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In Vitro and *In Vivo* Immunosuppressive Characteristics of Hepatocyte Growth Factor-modified Murine Mesenchymal Stem Cells

LI BIAN [1], ZI-KUAN GUO[2], HENG-XIANG WANG[3], JIN-SONG WANG[2], HUA WANG[2], QING-FANG LI[2], YUE-FENG YANG[2], FENG-JUN XIAO[2], CU-TSE WU[2] and LI-SHENG WANG[2]

[1]*Beijing 307 Hospital, The Academy of Military Medical Sciences, Beijing;*
[2]*Department of Experimental Hematology, Beijing Institute of Radiation Medicine, Beijing;*
[3]*Department of Hematology, General Hospital of Air Forces, Beijing, People's Republic of China*

Abstract. *Background: Previous data have proven that hepatocyte growth factor (HGF) is able to maintain the survival of mesenchymal stem cells (MSCs), rendering HGF gene modification as an optional strategy for MSC therapy. However, the question about whether gene-transferred MSCs (MSC/HGFs) exhibit more potent immunosuppressive activity remains elusive. Materials and Methods: Murine MSCs were isolated, culture-expanded and transfected by an adenovirus carrying human HGF cDNA (Ad-HGF). The transfection efficiency was evaluated by measuring HGF concentrations in the culture supernatants. An MHC-incompatible skin grafting model (C57BL→BALB/c) was used to observe if MSC/HGF transfusion could prolong the survival time of skin transplants compard to MSCs. Furthermore, their inhibitory effects on the proliferation of T lymphocytes elicited by Con A and the activation of CD11b+ cells in mixed lymphocyte reaction were compared with carboxyfluorescein diacetate succinmidyl ester labeling and flow cytometric techniques. Results: Ad-HGF was able to transfect mouse MSCs at high efficiency and administration of MSC/HGFs remarkably prolonged the mean survival time of skin grafts (16.73±0.57 days, p<0.01), compared with mice receiving MSCs (14.27±0.63 days), or saline (10.92±0.73 days). However, the presence of MSC/HGFs exhibited little additive impact on the suppression of T lymphocyte proliferation and activation of CD11b-positive and -negative cells in comparison with MSCs, though the inhibitory effects were evidently greater than with NIH3T3 cells and their Ad-HGF-modified counterparts. Conclusion: MSC/HGFs inhibit in vitro immune responses in a pattern similar to MSCs, but this gene modification might have beneficial effects for transplanted cells and damaged tissue.*

Correspondence to: Dr. Li-sheng Wang, Department of Experimental Hematology, Beijing Institute of Radiation Medicine, Beijing 100850, People's Republic of China. Tel: +861 066932245, Fax: +861 068158312, e-mail: wangls@nic.bmi.ac.cn

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Mesenchymal stem cells (MSCs) are generally defined as a cell population that has the potential to differentiate along multiple lineages such as chondrocytes, osteocytes, adipocytes and hematopoiesis-supporting stromal cells (1-2). This potential makes them an intriguing source of cells for cellular replacement therapy and tissue engineering. Recent data also suggest that MSCs are characterized by their immuno- and inflammation-suppressive activities both *in vitro* and *in vivo* by means of direct cell-cell contact and cytokine secretion (3-6), enhancing their therapeutic appeal in the setting of allogeneic bone marrow transplantation, cardiac ischemic diseases and other disorders (7-8). However, most MSCs have been found inanimate a few days after transplantation and their poor viability limits their reparative capacity and probably immunomodulatory activity *in vivo* (9-10).

Hepatocyte growth factor (HGF), originally identified as a mitogen for hepatocytes and a scatter factor, targets various cell types including MSCs, which have been shown to express c-met, the well-characterized receptor for HGF (11). Previous data have also demonstrated that HGF-modified mesenchymal stem cells (MSC/HGFs) exhibit effective protective activity against apoptosis elicited by anoikis *in vitro* (11). Furthermore, MSC/HGFs display more potent proangiogenic effects *in vivo* in the myocardial ischemic model, compared with their counterparts (12). Therefore, it is plausible to postulate that HGF-modification might allow MSCs to survive after transplantation and endow transplanted cells with a more potent capacity for tissue repair and immunosuppression.

However, there is still a paucity of information on whether HGF gene transfer could affect the immunosuppressive effects of MSCs, though recent data have demonstrated that HGF itself is able to inhibit the antigen-presenting capacity of dendritic cells (13), thereby probably ameliorating the severity of graft-*versus*-host disease in a murine model (14). In the present study, murine MSCs were culture expanded, transfected by an adenovirus vector carrying human HGF cDNA (Ad-HGF) and their *in vitro* antiproliferative activity on allogenic lymphocytes investigated. In addition, the *in vivo* immunosuppressive activity of transfused murine MSCs and MSC/HGFs was evaluated in a tail-skin grafting model transplanted across the MHC barrier. The study demonstrates that MSC/HGFs might display a more potent reparative rather than immunoregulatory activity, compared to their counterparts *in vivo*.

Materials and Methods

Mice. Normal inbred C57BL/6 (H2b) and BALB/c (H2d) female mice were purchased from the Laboratory Animal Center, Chinese Academy of Military Medical Sciences and were housed in conventional cages. All experiments in this study were performed in accordance with the Chinese Academy of Military Medical Sciences Guide for Laboratory Animals.

Isolation and culture-expansion of murine MSCs. Isolation and culture-expansion of murine MSCs were performed according to the protocols described in previous work (15). Briefly, the femurs and tibiae were collected from 2- to 3-week-old C57BL/6 female mice and the bone marrow was flushed out by drawing and expelling with a syringe. The compact bones were excised into chips of about 1 mm^3 and suspended in alpha-modified minimal essential medium (α-MEM) containing 1 mg/ml of collagenase II (Sigma-Aldrich, USA), followed by shaking for 2 hours at 37˚C. The released cells were discharged and the bone fragments were washed three times with α-MEM, followed by cultivation in α-MEM containing 10% FBS as in previous work (16). The cells at the third passage were used for the following experiments.

Transfection of MSCs and NIH3T3 cells by adenovirus particles. MSCs and NIH3T3 cells (purchased from ATCC) were harvested by trypsin digestion, plated into flasks and incubated overnight for cell attachment. The medium was changed for α-MEM without serum and recombinant adenovirus suspension was added at a multiplicity of infection (MOI) of 150:1 (virus particles *vs*. cells). Adenovirus particles were generated and purified as previously described (11). Two hours later, serum was supplemented and culture was maintained for 48 hours. Gene-modified cells, named as MSC/HGFs and NIH3T3/HGFs, respectively, were then collected for the experiments below.

Identification on the efficiency of adenovirus infection. An adenoviral vector carrying cDNA for enhanced green fluorescent protein (Ad-eGFP), which has the same framework as Ad-HGF, was used at a MOI of 150:1. Cells were observed by fluorescence microscopy at serial time points after transfection. Aliquots of MSCs (5×10^4) and MSC/HGF were seeded into 12-well plates with each well of 1 ml. Culture supernatants were collected and the levels of HGF were evaluated with ELISA as described in our previous work (11).

Tail skin grafting. MSCs or MSC/HGF (1×10^6) from female C57/BL were injected intravenously into BALB/c mice and sex-matched skin grafting was conducted as described elsewhere (17). Briefly, full-thickness tail-skin (around 0.5×0.5 cm^2) from C57/BL (allografting) or BALB/c (syngeneic grafting) mice was transferred into the sides of the recipient's tail, from which an equivalent amount of skin had been removed. Each mouse received two allografts and one syngeneic graft. The grafts were protected with a glass tube and monitored daily by visual inspection. A graft was considered rejected when more than 90% of it was necrotic and the grafts transferred to the tail on which the syngeneic skin had not survived were screened out for further analysis. On day 10, some of the skin grafts were removed for histological examination by H&E staining.

Carboxyfluorescein diacetate succinmidyl ester (CFSE) labeling. CFSE (Sigma, USA) cell labeling was conducted as previously described (18). Briefly, MSCs and spleen mononuclear cells from C57 and, BALB/c mice respectively, were harvested, suspended in phosphate-buffered saline (PBS) at a density of 10^7/ml and labeled with 5 μM CFSE for 15 minutes at room temperature in the dark. Equal volumes of fetal calf serum (FCS) were added and the labeled cells were incubated for a further 5 minutes. Cells were washed three times in cold PBS and used in the proliferation experiments below. Typically, the labeling proficiency was over 98% as analyzed with flow cytometry.

Lymphocyte proliferation assay. Aliquots of CFSE-labeled spleen mononuclear cells (1.0×10^6) were seeded into flasks (25 cm^2) that had previously been seeded with irradiated MSCs or NIH3T3s (5×10^4). The cells were incubated for 72 hours in RPMI-1640 containing 10% FBS and 20 μg/ml Con A. Suspended cells were then collected and their proliferation status was assessed by flow cytometric analysis. Briefly, at least ten thousand events were acquired on a FACSCalibur cytometer (Becton Dickinson) and the collected data were analyzed with Winmdi28 software after the population of interest was gated.

Mixed lymphocyte reaction (MLR) and flow cytometry assay. Aliquots of MSC and NIH3T3 cells (5×10^4) were plated into flasks (25 cm^2), transfected with Ad-HGF, maintained for 48 hours and co-cultured with BALB/c splenocytes (1.0×10^6) followed by irradiation at a dose of 20 Gy from a ^{60}Co source. CFSE-labeled spleen mononuclear cells (1.0×10^6) from C57/BL were added to the flasks and the culture was maintained for a further 48 hours. Suspended cells were harvested and stained with PE- or Percep-conjugated rat monoclonal antibodies against mouse CD11b and CD69 (BD Pharmingen, USA). Events were acquired with a FACSCalibur cytometer and the CFSE-positive cells were further analyzed with Winmdi28 software.

Statistical analysis. Data are presented as the mean ± standard deviation and those from skin grafting were analyzed with the log-rank test. A *p*-value less than 0.05 was considered statistically significant.

Results

Ad-HGF infects mouse MSCs at a high efficiency. Previous work has demonstrated that Ad-HGF can transfect rat MSCs at a high efficiency without resulting in cell damage (11). To address if this was the case in mouse compact bone-derived MSCs, an adenovirus vector carrying eGFP with the same

framework as Ad-HGF was used as an indicator. The results showed that nearly all cells were able to express exogenous protein at 48 hours after gene transfer when the MOI of 150:1 was empirically utilized, as assessed by fluorescence microscopy (Figure 1A and B) and flow cytometric analysis (Figure 1C). Further, ELISA showed that HGF secretion could be maintained for at least 8 days since the level of HGF in the culture supernatant from MSC/HGF was significantly higher than the baseline of MSCs (Figure 1D, 37.2±1.2 ng/mL/5×10^4 cells *vs*. 18.4±0.5 ng/mL/5×10^4 cells, $p<0.05$).

MSC/HGFs prolong skin survival in an MHC-incompatible skin grafting model. To address if HGF transfer could enhance the immunosuppressive effects of MSCs *in vivo*, skin grafting was performed in a mouse model (C57BL/6→BALB/c) with or without MSC pre-transfusion. The results showed that, as previously described (15), MSC delivery gave rise to a significant survival prolongation of allogeneic skin grafts (10.92±0.73 *vs*. 14.27±0.63 days, n=13 and 11 respectively, $p=0.0049$) (Figure 2A), and this prolongation was more significant in the MSC/HGF infusion group (16.73±0.57 days) than that in MSC-treated mice (Figure 2A, $p=0.0061$). Histological examination on day 10 showed intact epidermis in grafts from MSC- and MSC/HGF-transfused mice, though cellular infiltration was evident in the subcutaneous layer (Figure 2C and D). Moreover, it was observed that tissue necrosis in the subcutaneous layers occurred in mice without receiving cell therapy (Figure 2B), and the damage was lessened particularly by MSC/HGF treatment (Figure 2D), suggesting that local high concentration of HGF might ameliorate the severity of tissue damage by allo-reactive immunity.

HGF has little synergistc effect with MSCs on the lymphocyte proliferation elicited by Con A. To further investigate the why HGF-modified MSCs exhibit more protective activity on skin grafts, gene-modified MSCs and NIH3T3 were compared for their inhibitory effects on *in vitro* T lymphocyte growth stimulated by Con A by means of a membrane dye dilution assay. The results showed that the proportions of lymphocytes that had not experienced cell divisions were around 45% when co-cultured with MSCs or MSC/Ad-HGFs, while those in NIH3T3s or NIH3T3/Ad-HGFs were around 15% (Figure 3). However, the percentages of cells having experienced cell division once, twice or three times and more were comparable between MSC and MSC/HGF groups, as was also the case for NIH3T3s and NIH3T3/HGFs (Figure 3). Therefore, it may be deduced that HGF alone does not possess any antiproliferative activity on T lymphocytes.

HGF has little synergistic effect with MSCs on the suppression of monocyte and macrophage activation. Previous data demonstrated that the inhibitory effect of MSCs on the activation and maturation of antigen-presenting cells, including monocytes and dendritic cells, probably by activating the JAK/STAT signaling, represents a critical step in the immune modulation observed *in vitro* (19-20). Furthermore, HGF itself is able to suppress Th1 and Th2 responses by reducing the antigen-presenting activity of dendritic cells (13). CD11b is a marker for mouse monocytes that are a well-characterized component responsible for the activation and subsequent clonal expansion of T lymphocytes in MLR. Therefore, in the present study, CD69 expression was evaluated by flow cytometry to determine if HGF transfer could enhance the inhibitory effect of MSCs on CD11b+ cell activation. As shown in Figure 4, the presence of MSCs did reduce the percentages of CD69-positive cells among the allo-reactive population (CFSE-positive) compared with NIH3T3 cells, however, HGF transfer had little impact on their inhibitory action to both CD11b-positive and CD11b-negative cells, as was also the case when the activation status was compared between NIH3T3 and NIH3T3/HGF groups (Figure 4).

Discussion

An increasing number of reports have implied that transplantation-associated survival of MSCs is the determinant factor that predisposes their functions *in vivo*. Therefore many investigations have been performed to show whether the modification of MSCs could be feasible and practicable. These treatment strategies include the incorporation of exogenous genes such as *Akt* oncogene (9, 21), *integrin-linked kinase* (22), *angiopointin-1* (23) and *HGF* (11), and treatment with growth factors such as insulin-like growth factor types I and II (24-25) and HGF in our previous work (12).

MSCs are not only characterized by their multiple differentiation capacity into osteoblasts, chondroblasts, adipoblasts and hematopoiesis-supportive stromal cells, but also by their immune modulation activity both *in vitro* and *in vivo,* mainly through directing the development of antigen-presenting cells toward a suppressive phenotype (3-5). Interestingly, HGF is also an immune regulator which down-regulates the maturation and antigen-presenting capacity of dendritic cells (13). However, to date, little information has been available on whether HGF could provide synergistic effects on MSC-mediated immune suppression.

To address this issue, a skin-grafting model was utilized in this study and the results showed that the transplantation of gene-modified MSCs resulted in a definitely greater prolongation in the survival time of skin grafts compared with MSCs, though graft rejection occurred in all three groups. Furthermore, histological examination revealed that MSC and MSC/HGF therapy lessened tissue damage, but the infiltration of inflammatory cells was not remarkably avoided. Therefore, although not investigated here, the mechanisms by which

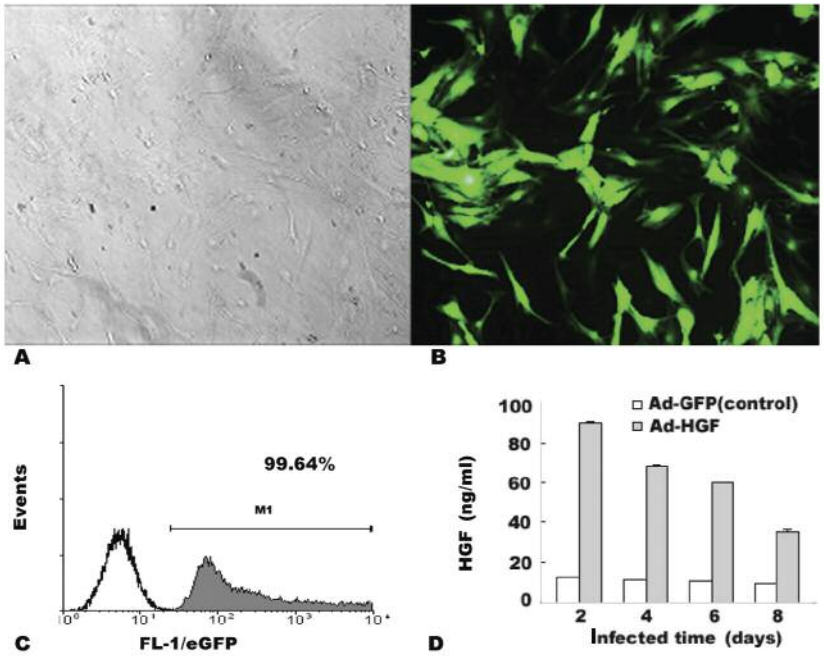

Figure 1. *Ad-HGF transfects mouse MSCs at a high efficiency. Murine compact bone-derived MSCs were infected by Ad-eGFP at a MOI of 150 and observed by fluorescence microscopy at 48 hours afterwards (A, B). In addition, transfected cells were collected for flow cytometric analysis as shown in C, in which open histograms show the background signal while shaded histograms show the events from MSC/eGFP. D, ELISA results for the determination of HGF concentrations from culture supernatants.*

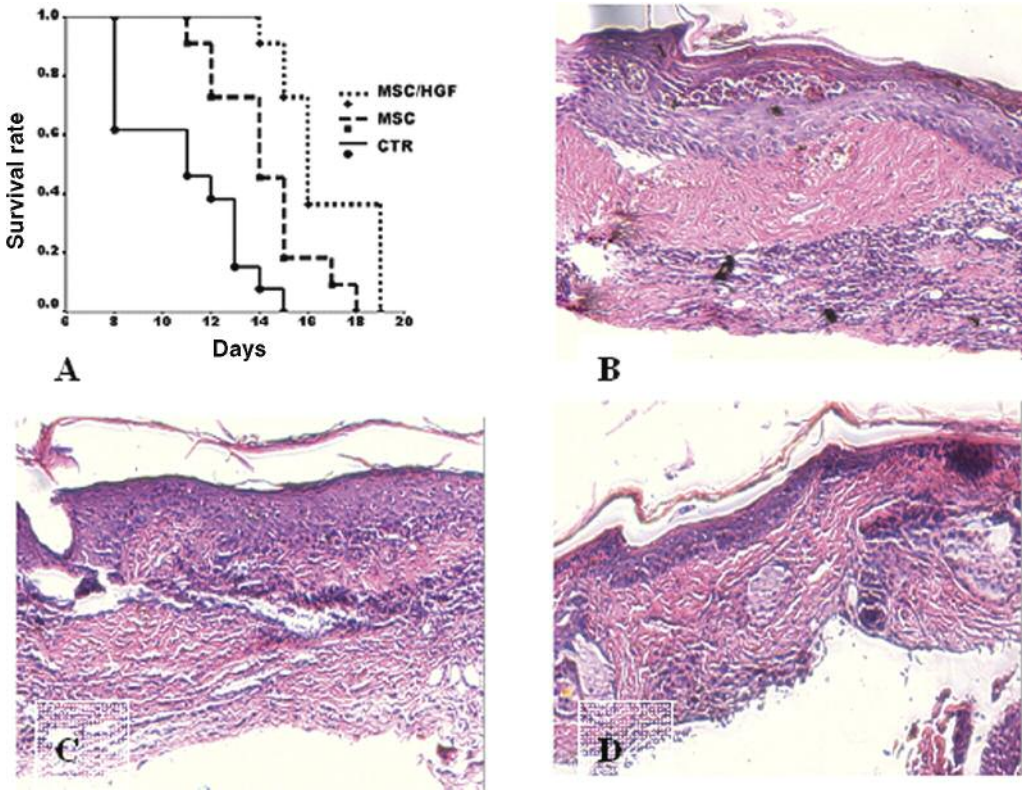

Figure 2. *Ad-HGF transfer prolongs the survival time of transplanted skin clips. Tail-skin was grafted from female C57/BL to BALB/c mice and the survival time of the grafts was documented (A). In addition, skin graft biopsies were obtained on day 10 post-transplant and are shown for one representative graft in each group (B-D, ×100). B, control; C, MSCs; D, MSC/HGF.*

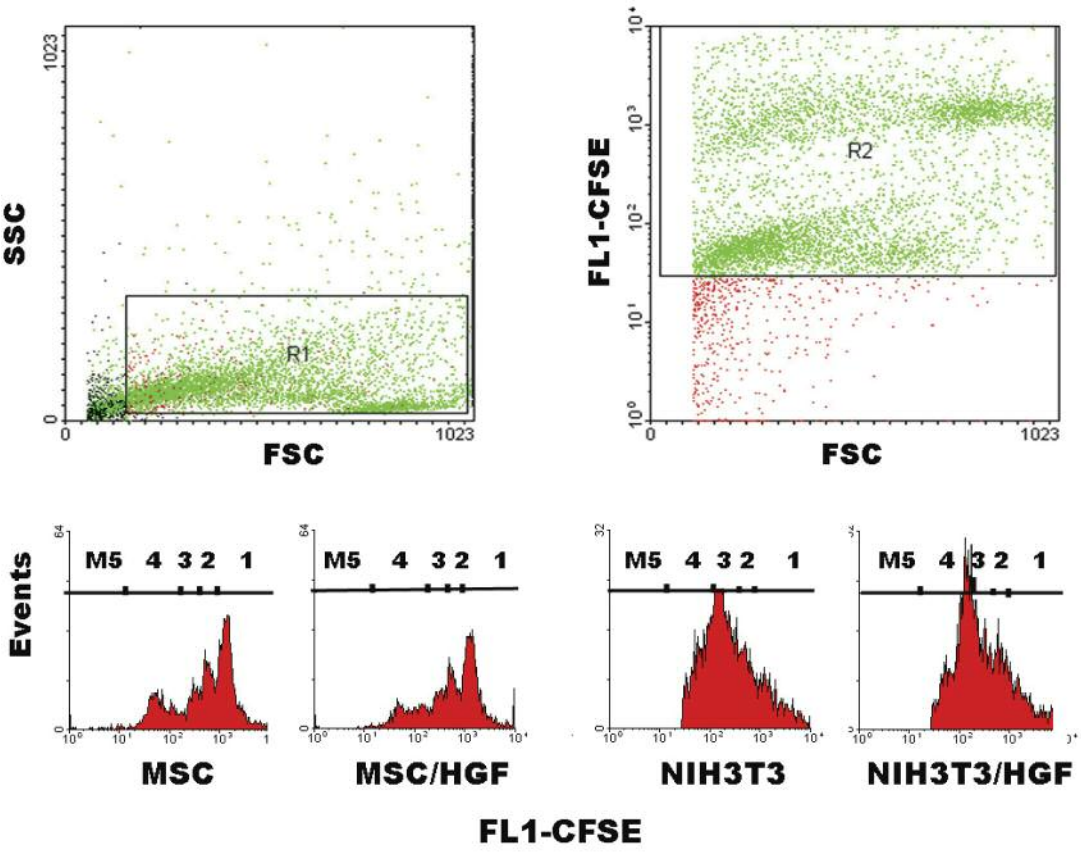

Figure 3. *Ad-HGF transfer has little effect on the antiproliferative activity of MSCs on allogenic lymphocytes. After event collection, cell debris was gated out according to the FSC and SSC signals (upper left) and CFSE-positive cells were gated (R2, upper right) for further analysis. Events in regions of M1-5 represent cells that had not experienced cell division, divided once, twice, three times or more and were negative for CFSE, respectively. The percentages of events in M1-5 were 46.0/20.7/12.4/20.8/0.66, 45.3/20.6/16.0/18.2/0.46, 16.1/14.2/26.1/46.3/0, and 13.7/12.9/25.7/48.7/0 respectively for MSC, MSC/ HGF, NIH3T3 and NIH3T3/HGF cells. The data are representative of two individual experiments.*

MSC/HGF treatment promoted grafted skin survival might include additive effects from HGF on immune suppression and amelioration in cell injury.

To search for the proof for this hypothesis, T lymphocyte transformation and mixed lymphocyte reaction experiments were performed to compare the inhibitory activity between MSCs and MSC/HGFs. Interestingly, MSCs and MSC/HGFs behaved similarly in the suppression of T lymphocyte proliferation and monocyte activation, suggesting that HGF transfer did not provide a clear additive role for MSC-mediated immune modulation *in vitro*, and that the prolonged survival time of skin grafts in MSC/HGF mice might be attributed to the protection of damaged tissues by HGF.

In fact, HGF has been shown to protect intestinal and thymic epithelial cell damage from irradiation and cytotoxicity from allogenic T-cell immunity in the setting of a bone marrow transplantation mouse model (14, 26). It could be also the case in the model of skin grafting, since HGF is a well-characterized antiapoptotic cytokine targeting various cell types. HGF combination, therefore, might promote the survival of both transplanted MSCs and grafted skin tissue, resulting in the prolongation of cytokine secretion by MSCs and the elevation of cytokine levels in the grafted tissue. This might account for the observation that MSC/HGFs were not able to enhance the immunosuppressive activity of MSCs *in vitro* but displayed a more protective activity on skin grafts in this MHC-incompatible model. In conclusion, a deep understanding of the mechanism for this phenomenon needs more detailed investigations, which would also promote MSC therapy for clinical practice.

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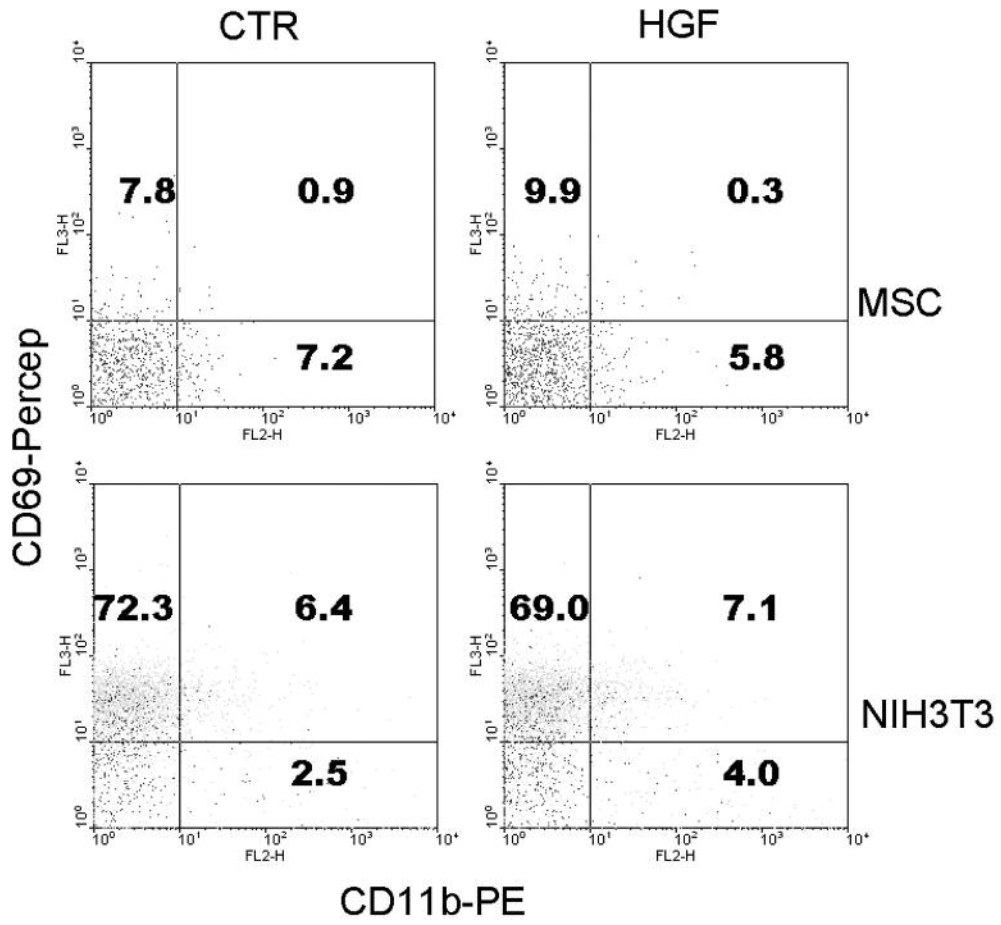

Figure 4. *HGF transfer into MSC and NIH3T3 cells exhibits little effect on the activation of CD11b+ cells. CFSE-positive cells were gated for CD11b and CD69 expression analysis. The percentages of different populations are given. The data are representative from two separate experiments.*

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