Biochemical and Biophysical Research Communications 426 (2012) 475–479

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Differentiating multipotent mesenchymal stromal cells generate factors that exert paracrine activities on exogenous MSCs: Implications for paracrine activities in bone regeneration

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ARTICLE INFO

Article history:
Received 16 August 2012
Available online 29 August 2012

Keywords:
Mesenchymal stem cells and paracrine activities
Mesenchymal stem cells
Bone morphogenetic protein 2
Bone regeneration

ABSTRACT

The mechanisms by which multipotent mesenchymal stromal cells (MSCs) contribute to tissue repair following transplantation into host tissues remains poorly understood. Current concepts suggest that, in addition to differentiation into cells of the host tissues, MSCs also generate trophic factors that modulate host tissue microenvironment to aid in the repair process. In this communication, we assessed whether factors secreted by MSCs undergoing osteogenic differentiation induce expression of osteoblast markers in exogenous MSCs as well as their migration. Murine MSCs were cultured in osteogenic medium, and at different time points, medium conditioned by the cells was collected and assessed for its effects on differentiation and migration of exogenous MSCs. In addition, we determined whether MSCs infused into mice femurs expressed genes encoding for factors predicted to play a role in paracrine activities. The results showed that MSCs maintained in osteogenic medium, secreted factors at specific time points that induced alkaline phosphatase activity (ALP) in exogenous MSCs as well as their migration. MSCs infused into mice femurs and retrieved at different days expressed genes that encoded predicted factors that play a role in cell differentiation and migration. Neutralizing antibodies to bone morphogenetic protein-2 (BMP-2) led to the decrease in ALP activity by exogenous MSCs. These data demonstrated that, as MSCs differentiate toward osteogenic lineage, they secrete factors that induce recruitment and differentiation of endogenous progenitors. These data reveal mechanisms by which donor MSCs may contribute to the bone reparative process and provide a platform for designing approaches for stem cell therapies of musculoskeletal disorders.

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1. Introduction

Emerging concepts suggest that, the therapeutic potential of multipotent mesenchymal stromal cells (MSCs) may not only be confined to their differentiation into cells of host tissues but also to production of trophic factors that may modulate host tissue microenvironment so as to facilitate regenerative process. Cell therapies are being investigated as potential alternatives for the treatment of various skeletal diseases [1–4]. In most cases, the intent is to replace or replenish the host tissue with new cells capable of carrying out functions of the host tissue cells [5,6]. The extent of donor cell contribution to differentiation into cells of host tissue or to production of factors that initiate regenerative process is not clearly understood. Studies in which MSCs were transplanted into children with severe forms of osteogenesis imperfect (OI) revealed that, the children who received cells exhibited acceleration in growth and reduction in the rate of bone fracturing [7,8]. The level of donor cell engraftment was however, extremely low and could not have accounted for the benefits observed. In related studies, transplantation of MSCs into animal models of infracted hearts led to the amelioration of the disease but differentiation of donor cells into cardiomycytes was not observed [9]. These findings led to the conclusions that, the therapeutic effects of the donor cells were not entirely due to the differentiation into cells of the host tissues but to other mechanisms. It was postulated that the donor cells secrete factors that modulate the microenvironment for endogenous cells to carry out the repair process or they exerted paracrine activities that enhanced reparative process. The paracrine activities have mostly been demonstrated in soft tissues but not in hard tissues.

We previously demonstrated that infusion of MSCs into femurs of a mouse model of osteogenesis imperfect resulted in new bone formation [10]. The cells that were responsible for the new bone

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http://dx.doi.org/10.1016/j.bbrc.2012.08.095

were not determined; but it was hypothesized that, the new bone was a result of a combination between the donor and endogenous cells. The process by which this was achieved was not determined but paracrine activities by the donor cells were suspected.

To explore whether paracrine activities play a role in bone regeneration, we examined the effects of factors produced by MSCs maintained in osteogenic differentiation medium on expression of osteoblast markers and migration of exogenous MSCs in an in vitro system assay. We report here that as MSCs differentiate toward osteogenic lineage, they produce factors that exert effects on exogenous MSCs or progenitors differentiation and migration. This process may define mechanisms by which donor cells contribute to bone repair and regeneration following transplantation.

2. Materials and methods

2.1. Cell isolation, maintenance and expansion

Murine derived MSCs were established from the bone marrow harvested from femurs and tibia of 8 week-old mice as described previously [10,11]. The BMSCs were cultured in T25 flasks in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin (P/S, v/v). After 4 days of culture, the medium was replaced with fresh medium and the cells were maintained in culture with passaging. At passage 3, the cells were transduced with a retrovirus carrying enhanced green fluorescent protein and Zeocin resistant genes.

2.2. Cell transduction

To aid in donor cell tracking in vivo, cell transduction was performed as described previously [10,11]. Briefly, cells were plated in six-well plates in DMEM supplemented with 10% FBS and maintained in culture to 60% confluence. The cells were then incubated in 1 ml of a high-titer DFG-retro virus carrying eGFP and Zeocin^r resistant genes in DMEM medium supplemented with 10% FBS [10–12]. The cells were incubated in the virus supplemented medium for 24 h. Thereafter, medium was replaced with fresh medium supplemented with 10% FBS. The cells were then treated with 2 more rounds of the virus containing medium at 24 h intervals. To select a population of cells expressing eGFP, transduced cells were maintained in DMEM supplemented with 10% FBS and 25 μg/ml of Zeocin. The EGFP + BMSCs were maintained in culture in presence of Zeocin until use.

2.3. Preparation of conditioned media

One million MSCs were cultured in osteogenic medium and the medium conditioned by the cells was collected at day 0, 5, 10, 15 and 20. Before each medium collection, cells were washed twice with 10 ml of PBS, and replaced with serum free medium which was collected 24 h later. The collected media were passed through 0.2 μm low protein binding filters and diluted 1:1 with cell culture media and stored at −80 °C until use. To prepare medium supplemented with neutralizing antibodies, BMP-2 (R&D, Minneapolis, MN) and VEGF (Santa Cruz, Santa Cruz, CA) neutralizing antibodies were added in the conditioned medium at concentration of 2.5 μg/ml and 5 μg/ml respectively. The media were then used for assessing activities of these factors.

2.4. Alkaline phosphatase activity

Murine MSCs were plated in 24 well plates and incubated in medium conditioned by differentiating MSCs collected at different days indicated above. Media were replaced every day, on day 7, Alkaline phosphatase activity (ALP) was determined in cell lysates using an alkaline phosphatase assay kit and as described previously [10].

2.5. Cell migration assays

Upper Boyden chambers with 8 mm pore membranes were seeded with MSCs at a density of 1–3 $\times 10^4$ cells per well, and 500 μl of concentrated conditioned medium collected by differentiating MSCs at 0, 5, 10, 15 and 20 days were added in lower chambers. After 6 h incubation, cells that migrated to the undersurface of the filters were stained by Giemsa and counted. Maintenance medium was used as a control.

2.6. Determination of potential paracrine factors secreted by differentiating MSCs in vitro by Western blotting

Western blotting was used to assess the expression level of selected paracrine factors. Total protein in matrix conditioned by differentiating MSCs at different time points was extracted with RIPA lysis buffer; proteins were separated by electrophoresis on 12% (wt/vol) polyacrylamide gels and electrophoretically transferred to PVDF membranes. Blots were probed with mouse anti-mouse VEGF, PEDF, Col1 and GAPDH (Santa Cruz Biotechnology, Santa Cruz CA), goat anti-BMP2 (R&D, Minneapolis, MN), Rabbit anti-Osteocalcin (Millipore Corp, Billerica, MA) followed by HRP-conjugated secondary antibodies. The blots were developed by SuperSignal West Pico substrate (Thermo Scientific). The relative intensities of the immunoreactive bands were analyzed with Quantity One software (Bio-Rad Laboratories) after densitometric scanning of the X-ray films using a Fluor-S Multimager (Bio-Rad). Images were calibrated against a reference autoradiography and given in relative density units. After autoradiography acquisition, the membranes were stripped and reprobed for 2 h at room temperature with anti-GAPDH antibody (Santa Cruz Biotechnology, Inc.) to normalize protein loading.

2.7. Infusion and retrieval of GFP marked cells into mice femurs

To determine whether MSCs secrete selected and predicted paracrine factors in vivo upon transplantation, cells were infused into femurs of mice and retrieved at different time points. Recipient mice were sub lethally irradiated prior to cell injection as described previously [10]. Four hours after irradiation, mice were anesthetized by subcutaneous injection of 1 ml of Ketamine and Xylazine (100 mg/ml, 2:1)/kg body weight. A tunnel was created within five mice femurs cavities via the femoral condyle using a 26 gauge needle; this was followed by insertion of a smaller gauge needle (30 gauge) attached to a syringe containing cells for injection. MSCs for injection were suspended in 20 μl of PBS (2×10^6 cells) and delivered within the bone cavity by slowly retracting the needle while depositing cells. Donor GFP+ cells were retrieved from recipient femur bones and marrow at 1, 3, 7, 14 and 28 days following cell infusion. Retrieved cells were expanded in culture in a medium supplemented with Zeocin for selection of GFP+ donor cells.

3. Retrieval of donor cells from the recipient mice

For cell retrieval from bone and marrow of recipient femurs, methods described previously were followed [11,13]. After marrow flush, femurs were cut into small pieces and placed in Petri dishes containing DMEM, 20% FBS, and 50 μg/ml ascorbic acid; cells were maintained as tissue explants. Marrow and bone derived cells were maintained separately in culture in DMEM, supplemented with

20% FBS, 50 μg/ml ascorbic acid, 1% (vol/vol) P/S. After 1 week of culture, 25 μg/ml Zeocin was added to select for the GFP+ donor cells.

4. FACS analysis and sorting for GFP+ cells retrieved from bone and marrow

The cells retrieved from marrow and bone of the recipient mice at 4 weeks were expanded in culture in presence of Zeocin for 7 days. The expanded cells were sorted by FACS for the GFP+ donor cells prior to their use for gene expression analysis.

4.1. Total RNA isolation and RT-PCR

RNA was extracted from 1×10^6 cells of expanded MSCs retrieved from recipient bone and marrow at different days shown above to assess expression of selected candidate genes (BMP-2, VEGF, SDF-1) by differentiating MSCs in vivo. Gene expression of candidate factors by differentiating MSCs in osteogenic medium at different time points was also assessed. Triplicate PCR reactions were amplified using specific primers and β-actin as a control for assessing PCR reaction efficiency.

5. Results

5.1. Induction of ALP activity in exogenous MSCs by factors in a medium conditioned by differentiating MSCs

We focused on early markers of osteoblasts differentiation; ALP activity. Upon incubation of exogenous MSCs in a medium conditioned by differentiating MSCs, the cells displayed ALP activity. The strongest activity was elicited by the medium conditioned by the cells at days 15 and 20 following induction of osteogenic differentiation (Fig. 1A). These data indicate that at these time points, differentiating MSCs produced factors capable of inducing differentiation of exogenous MSCs toward osteogenic lineage.

5.2. Cell migration

Factors produced by differentiating MSCs were also assessed for induction of exogenous MSCs migration in an in vitro assay system. The results revealed that, as the cells differentiated toward osteogenic lineage, they secreted factors that were chemoatractant to exogenous MSCs. Medium harvested at days 5, 10 and 15 showed the strongest activity (Fig. 1B). The data demonstrate that factors that play a role in cell chemoattraction are secreted by differentiating MSCs at specific time points during osteogenic differentiation.

5.3. Assessment of secreted factors

To determine the nature of the factors that exhibited paracrine activities toward exogenous MSCs, we focused on predicted factors known to play a role in cell differentiation and migration. Examination of the medium conditioned by the differentiating cells showed that by day 1, BMP-2 was being synthesized although at low levels; expression of this factor increased in culture and was maximally expressed beginning from day 5 up to day 15 and then began to decline (Fig. 2A and B). Pigment epithelial derived factor (PEDF), a novel factor expressed by MSCs was detected at low levels at day 0 and was maximally expressed at day 10 (Fig 2A and B). Published reports suggest that this factor may a play a role in cell differentiation and migration [14,15]. Absence of PEDF expression has been found to lead to defects in OI type VI whose major characteristic is poorly mineralized bone matrix [16,17]. Vascular endothelial growth factor (VEGF) increased in expression during MSCs differentiation and was maximally expressed at day 10 and then began to decline (Fig 2A and B). Fig. 2B, shows time course synthesis of BMP-2, PEDF, VEGF, osteocalcin (OCN) and type I collagen by MSCs maintained in osteogenic medium. As shown in Fig. 2B, there is gradual increase in synthesis of these factors followed by a decline during osteogenic differentiation. These data imply that upon engraftment in bone, MSCs undergo differentiation; during this process they secrete factors that exert an effect on endogenous progenitors. Some of the factors play a role in recruiting new progenitors; some other factors play a role in the induction of progenitor differentiation.

5.4. MSCs transplanted in vivo secrete factors that may play a role in paracrine activities

To determine if donor MSCs secrete factors that play a role in paracrine activities in vivo, donor cells infused into mice femurs were retrieved at different days and were then assessed for expression of selected factors by PCR. The data showed that donor cells retrieved from recipient femurs expressed genes encoding BMP-2, VEGF and SDF-1 (Fig. 3); PEDF was not assessed. These data indicate that upon transplantation, donor cells secrete factors that display paracrine activities in vivo thus influencing the reparative process of the host tissue.

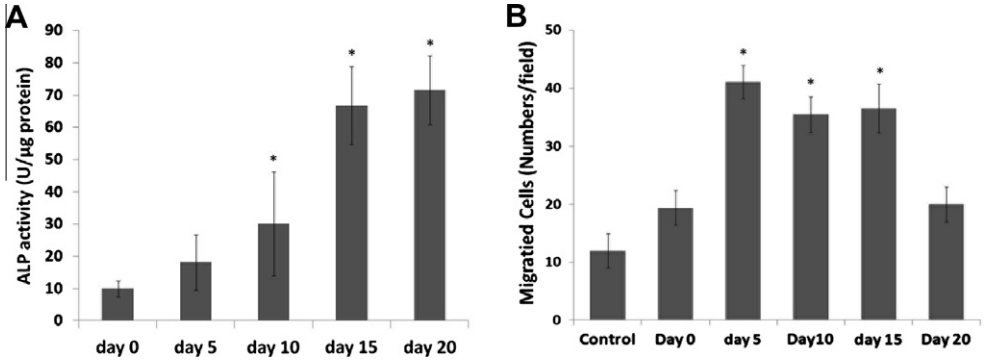

Fig. 1. ALP activity and migration of exogenous MSCs induced by incubation in medium conditioned differentiating MSCs. (A) ALP activity in exogenous MSCs. (B) Migration of exogenous MSCs to medium conditioned by differentiating MSCs. Medium was harvested at the indicated days of differentiation (*X* axis) and added to the exogenous MSCs to assess ALP activity and cell migration. Medium harvested from day 15 to day 20 showed the highest induction of ALP activity. Medium conditioned by differentiating cells from day 5 to day 15 showed the highest induction of cell migration. ALP activity was assessed following 7 day incubation of exogenous MSCs in the conditioned medium. Cell migration was performed using Boyden assay chambers and migrated cells were assessed by counting.

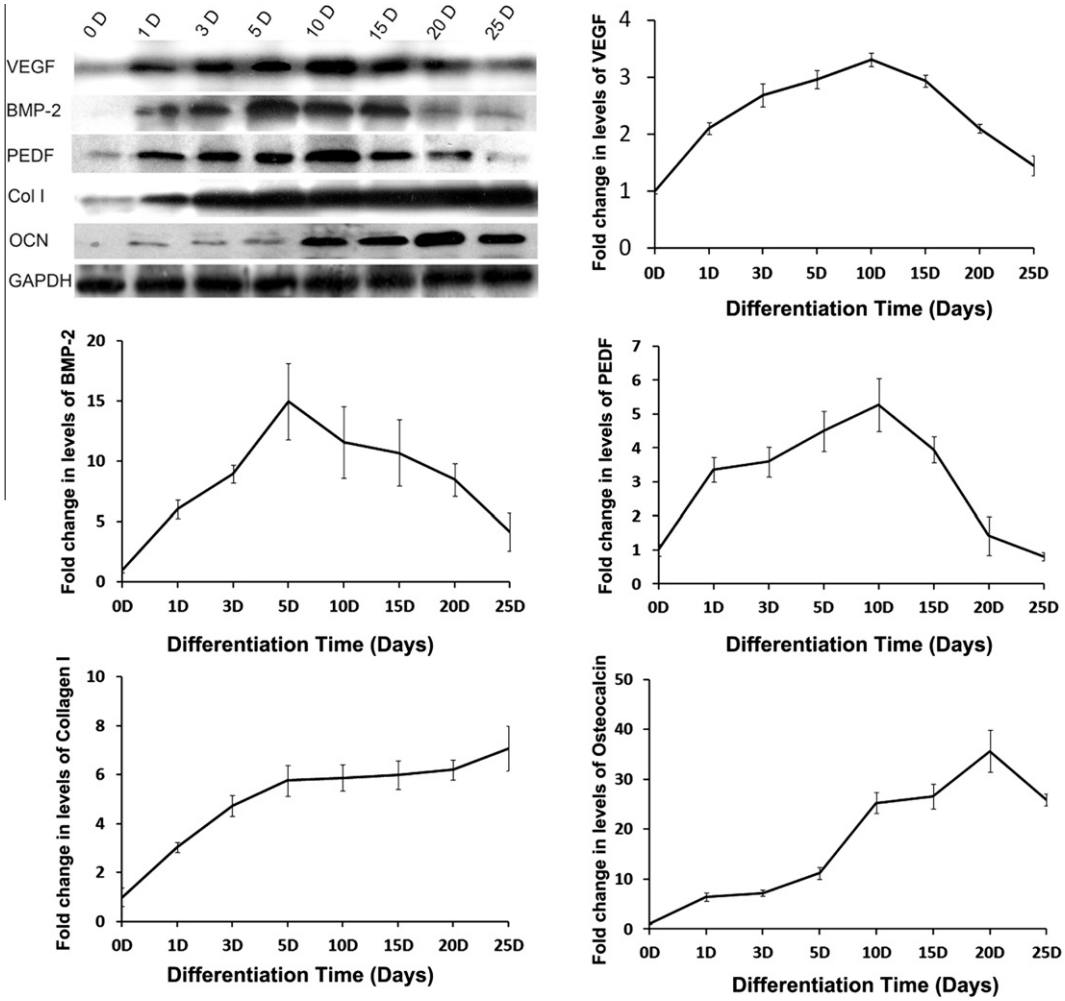

Fig. 2. Analysis of selected and predicted paracrine factors synthesized by MSCs at different days of differentiation. A Western blot of the suspected paracrine factors by differentiating MSCs. BMP-2 synthesis was maximal beginning at day 5 and continued up to day 15 and then began to show a decline. VEGF appeared to be synthesized throughout the differentiation period with a slight increase at day 10. PEDF synthesis appeared maximal at day 10 and showed a steady decline to day 25. Time course synthesis for each of the factors and other bone specific proteins are shown. Synthesis of collagen I (Col1) osteocalcin (OCN) are also shown.

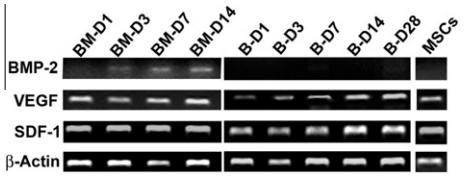

Fig. 3. Analysis for expression of predicted paracrine factor genes by MSCs infused into mice femurs. BMP-2 expression was detected beginning at day 3 in bone marrow (BM) following cell infusion into femurs. There was no expression of BMP-2 by donor cells in bone (B) at all the time periods assessed. VEGF was expressed by donor cells in bone marrow beginning at day 1 through day 14. Expression of VEGF by donor cells in bone appeared to increase with extended period. SDF-1 was equally expressed at all time periods by donor cells in bone marrow and bone. MSCs marked with GFP and Zeocin resistant genes were infused into femurs and retrieved from bone marrow and bone at specified days. BM (Bone marrow), B (Bone). D1-28 days at which donor cells were retrieved.

5.5. Blocking of BMP-2 and VEGF to assess their effects on differentiation and migration of exogenous MSCs

To test whether BMP-2 expressed by differentiating MSCs played a role in exogenous MSCs differentiation, BMP-2 activity was blocked using neutralizing antibodies to the protein. Exogenous MSCs incubated in conditioned medium supplemented with neutralizing antibodies to BMP-2, reduced ALP activity in the exogenous MSCs (Fig. 4A). The reduction in activity was however not returned to the control level suggesting that there are other factors that play a role in MSCs differentiation toward osteogenic lineage. The data demonstrated that BMP-2 was partly responsible for the induction of ALP activity in exogenous MSCs. Neutralizing antibodies to VEGF did not have any effect on cell migration suggesting that VEGF may not play a role in cell migration at least in vitro (Fig 4B).

Taken together, the results indicate that, during MSCs differentiation toward osteogenic lineage, they secrete factors at specific time points that exert paracrine activities. Through this process, donor cells contribute to the reparative process of the host tissues.

6. Discussion

The data reported in this communication showed that MSCs differentiating toward osteogenic lineage secrete factors that have an influence on exogenous MSCs activities. In this communication, we have shown that donor MSCs differentiating toward osteogenic linage generate factors that induce differentiation and migration of exogenous MSCs. These findings are quite significant for understanding application of MSCs in bone repair and regeneration. Clinical trials carried out to assess efficacy of MSCs to treat children with severe forms of OI, showed that, the children who received

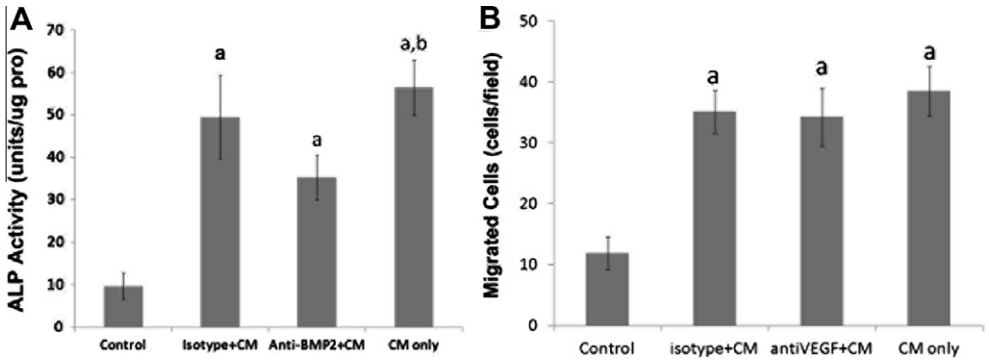

Fig. 4. Neutralizing antibodies to BMP-2 and VEGF to assess their paracrine activities in cell differentiation and migration. (A) Neutralizing antibodies to BMP-2 in a medium conditioned by differentiating MSCs decreases ALP activity in exogenous MSCs. (B) Neutralizing antibodies to VEGF in a medium conditioned by differentiating MSCs have no effect on exogenous MSCs migration. Control = Exogenous MSCs incubated in regular maintenance medium; Isotype + CM = Cells incubated in a conditioned medium containing nonspecific antibody to BMP-2 or VEGF; anti-BMP-2 and VEGF + CM = Cells incubated in a medium conditioned by differentiating MSCs containing antibody to BMP-2 or VEGF; CM = Cells incubated in a medium conditioned by differentiating MSCs without neutralizing antibodies. Data show that inhibiting BMP-2 activity decreases ALP activity expression.

the transplant exhibited increased growth and the reduction in the rate of bone fracturing [7,8]. The level of donor cells engraftment was however, low and could not account for the benefits observed. These findings led to the speculation that donor MSCs contribute to the regenerative process not only by differentiation into cells of the host tissue but by other mechanisms as well. The present findings have shown that as donor cells differentiate toward osteogenic lineage, they at specific time points secrete factors that play a role in induction of endogenous progenitor cell differentiation and recruitment. Through this mechanism, the donor cells contribute to the reparative process and these findings could explain previous observations.

Bone morphogenetic protein 2 (BMP-2) secreted by MSCs played a role in differentiation of exogenous MSCs as demonstrated by neutralizing antibodies experiments. Vascular endothelial growth factor is known to play a role in angiogenesis via recruitment of endothelial cells thus influencing cell migration [18]. PEDF is novel factor identified here to be secreted by MSCs during osteogenic differentiation, its role in MSCs differentiation has not been demonstrated in MSCs but may play a role in mineral deposition. Stromal cell derived factor (SDF-1) with its receptor CXCR4 are believed to play a role in cell trafficking. Expression of SDF-1 in vivo by donor MSCs suggests that this factor may play a role in progenitor cell recruitment [19].

In summary, the present findings reveal that, as MSCs differentiate toward osteogenic lineage, they secrete factors that exert paracrine activities on osteoblasts progenitors. Through this process, donor cells facilitate differentiation and recruitment of progenitors thus contributing to the bone repair and regeneration of the host tissue. These findings provide a basis for designing approaches for MSCs applications in various scenarios of bone repair and regeneration.

Acknowledgment

The work was supported by in part by Tobacco Cure Grant to CN and Brittle Bone Foundation Fellowship to FL.

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