantly higher percentage of BrdU$^+$ nuclei among CSCs, in presence of CM derived from HMGB1-treated cFbs ($6.90\pm1.96$%) compared either to CM from untreated cFbs ($1.93\pm0.41$%) and HMGB1 added to the culture medium ($1.20\pm0.79$%) (Figs. 5C, D). Similar results were obtained when total cardiac murine mononuclear cells were co-cultured with cFbs either in the presence or in the absence of HMGB1. This approach was used to guarantee the constant production of growth factors, cytokines and chemokines by HMGB1-stimulated cFbs. Co-culture experiments were performed using a Transwell permeable support, which allows the free diffusion of solutes preventing the physical contact of cells plated at the bottom of the two chambers. After 48 h co-culture without HMGB1, c-kit$^+$ cell percentage was $0.25\pm0.05$%. HMGB1 administration rescued the percentage of c-kit$^+$ cells to $0.43\pm0.04$%. Interestingly, c-kit$^+$ cells were not detected when cultured in medium containing 10 ng/ml of HMGB1 in the absence of cFbs (data not shown). At longer time points in culture, no further increase in c-kit$^+$ cell proliferation was observed. It is noteworthy that the CM did not contain serum and it was not concentrated; therefore, factors released could not be sufficient to sustain proliferation for longer times.

Finally, we evaluated the effect of CM from HMGB1-stimulated cFbs on human cardiac explant cultures. The same number of human auricle fragments (5–6 in a 35 mm plate), dimensionally comparable

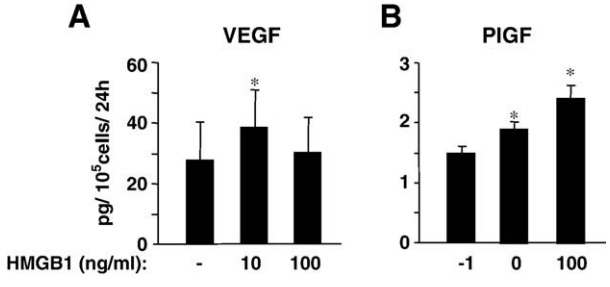


Fig. 4. HMGB1 enhances VEGF and PlGF secretion by cFbs. CFbs were cultured in EGM-2 and then transferred in starvation medium (EBM). HMGB1 was added at 10 and 100 ng/ml. Supernatants were collected after 3 days and analysed by ELISA. (A) HMGB1 (10 ng/ml) treatment significantly increased VEGF concentration in cFbs supernatants compared to untreated cFbs (−); ($n=6$ $p<0.05$). (B) PlGF secretion was significantly higher in 10 and 100 ng/ml HMGB1-stimulated cFbs, compared to unstimulated; $n=4$, *$p<0.05$ vs untreated cFbs.

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(1–1.5 mm$^2$), were cultured as explants. After 2–3 weeks, a population composed by suspended cells different in size and shape emerged from cultured fragments (Fig. 6A). At that time 10 ng/ml HMGB1 were added to the culture. After 4 days round bright cells were harvested from the culture and the percentage of c-kit$^+$ cells determined by FACS analysis. The administration of 10 ng/ml of HMGB1 to the explants increased by ~38% the percentage of c-kit$^+$ cells (Fig. 6B).

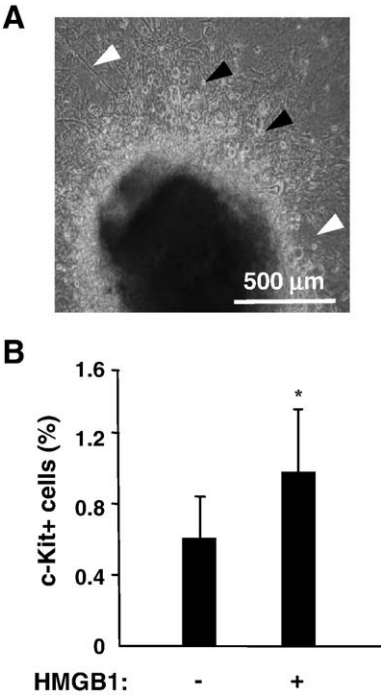


Fig. 6. Conditioned medium of HMGB1-treated cFbs enhances human c-kit$^+$ cells recovery from human cardiac explants. (A) Phase micrograph of human auricle fragments. CFbs (white arrows) and small bright round cells (black arrows) emerging from the sample are indicated. (B) FACS analysis of c-kit cells in the explant cultures untreated (−) and treated with 10 ng/ml HMGB1 (+); ($n$=7, *$p$<0.05).

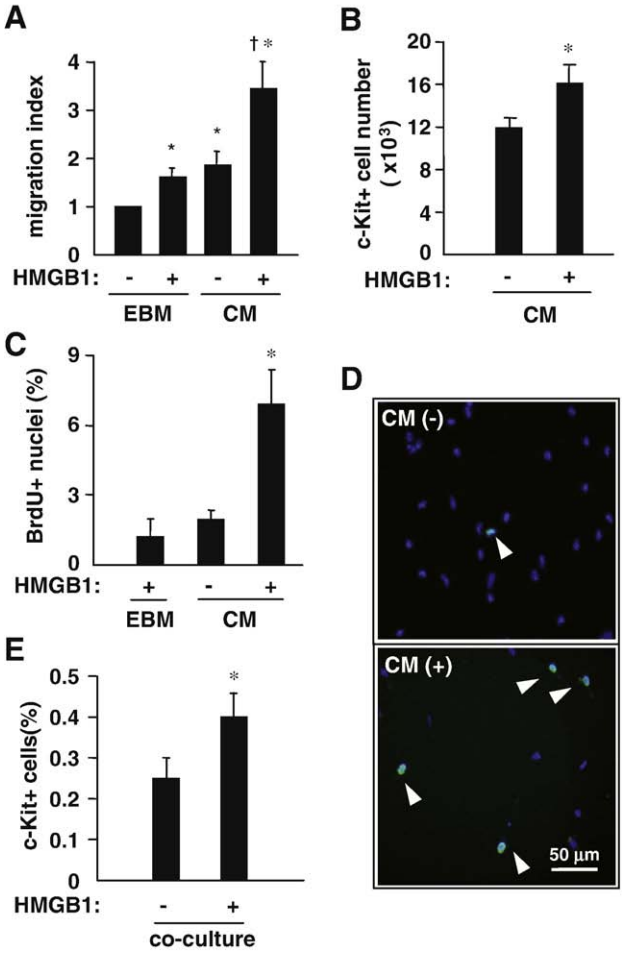


Fig. 5. Conditioned medium of HMGB1-treated cFbs induces murine CSC migration and proliferation. (A) 2×10$^4$ murine c-kit$^+$ cells were placed in EBM in the upper compartment of the modified Boyden chambers. In the lower compartment of the chamber were added: EBM without (−) and with 10 ng/ml HMGB1 (+), CM from untreated (−) and 10 ng/ml HMGB1-treated (+) cFbs. The data are expressed as the fold increase in the number of migrated cells relative to the number of migrated cells in the absence of factor (migration index) and are the means SE of at least 4 independent experiments performed in duplicate. *$p$<0.05 vs EBM (−); †$p$<0.05 vs EBM (+) and CM (−). (B) Murine c-kit$^+$ were cultured in the presence of CM from untreated (−) and 10 ng/ml HMGB1-treated (+) cFbs. After 48 h cells were harvested and counted. (C) Quantification of BrdU expressing c-kit$^+$ cells. Cells were cultures as in (B) and BrdU added the last 24 h ($n$=3; *$p$<0.05) (D) Representative BrdU staining. Blue light fluorescence (arrowheads), BrdU$^+$ cells; blue fluorescence, Hoechst 33342 of nuclei. Bar=50 µm. (E) Bar graph of flow cytometric measurements of c-kit$^+$ cells in the total non-myocyte cardiac fraction after 48 h co-culture with untreated (−) and HMGB1-treated (+) cFbs. The percentage of c-kit$^+$ cells in the non-myocytes fraction of cardiac murine cells detected by FACS analysis was 0.36±0.27% before co-culture experiments. $n$=3, *$p$<0.05.

### 3.5. Conditioned medium obtained from HMGB1-treated cFbs stimulates CSC differentiation toward the endothelial phenotype

The detection of angiogenic factors in the CM of HMGB1-treated cFbs prompted us to evaluate the effect of CM on CSC differentiation toward the endothelial phenotype. Murine cardiac c-kit$^+$ cells were cultured on fibronectin-coated dishes either with CM of untreated- or HMGB1-treated cFbs. After 7-day, c-kit$^+$ cells acquired the endothelial phenotype, as shown by their capacity to uptake 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labeled acetylated LDL (Ac-LDL-DiI) (Figs. 7A, B). The number of Ac-LDL-DiI$^+$ cells were significantly higher in presence of CM from HMGB1-treated cFbs compared either to CM from untreated cFbs or HMGB1 added directly to the culture medium (Figs. 7A, B). These last two culture conditions did not modify the fibronectin-induced endothelial differentiation.

## 4. Discussion

HMGB1 is a pleiotropic molecule with a role in tissue repair. We previously showed that HMGB1 administration in the ventricular wall of the infarcted mouse heart resulted in cardiac c-kit$^+$ cell activation and myocardial regeneration [17]. In the present study we demonstrated that cardiac c-kit$^+$ cell proliferation induced by HMGB1 occurred, at least in part, via a paracrine mechanism mediated by cFbs. These cells represent the two-

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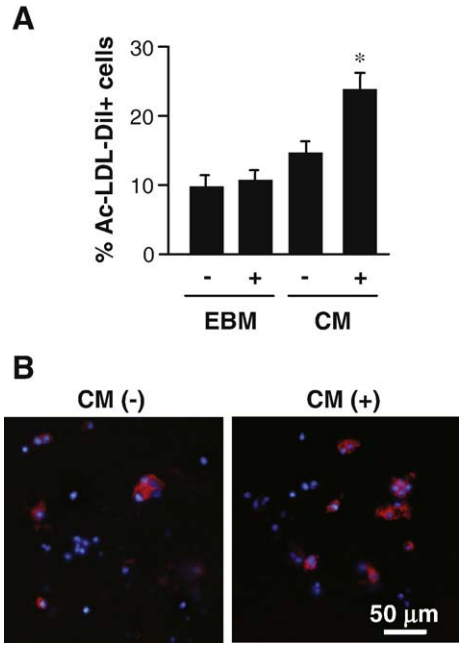


Fig. 7. Conditioned medium of HMGB1-treated cFbs promotes endothelial differentiation of murine c-kit$^+$ cells. Purified c-kit$^+$ cells were cultured in EBM for 6 days either with CM from untreated (−) and 10 ng/ml HMGB1-treated (+) cFbs or with EBM either in absence (−) or in presence of HMGB1 (+). Bar graph indicates the percentage of c-kit$^+$ derived-Ac-LDL-DiI$^+$ cells on total nuclei ($n=5$ *$p<0.05$ vs all culture conditions). (B) Representative immunofluorescence of c-kit$^+$ derived-Ac-LDL-DiI$^+$ cells (red fluorescence) cultured in CM from untreated (−) and 10 ng/ml HMGB1-treated (+) cFbs. Blue fluorescence, Hoechst 33342 of nuclei.

third of non-myocyte cardiac cell population [20] and they are particularly resistant to ischemia-induced cell death [33]; therefore cFbs represent a cell population which may contribute to sustain a paracrine action. Primary cFbs spontaneously differentiate into MyoFbs [27,28,34]. *In vivo* MyoFbs are involved in scar formation, being part of granulation tissue where they secrete new collagen and matrix proteins [19], and in scar shrinkage as a consequence of their contractile properties [29]. MyoFbs are normally absent in the healthy heart and still absent during the ischemic and necrotic phase which follow myocardial infarction. In our previous study, HMGB1 was administered early after MI (4 h), when MyoFbs were not yet present [17]. Therefore, to study HMGB1-induced paracrine action on cFbs, we developed culture conditions to prevent cFbs differentiation into MyoFbs. CFbs were identified for the expression of vimentin, α-SMA and the surface fibroblast antigen. We found that the Endothelial Growth Medium (EGM-2) drastically reduced the appearance of the MyoFb phenotype, in terms of flattened large cells with smooth muscle actin organized to form stress fibers. In light of this evidence, we speculated that the presence of growth factors including VEGF, bFGF and IGF-1 in this medium could account for the observed effect. EGM-2-cultured cFbs retained the ability to differentiate into MyoFbs, demonstrating that EGM-2 was efficient in maintaining these cells in a more undifferentiated state. Notably, cFbs were highly responsive to HMGB1 and levels of the HMGB1 receptor RAGE were detected in cFbs while this receptor was down-regulated in MyoFbs. HMGB1 induced cFb migration but did promote their proliferation and differentiation into MyoFbs. Importantly, HMGB1 significantly increased, cytokine, chemokine and growth factor secretion by cFbs. At functional level, the supernatant of HMGB1-stimulated cFbs enhanced CSC migration, proliferation and endothelial differentiation compared to HMGB1 alone added to the stem cell culture medium. It is likely that the effects of conditioned media on CSC are due not to a single factor but to the synergistic interaction of a multitude of molecules. Specifically, in the presence of HMGB1, cFbs released VEGF and PlGF, two angiogenic factors also involved in stem cell recruitment to the site of injury [35–37] and in their differentiation into endothelial cells. These factors may modulate CSC functions. Accordingly, a subpopulation of CSCs express the VEGF and PlGF receptors Flk-1 and Flt-1 and CM from HMGB1-stimulated cFbs enhanced fibronectin-induced endothelial differentiation of CSC. Interestingly, both VEGF and PlGF administration in infarcted heart restore cardiac function improving neovascularization and myocardial tissue viability [36,38,39]. Whether these effects are mediated by CSC activation remains to be investigated.

The supernatant of HMGB1-treated cFbs, also showed increased levels of GM-CSF, which has been involved in EPC mobilization [40] and hematopoietic progenitor cell proliferation [41]. According to the proinflammatory role of HMGB1, significant amount of TNF-α, IL-1β, IL-1ra and MIPFS-1α were also released by cFbs stimulated with HMGB1. The inflammatory cascade plays an important role following myocardial infarction and repair. However, attempts to modulate inflammation have been unsuccessful since cytokines and chemokines which may have a deleterious role in the early phase of infarction are regulators of cardiac repair in the late phase [42]. In spite of the increased production of proinflammatory mediators by HMGB1-stimulated cFbs *in vitro*, the direct HMGB1 administration to the infarcted hearts, did not enhance inflammatory cell recruitment [17]. Notably, in the supernatant of HMGB1-stimulated cFbs increased levels of IL10 and IL4, which are involved in the suppression of acute inflammation following myocardial ischemia [43,44], were also detected. Therefore, a balance between secreted pro- and anti-inflammatory mediators by cFbs following HMGB1 delivery to infarcted heart, could account for the lack of enhanced inflammatory response *in vivo*.

Although we have demonstrated that cardiac c-kit$^+$ cells may be activated *in vitro* by a paracrine mechanism, we cannot exclude that the proliferative effect may depend *in vivo* at least in part to cell-to-cell direct contact. Recently it has been shown that cFbs are present in cardiac stem cell niches [23], where they form junctional connection with CSCs and possibly support their activity. Therefore, HMGB1-induced cardiac tissue regeneration might result from the paracrine CSC activation within the cardiac niches located in the border zone, where HMGB1 was delivered [17].

In conclusion, in the present study we provided the first evidence for HMGB1-induced paracrine regulation of cardiac c-kit$^+$ cell function. Via this mechanism, HMGB1 may support and accelerate the regeneration of ischemic tissues.

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