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Pre-stimulation with FGF-2 increases in vitro functional coupling of mesenchymal stem cells with cardiac cells

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ABSTRACT

The functional coupling of transplanted cells with host myocardial cells is a significant challenge in mesenchymal stem cell (MSC) cardiomyoplasty being related to cell survival and therapeutic outcomes. Priming of MSCs with growth factors has been reported to improve their therapeutic efficacy through gap junction-mediated mechanisms. However, the expression pattern of Connexin43 (Cx43) in growth factor-stimulated MSC was not previously addressed. In this study we investigated how the pre-treatment with growth factors modulates MSC ability to integrate into the host tissue after transplantation, with particular focus on the expression of Cx43 and its cellular distribution.

Our results showed that stimulation of MSCs with IGF-1, FGF-2, but not TGFβ, increased the level of Cx43 at both mRNA and protein levels. IGF-1 stimulation resulted in a shift of the fibroblast morphology into an epithelial morphology in several well-defined areas of stimulated cells. Confocal microscopy examination revealed that the increase of Cx43 was restricted to the epithelial-like cells and did not occur in other cells. In variance, FGF-2 induced a rod-shape morphology of every single cell, which achieved an extremely low cell index. FGF-2 stimulation also induced a time-dependent increase in Cx43, with a regular distribution pattern in all cells. Dye transfer assay coupled with confocal microscopy and flow cytometry analysis demonstrated functional in vitro cell coupling between FGF-2-stimulated MSCs as well as between FGF-2-stimulated cells and H9c2 cardiomyoblasts, a scenery that mimick MSC transplantation into the myocardium.

We conclude that the stimulation of MSCs with FGF-2 prior to transplantation may facilitate their access among the myocardial cells and increase the functional coupling between transplanted and host cells.

1. Introduction

Mesenchymal stem cells (MSCs) have become one of the most useful tools in the field of regenerative medicine for myocardial reconstructive therapy [1]. Despite a still misunderstanding of cell fate after transplantation, it is nowadays generally accepted that MSCs exerted cytoprotective effects by active secretion of paracrine factors, rather than by differentiation into cardiomyocytes [2–4].

A major drawback in MSC cardiomyoplasty is the low rate of cell survival after transplantation, which could be directly linked to the improper functional integration within the host's myocardium. As an essential component of the functional coupling between host and transplanted cells, gap junctions residing at cell–cell borders are constituted by integral membrane proteins that enable the direct cytoplasmic exchange of ions and small metabolites between adjacent cells [5]. In mammals, gap junctions are formed by multiple combinations of around twenty connexin proteins, with connexin 43 (Cx43) being the most abundant connexin in the human body and the major ventricular gap junction protein in the heart [6,7].

Several studies have previously shown that priming of MSCs with growth factors before transplantation improved their therapeutic efficacy and cytoprotective effects in part through gap junction-mediated mechanisms [8]. Therefore, a better understanding of how growth factors modulate the stem cell engraftment into the myocardium might be very important for the development of novel cell therapy-based strategies aiming cardiac regeneration.

In this study, we investigated how the pre-treatment with growth factors modulates MSC ability to integrate into the host

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tissue after transplantation, with particular focus on the expression of Cx43 and its cellular distribution. We provided evidence that IGF-1 and FGF-2 induced important upregulation of Cx43 protein that assembled into functional gap junctions. However, while the increase in Cx43 level induced by IGF-1 was transient and only restricted to certain cells within treated culture, FGF-2 induced robust and sustained overexpression of Cx43, which may offer advantage in cell therapy for myocardial regeneration. These results broaden our understanding of stem cell modulation by growth factors and draw attention on care that must be considered when a treatment scheme is designed for enhancing the effectiveness of transplanted cells.

2. Materials and methods

2.1. Cell culture

MSCs were isolated from mouse bone marrow as previously described [3] and used between the 8th and 13th passages. The routine culture procedure consisted of cell seeding on 0.1% gelatin-coated dishes in complete growth medium (low glucose DMEM with 10% MSC-qualified FBS) at a density of 5000 cells/cm^2 and trypsinization (for re-plating or analysis) after 5 days, before cells getting confluence. Stimulation with growth factors was achieved by supplementing the complete growth medium with either 10 ng/ml TGFβ1, 50 ng/ml IGF-1, or 50 ng/ml FGF-2 for the whole 5 day-culture period or for the last 3 h or 24 h before analysis.

2.2. xCELLigence assay

The effect of growth factors on the MSC index was monitored with xCELLigence System (Roche Applied Science). Briefly, the cells were seeded on 16-well E-plates (2000 cells/well) in complete growth medium in the presence or absence of growth factors and cell index was recorded for 5 days at every 10 min for the first 3 h of culture and every 1 h thereafter. Control consisted in MSCs grown for 5 days in complete growth medium in the absence of any growth factor.

2.3. Gene expression analysis

Total RNA was extracted using the RNeasy Micro kit (Qiagen), and cDNA was synthesized from 1 μg of total RNA by using oligo (dT) primers and MMLV reverse transcriptase (Life Technologies). Real time RT-PCR was performed by using a Light Cycler 480 II Real-Time PCR system (Roche Applied Science). The DNA sequence of each primer can be provided on request. The comparative C_T method was used to quantify the results and GAPDH was used for internal normalization.

2.4. Western blot assay

Cell lysates were obtained by scraping the cells in hot Laemmli's buffer containing complete protease inhibitor cocktail (Roche Applied Science). Equal amounts of protein were subjected to SDS-PAGE followed by transfer onto nitrocellulose. After blocking, the membranes were incubated overnight at 4 °C with anti-Cx43 (Merck Millipore; dilution of 1:5000) or anti-β-actin (Sigma–Aldrich; dilution of 1:4000), followed by HRP-conjugated goat anti-mouse/rabbit IgG (R&D Systems; dilution of 1:500) for 1 h. Antigen-antibody complexes were visualized by chem-iluminescence reaction (ImageQuant LAS4000 system). The protein expression was quantified by densitometry with ImageJ software.

2.5. Fluorescence microscopy

Cells grown on cover slides were fixed with 4% PFA for 10 min and then permeabilized with 0.1% Triton X-100 for 3 min. All solutions were prepared in PBS and all steps were performed at room temperature (RT). Non-specific binding was blocked by incubating the cells for one hour with 5% goat serum. Cells were stained with polyclonal rabbit anti-mouse Cx43 (Merck Millipore; dilution 1:50) for one hour and then incubated with secondary antibody goat anti rabbit IgG-NL637 (Merck Millipore; dilution 1:100) for 30 min. Mounted cover slides were examined under a Leica TSC SP5 confocal microscope.

2.6. Flow cytometry analysis

Trypsinized cells were resuspended in PBS containing 2 mM EDTA and 2% FBS (PBS-EDTA-FBS), fixed in 4% PFA for 15 min and then permeabilized with 0.2% Triton X-100 for 10 min at 4 °C. Aliquots of 200 μl containing 10^5 cells were incubated with polyclonal rabbit anti-mouse Cx43 (Merck Millipore; dilution 1:100) for one hour at RT. Washed cells were further incubated with goat anti rabbit IgG-FITC (Merck Millipore; dilution 1:100). Cells were resuspended in 400 μl PBS-EDTA-FBS and analyzed by flow cytometry (20,000 events/sample) using a Gallios flow cytometer (Beckman Coulter). Acquired data were analyzed using Summit v4.3 software (Cytomation Inc.).

2.7. Gap junction intercellular communication assay

After stimulation with growth factors, cells were double labeled with CMTPX and Calcein-AM (Life Technologies) by simultaneous incubation with 5 μM CMTPX and 5 μM Calcein-AM for 30 min in serum-free medium at 37 °C. A mixture of double labeled and unlabeled cells containing similar treated cells at a ratio of 1:10 (labeled vs. unlabeled) was seeded on culture plates at high density (to form a confluent monolayer of cells) for three hours before microscopic evaluation of Calcein transfer from a labeled cell into neighboring unlabeled cells. The same experimental design was used for co-culture of MSCs (labeled) with the cardiomyoblasts cell line H9c2 (unlabeled), except that H9c2 cells were seeded onto culture plates 24 h before MSC addition. Dye transfer was analyzed by visualization in life imaging under fluorescence microscope (Nikon Eclipse TiE) and by flow cytometry.

2.8. Statistics

The results were expressed as the mean ± S.E.M. of at least three experiments. Statistical analyses were performed using GraphPad Prism 5.0 and one-way ANOVA followed by Bonferroni's post-test was used to assess statistical significance ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$).

3. Results

3.1. Tracking the global morphological changes of MSC culture during in vitro stimulation with IGF-1, TGFβ1, or FGF-2

MSCs were cultured for 5 days in complete medium supplemented with 10 ng/ml TGFβ1, 50 ng/ml IGF-1 or 50 ng/ml FGF-2. These concentrations were chosen based on a careful review of the literature. Rough examination under phase contrast microscopy revealed overall specific morphological changes induced by each individual growth factor. Particularly, TGFβ1 stimulation resulted in cell alignment in swirls, IGF-1 partially induced an epithelial phenotype, while FGF-2 induced a fibroblast-like morphology with

all cells being converted into thin rod-shaped by the end of the treatment (Supplementary Fig. 1). These morphological changes were also followed in real time with xCELLigence system. Remarkably, despite their well-known mitogenic effects, declines of cell index were noted in MSC cultures after exposure to any individual growth factor. Furthermore, while the decline of cell index was slightly or moderately for IGF-1 or TGFβ1 treatment, in comparison to control cells, the addition of FGF-2 resulted in dramatic collapse of cell index, which might have been taken as a cytotoxic, rather than proliferative, effect of FGF-2 on MSCs (Fig. 1a). The proliferative effect of growth factors was certified by cell counting, confirming that the stimulation with either IGF-1 or FGF-2 increased the proliferation rate of MSCs (Supplementary Fig. 2). As variations in cell index reflect not only changes in cell number but also in cell adhesion and/or spreading extent, which can all influence the cell impedance, these data pointed out that the morphological changes induced by growth factors, and in particular by FGF-2, masked their proliferative effect when expressed in terms of cell index resulted from impedance measurements. These important changes in MSC morphology may impact tissue architecture after transplantation.

3.2. Impact of growth factor stimulation on connexin43 level in MSCs

An important pre-requisite for therapeutic efficacy of stem cells in cardiac regeneration therapy is their ability to survive after transplantation by engraftment into the myocardium. As gap junctions play essential roles in the electromechanical coupling with the host myocardium, we focused on the levels of Cx43 gap junction protein in MSCs and the ways by which growth factor treatment modulate these levels.

First, a spontaneous upregulation of Cx43 gene expression during normal culture was noticed (Supplementary Fig. 3). Thus, quantitative RT-PCR analysis showed that MSCs had a low level of Cx43 transcripts one day after plating, which doubled in preconfluent cells (3 days after plating) and so once again at the time they get confluence (5 days after plating). Besides this naturally occurring increase in Cx43 gene expression with increasing cell density, Cx43 mRNA level was significantly upregulated after 5-day culture in the presence of IGF-1 or FGF-2 (1.5 folds or 2.3 folds in the presence of IGF-1 or FGF-2 respectively, over the control cells), while TGFβ1 decreased the gene expression level (Fig. 1b). Still at the protein level, stimulation of MSCs with IGF-1 or FGF-2 resulted in the increase in Cx43 level (4 ± 0.6 folds and 9.6 ± 1.1 folds increase induced by IGF-1, respectively FGF-2), while TGFβ1 stimulation significantly reduced the protein level (Fig. 2c). These results were further supported by immunofluorescence staining of Cx43 in MSCs, which revealed the downregulation of protein in MSCs cultured for 5 days in the presence of TGFβ1 and the upregulation of protein after 5 day-culture in the presence of IGF-1 or FGF-2 (Supplementary Fig. 4). These data suggest that stimulation of MSCs with IGF-1 or FGF-2 might enforce the electromechanical coupling with other Cx43-expressing cardiac cells (local cardiomyocytes and cardiac fibroblasts) after transplantation into the infarcted myocardium.

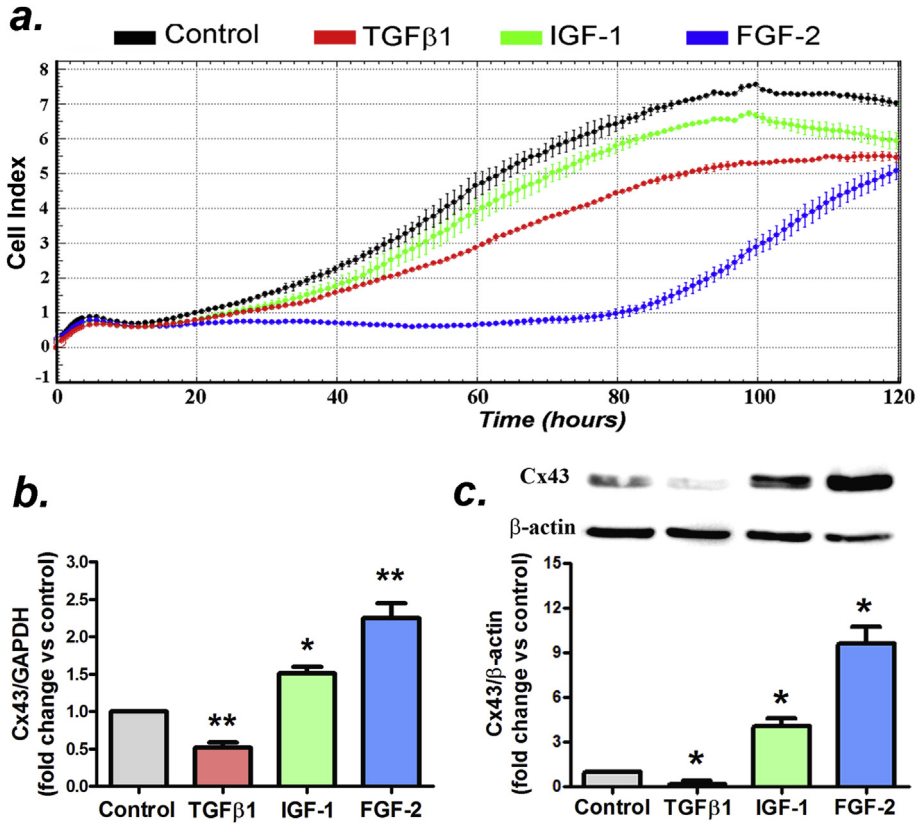

Fig. 1. Impact of growth factors on Cx43 in MSCs. a) Real-time monitoring of cell index of MSCs grown in the presence of growth factors. The plot illustrates a representative experiment of three independent experiments and error bars (standard deviation) come from duplicate samples; b) Real-time PCR quantification of variations in Cx43 gene expression in MSCs stimulated for 5 days with TGFβ1, IGF-1, FGF-2, as compared to control cells (unstimulated cells). c) The level of Cx43 protein in MSCs grown for 5 days in the presence of TGFβ1, IGF-1 or FGF-2, as determined by Western blot. The blot image is a representative image of five experiments and the morphometric analysis was done by averaging data (mean ± SEM) from at least three independent experiments. (b and c): *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ as compared with control.

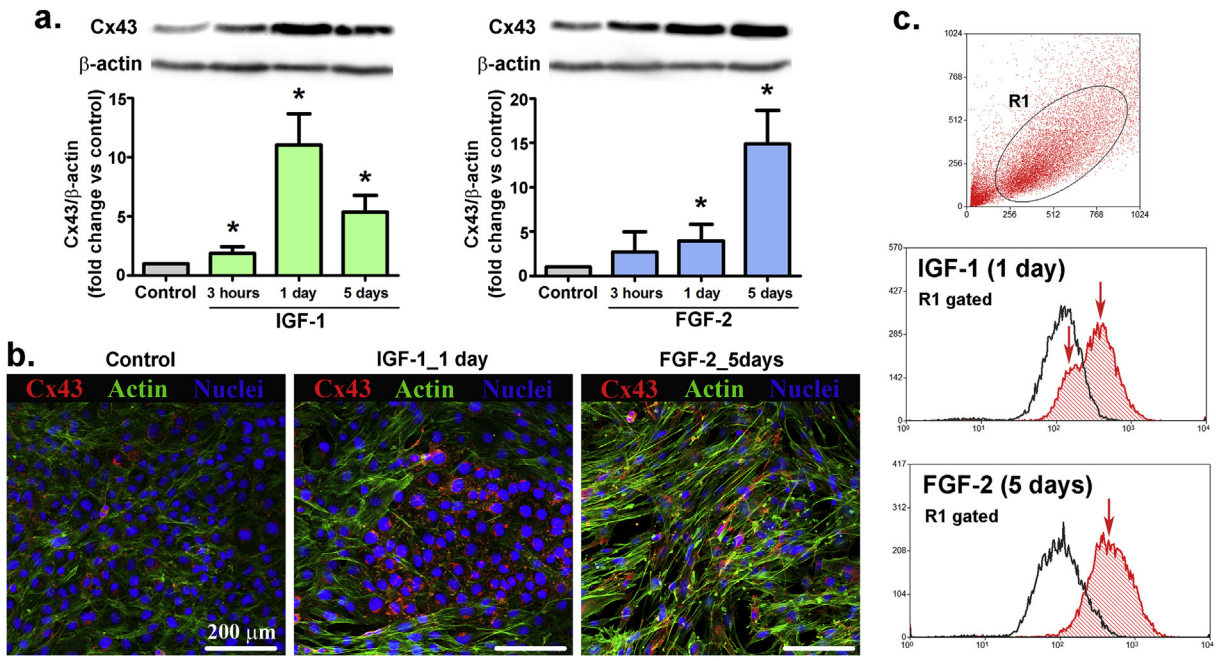

Fig. 2. Dynamics of Cx43 increase in MSCs after stimulation with IGF-1 and FGF-2. a) Cx43 protein level in MSCs treated for 3 h, 1 day and 5 days with IGF-1 and FGF-2, as determined by Western blot. The blot images are representatives of three independent experiments and the morphometric analysis were done by averaging data (mean ± SEM) from all independent experiments. *$p < 0.05$ versus control. b) Immunofluorescence images illustrating the pattern of Cx43 (red) in MSCs stimulated with IGF-1 or FGF-2. Cells were counterstained for β-actin (Plaloidin-FITC, green) and nuclei (Hoechst 33258, blue). c) Flow-cytometry analysis of Cx43 in MSCs stimulated for indicated times with IGF-1 or FGF-2. Note that FGF-2 induced a uniform upregulation of Cx43 protein, while only a sub-population of IGF-1-stimulated cells overexpressed Cx43. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Connexin 43 expression pattern in MSCs stimulated with IGF-1 and FGF-2

We next followed the dynamics of Cx43 increase in MSCs after stimulation with IGF-1 and FGF-2. To this aim, the cells were allowed to grow in culture for 5 days and the growth factor was added during the last 3 h or 1 day of culture. As controls, cells incubated in complete medium in the absence of growth factors and cells cultured for 5 days in medium supplemented with respective growth factors were used. The results (Fig. 2a) showed that three-hour exposure of cells to IGF-1 was enough to significantly increase Cx43 level (1.9 ± 0.3 fold increase vs control) and the maximum increase was apparently obtained when cells were stimulated with IGF-1 for 24 h before harvested for investigation (11 ± 2.6 for 24-h stimulation versus 5.4 ± 1.4 fold increase in 5-day stimulation). In variance, stimulation of MSCs with FGF-2 induced an increase in Cx43 level in a time-dependent manner, with the highest level of Cx43 obtained when cells were constantly exposed to FGF-2 for whole culture period (14.9 ± 3.8 folds increase in Cx43 levels in 5-day stimulated cells), versus 4 ± 1.9 folds increase in 1-day stimulated cells (Fig. 2a).

Surprisingly, immunofluorescence analysis of MSCs after 1-day stimulation with IGF-1 revealed an uneven expression of Cx43, in contrast to the robust and sustained Cx43 overexpression observed after FGF-2 stimulation. Thus, only cells emerged into an epithelial phenotype after IGF-1 stimulation showed an increased level of Cx43, with a typical distribution onto cell membranes (Fig. 2b). These results were further validated by flow cytometry analysis that confirmed the appearance of two morphologically distinct cellular sub-populations within MSC culture stimulated with IGF-1 (Fig. 2c). Thus, while FGF-2 stimulation resulted in a uniform increase in Cx43 level in all cells, only a sub-population of cells stimulated with IGF-1expressed higher Cx43 level in comparison to control cells.

3.4. Functionality of gap junctions in growth factor-stimulated MSCs

Although FGF-2 induced a robust increase in Cx43 that encompassed all cultured cells, its distribution appeared in a scattered pattern throughout the cells rather than as gap junction plaques typically noticed in cardiomyocytes. This rose up questions about the functionality of gap junctions in MSCs, which was further evaluated by the capacity of fluorescently-labeled cells to transfer the small dye into adjacent unlabeled cells.

To this aim, growth factor-stimulated or un-stimulated MSCs were double labeled with CMTPX (red label) and Calcein-AM (green label) and then mixed with similar-treated unlabeled cells at a ratio of 1:10 (labeled vs. unlabeled). Cells were re-plated on cell culture-treated cover-slides at high density and examined three hours later by live fluorescence imaging. The presence of a functional gap junction between a labeled and an unlabeled cell would normally give rise to two adjacent cells, one of which is double labeled with green and red (yellow in merged image) and the other one is green labeled only (due to the gap junction-mediated transfer of Calcein from the labeled cell). Our results showed that untreated MSCs developed functional gap junctions only to a relative low level, demonstrated by the presence of only few cells stained green (Fig. 3). However, both short-term (1 day) and long-term (5 days) stimulation with either IGF-1 or FGF-2 enhanced gap junctional communications between cells, as revealed by the presence of a higher number of cells stained with green fluorescent calcein only (Fig. 3). Consistent with the dynamics of Cx43 increase in IGF-1 and FGF-2-stimulated cells, 1-day stimulation with IGF-1 and 5-day stimulation with FGF-2 apparently produced highest levels of functional junctions between cells.

To rank between these two stimulations in a more closely mimicked clinical setting, an in vitro model of cell therapy for myocardial regeneration was designed, in which double-labeled

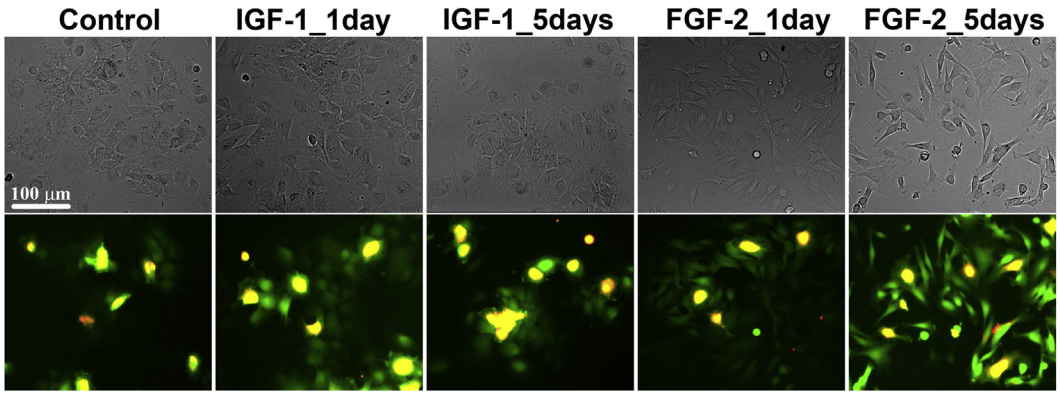

Fig. 3. Functionality of gap junctions in MSC–MSC interactions after stimulation with IGF-1 or FGF-2. Phase contrast microscopy (upper images) showing the high density of the cultured cells and fluorescent microscopy (lower images, merged of green and red channels) showing the spreading extent of green fluorescence from double labeled cells (yellow) into the neighboring unlabeled cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

growth factor-stimulated MSCs were mixed with cardiomyoblasts (H9c2 cell line) in 1:10 ratio and functionality of gap junction was quantitatively assessed three hours later by flow cytometry analysis. The results demonstrated that both stimulations increased the intercellular communication between cardiomyoblasts and stem cells, in comparison to untreated MSCs (Fig. 4a). However, quantifying of green fluorescent cardiomyoblasts, as a direct measure of functional communication, revealed that 5-day stimulation of MSCs

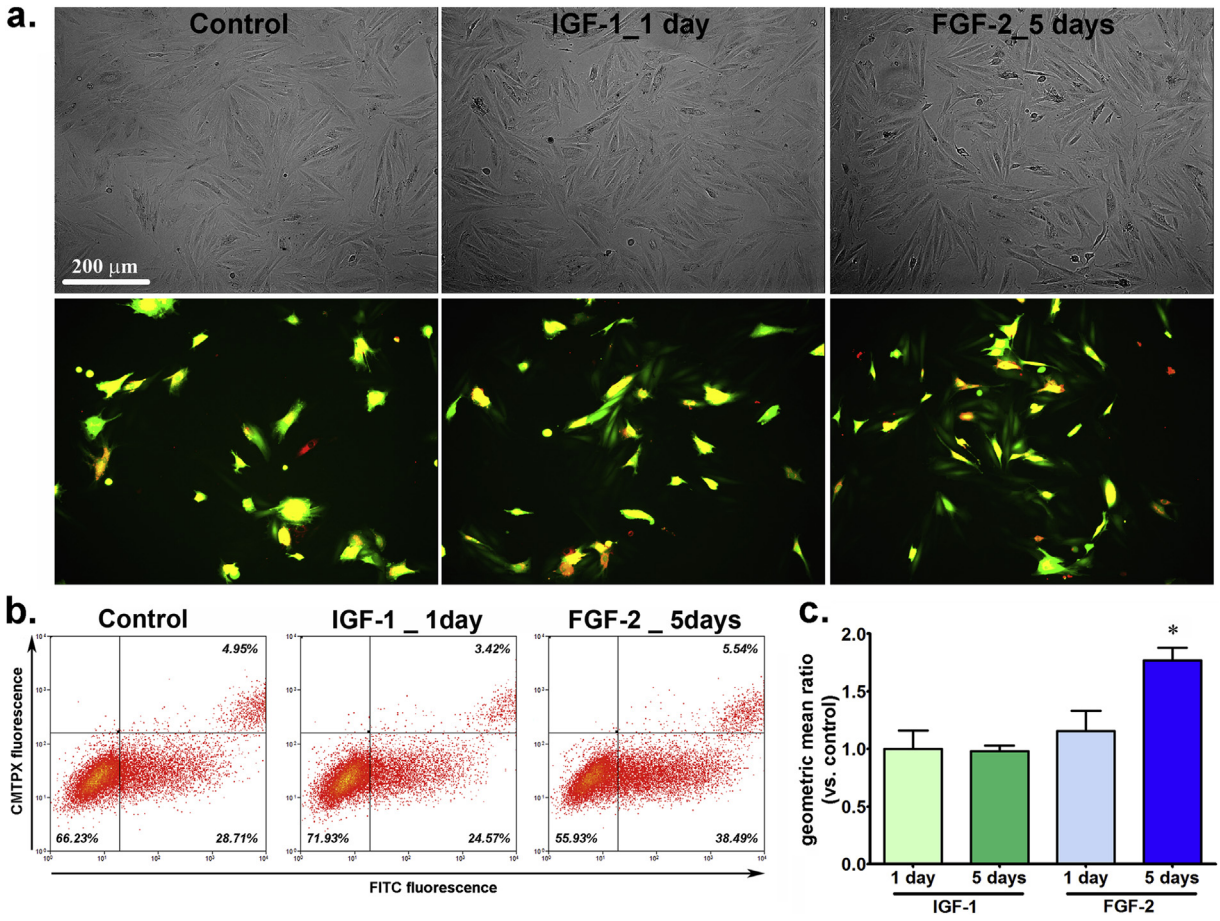

Fig. 4. Functionality of gap junctions in MSC-cardiomyoblast interactions after stimulation with IGF-1 or FGF-2. a) Phase contrast microscopy (upper images) showing the high density of the cultured cells and fluorescent microscopy (lower images, merged of green and red channels) showing the spreading extent of green fluorescence from double labeled cells (yellow) into the adjoining unlabeled cardiomyoblast cells. b) Flow-cytometry analysis of the functionality of the gap junctions after MSC stimulation with IGF-1 or FGF-2. Histograms shows the double labeled MSC population (upper right, contributing for around 5% of the total cells), unlabeled cardiomyoblasts (lower left), and green-labeled cardiomyoblasts (lower right) produced as a result of functional communication with double labeled MSCs. c) Quantitative analysis of intercellular communication between MSCs and cardiomyoblasts expressed as geometric mean ratio of green fluorescence of cardiomyoblast population in coculture with stimulated MSCs versus unstimulated (control) MSCs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with FGF-2 promoted a higher dye transfer from stimulated MSCs into local cardiac cells than 1-day stimulation with IGF-1 (Fig. 4b). Thus, the percentage of Calcein-positive cardiomyoblasts detected after interaction with FGF-2-stimulated MSCs was higher than after interaction with IGF-1-stimulated or un-stimulated MSCs. Furthermore, when expressed as a geometric mean ratio of green fluorescence of cardiomyoblast population in cocultures with stimulated MSC vs unstimulated (control) MSCs, it appeared that 5-day stimulation of MSCs with FGF-2 before in vitro transplantation induced the best engraftment of MSCs among the cardiac cells (1.8 ± 0.1 in cardiac cells co-cultured with FGF-2-stimulated MSCs vs. 1.2 ± 0.2 in cardiac cells co-cultured with IGF-1-stimulated MSCs). Thus, the 5-day stimulation of MSCs with FGF-2 not only increased the expression of Cx43, but also enhanced intercellular communication between adjacent cells and might provide better engraftment of cells into the myocardium.

Overall, these results concluded that the presence on an extra-amount of FGF-2 during MSC culture significantly increased the level of Cx43, which may offer advantage in cell therapy for myocardial regeneration, by bettering the engraftment of exogenous cells within host myocardial cells.

4. Discussion

Pre-treatment of MSCs with growth factors to enhance gap junction-mediated integration into the host tissue is a feasible strategy to increase therapeutic efficacy of cell therapy for myocardial regeneration [9]. Here we report that:

(i) Stimulation with FGF-2, or IGF-1, but not TGFβ1, induced an increased level of Cx43 protein in MSCs in vitro;
(ii) IGF-1 induced a transient and uneven increase in Cx43 protein, which failed to produce benefits in terms of functional intercellular communications between MSCs and cardiomyogenic cells;
(iii) extra-amounts of FGF-2 added in culture medium during MSC growing produced a stable and robust increase in Cx43, which drove an improved functional coupling of MSCs with myocardial cells.

Efficient engraftment of exogenously delivered cells within the host cardiac tissue encompasses not only morpho-functional connections, but also cell–cell coupling that allows propagation of action potential across the heart [5]. It is important to note that the release of ATP or glutamate, which could serve as paracrine signaling, has been attributed to Cx43 hemi-channels [10]. On the other hand, the transplantation of cells with inappropriate level of Cx43 expression created heterogeneities that predisposed the heart to reentrant arrhythmias [11].

Besides its well-recognized role in cell–cell communication, Cx43 may also modulate cell migration, as demonstrated in astrocytes and prenatal neuronal stem cells [12,13]. Interestingly, Desai et al. demonstrated that FGF-2 induced a migratory phenotype in adipose-derived MSCs, which was correlated with up-regulation of the anti-adhesive extracellular protein Tenascin-C, and the pro-migratory cytoskeletal protein Vimentin, along with the loss of focal adhesions and stress fibers [14]. Our results corroborate with these data by showing a decrease in MSC index as a consequence of FGF-2 stimulation, as well as a robust and uniform upregulation of Cx43, which together may facilitate the spreading and functional coupling of transplanted cells among the myocardial cells to improve the direct and/or paracrine benefits of MSC transplantation. The efficiency of FGF-2-stimulation was certified by a higher efficacy of dye transfer between stimulated MSCs and cardiomyoblasts in culture, in a model mimicking in vivo setting. IGF-1 also induced the upregulation of Cx43 in MSC culture, but the uneven distribution at cellular level rendered the IGF-1-stimulated cell culture heterogeneous, with no benefits in term of functional communication with cardiac cells.

Gap junctions are also obligatory features of endocrine glands, which ensure the cross-talk between the secretory cells. Furthermore, several hormones are involved in the transcription, mRNA stability, translation and cytoplasmic trafficking of connexins, as well as in regulation of cell–cell channels formed by these proteins [12]. Considering that the endocrine mechanisms may also be involved in mediating the therapeutic effects of MSCs when transplanted at distant sites from damaged tissues [15–17], these data bring forward another aspect of the importance of high expression level of Cx43 in MSCs.

The insights of Cx43 overexpression by growth factor-stimulated MSCs were not resolved in our experiments. However, our data indicate that stimulation of MSCs with different growth factors before transplantation activate different mechanisms modulating Cx43 protein expression. A short pre-stimulation of stem cells with growth factors may induce suboptimal cardioprotective effects, as previously reported [9]. Finding the optimal combination and dose of growth factors to be delivered at the right moment remain mandatory for this therapeutic strategy to have real chances to succeed in a clinical scenario.

Overall, our findings provide a framework for understanding the mechanisms lying behind MSC stimulation with growth factors in order to improve the treatment regimen of MSC before transplantation. It is important to emphasize that although growth factors increase the overall therapeutic efficacy of MSCs, they might have different regulatory mechanisms for the same target proteins, and the understanding of these disparities could eventually lead to better therapeutic strategies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.055.

Transparency document

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