Dopamine Mobilizes Mesenchymal Progenitor Cells Through D2-class Receptors and Their PI3K/AKT Pathway

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Running head of the title: Catecholamines mobilizes mesenchymal stem cells

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Key words
Mesenchymal Stromal Cells, Stem Cell Mobilization, dopamine, Catecholamines, Stem Cell Niche

ABSTRACT

As the nervous system exerts direct and indirect effects on stem cells mobilization and catecholamines mobilize hematopoietic stem cells (HSCs), we hypothesized that dopamine might induce mesenchymal progenitor cells (MPCs) mobilization. We show that *in vitro* dopamine induced MPCs migration through D2-class receptors, and their alternative PI3K/Akt pathways. Also, administration of catecholamines mobilized *in vivo* colony forming unit-fibroblast (CFU-F) in mice injected with dopamine. In contrast, *in vitro* and *in vivo* MPCs migration was suppressed by D2-class receptors antagonists consistent with this signaling pathway implication. In humans, patients treated with L-dopa or catecholaminergic agonists showed a significant increase of a MPC-like population ($CD45^-CD31^-CD34^-CD105^+$) in their peripheral blood. These findings reveal a new link between catecholamines and MPCs mobilization and suggest a novel use for D2-class receptors agonists in the potential clinical mobilization of MPCs.

INTRODUCTION

Over the last two decades, the field of MPCs has progressed rapidly from the laboratory to the early clinical trials for a wide range of diseases [1]. Overall, the types of reagents and methods that helped the field of HSCs research can guide, at least in part, what is needed for the MPCs field. It is well known that HSC continuously egress out from the bone marrow (BM) to the circulation under homeostatic conditions and the sympathetic nervous system regulates the hematopoietic niche in BM [2]. At present, the hypothesis of MPCs circulating through the peripheral blood (PB) in steady state is controversial and the molecular mechanisms are very poorly understood [3]. However, there are numerous studies that show MPCs recruitment to damaged tissue under injury conditions, released into circulation from remote tissues and, theoretically, stimulate MPCs to leave their niche [4]. In this regard, hematopoietic and endothelial progenitors also increase transitorily their number into peripheral blood after a traumatic event or pharmacological treatment [5].

In stress situations high amount of catecholamines are secreted into PB. In these situations catecholamines mobilize HSCs [2, 5, 6]. and it is controversial their effects on endothelial cells [6, 7]; conversely the knowledge on MPCs mobilization by catecholamines is scarce. Spiegel *et al* they proposed the existence of a "brain-bone-blood triad" where some signals are delivered to the HSCs pool directly, while other effects are exerted indirectly on niche-supporting stromal cells [8]. Recently, it has been demonstrated that MPCs in BM constitute an intrinsic element of the stem niche, in a close physical association with HSCs, implying a neural and hormonal regulation [9].

The actions of dopamine are mediated by D1 and D2 classes of dopamine receptors. Usually dopamine receptors activation is mediated by adenylyl cyclase, however also could be mediated by transactivation of tyrosine kinase receptors [10]. These receptors are able to active several downstream signal networks, as the phosphoinositide 3-

kinase (PI3k)/Akt signaling pathway, which is a survival pathway that regulates cell proliferation, apoptosis, differentiation and migration [11]. Recently it has been described that several PI3K/Akt stimulators increase MPCs migration, although the downstream target proteins of the PI3K/Akt pathway in MPCs migration is not well understood [12-15].

Here, we describe the expression of dopamine receptors in human MPCs and their function in the migration of these cells. Dopamine induced D2-class receptors activation in MPCs, through the non-canonical PI3-kinase/Akt activation signaling. We also tested *in vitro* and *in vivo* this hypothesis, using a mouse model and also in PB from human patients. In conclusion, our results suggest that catecholamines can mobilize MPCs into PB, in mice and humans.

METHODS

Culture of hMPCs from peripheral blood.

Mobilized PB samples were obtained from "Niño Jesús" Hospital (Madrid, Spain), after informed consent from healthy donors. Samples were centrifuged in a density gradient using Ficoll-Paque (Amersham) to obtain the mononuclear cells (MNC). In experiments where we tested matrix, non-treated culture flasks were cover with matrix: 7.5 μg/cm^2 of matrigel (BD Biosciences), 6.25 μg/cm^2 of fibronectin (R&D Systems), 6.25 μg/cm^2 of vitronectin, 0.1 mg/mL of Cultrex Basement Membrane Extract (R&D Systems) or 10% of dextrose-gelatin-veronal (DGV; Lonza). MNC were incubated in commercial MPC-specific medium: BMMPC (Lonza), NH Expansion Medium (Miltenyi), Stemline MPC Expansion Medium (Sigma) or DMEM (Sigma) plus 10-20% of FBS. In cultures where growth factors were used, their concentration was 10ng/ml. Individual cell cultures were we obtained a homogeneous PB-MPCs at fourth passage were defined as positive.

Human peripheral blood-derived MPCs characterization.

Human PB-MPC were characterized following The International Society for Cellular Therapy (ISCT) position statement [16]. In coculture experiments, CD3+ cells were isolated using immunomagnetics methods (Miltenyi Biotec). PB-MPC and T lymphocytes were co-cultures for 96 hours and were co-stimulated with 10 μg/ml of anti-CD3 and 1μg/ml of anti-CD28 antibodies (BD Bioscience). In mitogen-activated proliferation assays, they were performed by incubating 1x10^5 MNC with 10μg/ml of PHA. The cells were harvested on day 3 and T cell activation and cell cycle was measurement by flow cytometry.

Human PB-MPCs *in vivo* immunomodulation assays.

A model of BM haploidentical transplantation was conducted by transplanting BM cells from C57Bl/6 (H2$^{b/b}$) mice into B6D2F1 (H2$^{b/d}$) recipients as previously we reported [17].

Briefly, female mice B6D2F1 recipients were irradiated with 9 Gy of x-ray and injected with $5x10^6$ BM cells mixed with $2x10^6$ T cell from spleen. After 0, 7 and 14 days, the recipients were injected with $1X10^4$ hPB- or hBM-MPCs. Procedures were approved by Animal Experimentation Ethical Committee according to all external and internal biosafety and bioethics guidelines.

***In vitro* cell migration assays**

In migration experiments transwells were coated with 0.1% gelatin (Sigma). 5×10^4 MPCs were transferred to transwell chambers with 8 mm pore filters (BD Biosciences). Cells were incubated in the presence of 50 μM dopamine, 50 μM bromocriptine, 50 μM SKF-38393, 50 μM eticlopride, 50 μM SCH-23390 or 400 μM AS-604850 in the bottom chamber for 24 hours. Treatment with eticlopride, SCH-23390 and AS-604850 was performed by pre-incubating the cells with these reagents for one hour. Migrated cells were fixed and stained with crystal violet. For statistical analysis, cells were manually counted in 10 high-power fields (HPFs) and each experiment was repeated three times (n=6).

Measurement of intracellular cAMP levels in human MPCs.

cAMP levels in human PB-MPCs were measured using the Cyclic AMP XP Assay Kit (Cell Signaling Technology) according to manufacturer protocol. Briefly, $50x10^6$ cells were incubated in serum-free DMEM in presence of 50 μM of dopamine for 10, 20 and 30 min. Each experiment was repeated three times (n=6) and statistical significance was determined according to the Wilcoxon test.

Western blot analysis

Proteins were extracted with SDS sample buffer and separated by 10% SDS–PAGE and blotted on PVDF membranes (Bio-Rad). Primary antibodies were rabbit monoclonal anti-AKT1 (phospho-S473) antibody, 1:1000 dilution (Epitomics) and mouse monoclonal anti-β-actin, clone AC-15, 1:5000 dilution (Sigma). Secondary antibodies were

polyclonal goat anti-rabbit and anti-mouse immunoglobulins/HRP, 1:3000 dilution (DAKO).

Murine mobilization experiments.

FVB/NHanTMHsd female mice, between 8 and 12 weeks of age, were treated with different drugs. Epinephrine was diluted in water and administered intraperitoneally (i.p.) at a dose of 2mg/kg. Dopamine (Grifols) was diluted in 0.9% saline and administered i.p. at a dose of 50 mg/kg. The control group was inoculated i.p. with 0.9% saline. The G-CSF (200 μg/kg; Amgen) was diluted in 5% dextrose solution and administered subcutaneously. Dopamine receptors antagonist, eticlopride (10mg/kg), U99194 (20mg/kg) and SCH23390 (10mg/kg) were diluted in water and administered subcutaneously 30 minutes before of dopamine inoculation. All drugs were administered for 4 consecutive days, every 24 hours. The protocol was approved by Animal Experimentation Ethical Committee.

Blood was drawn by cardiac puncture. MNC were resuspended in DMEM plus 20% FBS and plated at 650,000 MNC/cm^2 on plates coated with fibronectin. The spleens were dispersed mechanically in 5 ml PBS by passing the cell suspension of 70 μm filter. To obtain cell from BM, femur and tibia of mice were collected. The count of CFU-F was performed at 7 days of culture.

Flow cytometry analysis.

In vitro human PB-MPCs cultures were analyzed by flow cytometry as previously we described [18]. Basically, cultured cells were incubated with appropriate monoclonal antibodies (supplemental Table 1), and, as a negative control, we used cells incubated with isotype immunoglobulins. For T cell activation analysis, cells were staining with anti-human antibodies (supplemental Table 1). Moreover, for cell cycle studies of T lymphocytes, $2x10^5$ cells were fixed and resuspended in 1ml of PBS containing 50 μg/ml

of propidium iodure (PI) and 200 µg/ml RNase. Cell were incubated during 30 min at 37ºC and analyzed by flow cytometry in a FACSCalibur cytometer (BD Biosciences).

Peripheral blood from 26 healthy volunteers and 56 patients with a cerebral stroke were examined obtained and analyzed. The study protocol was approved by Ethic Committee and patients have given written consent regarding participation in the study. MNC were obtained using Ficoll-Hypaque and $2x10^6$ cells were incubated with fluorochrome-conjugated monoclonal antibodies (Supplemental Table 1), washed and analyzed.

Immunostaining

Tissues were fixed in 10% neutral formaline and cells in 4% formaldehyde. After immunoperoxidase staining, it was visualized using the Vectastain ABC Kit (Vector Laboratories). Cells were counterstained with hematoxylin and placed in histoclear (National diagnostic) and mounted. Images were taken using a light microscope and digital camera (Nikon).

For the immunofluorescence staining, cells were grown on glass chamber slides (Thermo Fisher Scientific), stained with specific primary antibodies and nuclei cells were counterstained with Dapi. For negative controls cells were incubated with isotype matched control antibodies. Vectashield mounting media (Vector Laboratories) was added and images were taken using a confocal microscope (Leica).

***In vivo* transplantation of subcutaneous ossicles generated with PB-derived human subpopulations.**

Cell subpopulations were isolated from human mobilized peripheral blood using immunomagnetic MACS microbeads. $1x10^5$ CD45-CD31-CD34-CD105+ and $1x10^6$ CD45-CD31-CD34-CD105- cells were used to prepare each implant. Briefly, 40mg of

HA/TCP powder (Biomatlante) were deposited in a 50mL falcon tube and washed with 1mL of DMEM culture medium. Fresh cells were mixed with HA/TCP powder and centrifuged (1000 rpm, 5 minutes). Homogeneous cell seeding was checked and then ceramic/MSC compound was cultured overnight. Culture medium was carefully removed and culture medium with 35μg of BMP2 (Noricum) and fibrin were incorporated in order to prepare final ossicles. Finally, the ossicles were implanted s.c. under the dorsal skin of anesthetized NOD/SCID mice and one months later, the ossicles were harvested for their analysis.

RESULTS

Obtaining human MPCs from peripheral blood mobilized with G-CSF.

The frequency of MPCs in peripheral circulation seems to be very rare in steady-state conditions. Even using mobilizing agents, such as G-CSF, the peripheral blood-derived-MPCs (PB-MPCs) usually have a limited expanding potential [19]. In order to improve the protocol for obtaining human PB-MPC, we performed a combination of cell cultures with the following variables: source of samples, initial cell density, culture medium, matrix surface and growth factors supplement. As it is well known, human PB-MPCs do not form classical Colony Forming Units-Fibroblast (CFU-F), so we defined a semi-quantitative method: a cell culture was defined as positive when we obtained a homogeneous culture of hPB-MPCs and it could be expanded, at least during four passages. Our results showed that the initial cell density is critical for obtaining hPB-MPCs. Thus, the best result was obtained with an initial concentration of $1.25x10^6$ MNC/cm^2 (Supplementary Figure 1A), regardless of other parameters. Also, as shown in Figure 1A, there were differences among substrates, being fibronectin the most effective. In addition, as expected, fresh mobilized-MNC resulted in a higher hPB-MPCs production in all experiments. Finally, these hPB-MPCs could be *in vitro* expanded for a long time, maintaining a normal karyotype (Supplementary Figure 1B).

Characterization of human peripheral blood-derived MPCs.

The ISCT published their minimal criteria for defining a human MPCs [16], and also later they suggested the use of purified immune cells populations and well validated mice models to test immunoregulatory properties [20]. Using our optimized protocol we successfully isolated and expanded hPB-MPCs. First, a mixed and heterogeneous population was observed under light microscopy at 48 hours of culture but a more homogenous population could be observed after 2-3 passages. These cells were spindle-shaped, proliferated with a well-spread attached morphology and was able to differentiate (Figure 1B). Also the phenotype of PB-MPCs was determined by flow

cytometry (Figure 1C). In summary, our results indicated that we were able to obtain real hMPCs from human mobilized peripheral blood.

Immunomodulatory properties of human PB-derived MPCs.

We decided to study the immunomodulatory properties according to ISCT guidelines [20]. We isolated $CD3^+$ T-cells from PB-derived human mononuclear cells using immunomagnetic beads and these cells were mixed *in vitro* with hPB-MPCs at different ratios. CD3+ cells were activated using anti-CD3 plus anti-CD28 monoclonal antibodies. As shown in Figure 1D, the percentage of CD3+CD69-CD25+ was reduced when PB-MPCs were presented in the culture. In addition, hPB-MPCs were able to inhibit lymphocytes cell cycle in a dose dependent manner (Figure 1E). Similar results were obtained when we analyzed co-cultures of hPB-MPCs with T lymphocytes activated with phytohemmaglutinine (supplemental Figure 1).

Moreover, MPCs have been efficiently used for the control of graft-versus-host disease (GvHD) in murine models and also in human clinical trials [21, 22]. Thus, we used a xenogenic GvHD mice model for testing the immunomodulatory properties of hPB-MPCs (Figure 1F). hPB-MPCs protected the mice from lethal GvHD at the same level as hBM-MPCs did. These results indicate that hPB-MPCs were also able to be immunomodulators in an *in vivo* model.

Human mesenchymal progenitor cells express dopamine receptors.

In order to test the role of dopamine in mobilization of MPCs into peripheral blood we studied the *in vitro* expression of dopamine receptors in human BM- and PB-derived MPCs by immunocitochemistry (Figure 2). We found that steady state hBM- and hPB-MPCs expressed the six subtypes of dopamine receptor, indicating a potential role of dopamine in their cellular homeostasis.

Dopamine induces *in vitro* migration of hBM and hPB-MPCs through D2-class receptors via PI3K/Akt pathway.

We used an *in vitro* migration assay to investigate the ability of dopamine to induce migration of hMPCs. As shown in Figure 3A, the basal migration observed in hMPCs in the presence of medium alone was increased by the addition of dopamine. Our results indicated that this increase was higher in human MPCs from PB (2.9-fold) than from BM (2-fold) and also that serum starvation induced a higher increase in MPCs migration in both types of cells (5.3-fold and 2.8-fold in cells from PB and BM, respectively) (Figure 3B).

Next, we decided to study the type of dopamine receptor involved in the observed migration. We used agonists and antagonists for D1R and D2R receptors and assessed their effects on hMPCs *in vitro* migration (Figure 3C). SKF-38393, a D1R-class agonist, had no effects on hMPCs migration, nor did SCH-23390, a D1R-class antagonist. These results indicate that dopamine hMPCS migration seems not to be directed by D1-class receptors. However, bromocriptine, a D2R-class agonist, was found to clearly increase hMPC migration. In addition, eticlopride, a D2R-class antagonist was able to block both dopamine-induced and basal migration in BM- and PB-MPCs. Taken together these results indicate that the increased *in vitro* migration induced by DA occurs mostly through D2-class receptors.

The classical pathway of D2R activation induces inhibition of adenylyl cyclase (AC). However, we did not see changes in AC activity of MPCs after dopamine treatment (figure 3D) indicating the activation of an alternative signaling pathway. In this sense, D2R-class activation also could be mediated by transactivation of a tyrosine kinase receptor [10]. These receptors are able to active several downstream signal networks, as the PI3k/Akt signaling pathway [11]. To assess the function of the PI3K pathway in dopamine-induced BM- and PB-MPCs migration, we incubated hMPCs in transwell chambers with dopamine, in the presence of AS-604850, a PI3K inhibitor. As shown in

Figure 3D, cell migration was significantly suppressed when MPCs were incubated with AS-604850, as the number of migrated cells was decreased beyond the basal migration. In addition, hMPCs were incubated with dopamine from 1 to 40 minutes and we studied the phosphorylation of Akt. As is shown in Figure 3F the amount of phosphorylated Akt increased significantly with dopamine incubation. In sum, our data suggest that dopamine-induced MPCs migration is mediated via the PI3K/Akt pathway.

***In vivo* mobilization of murine MPCs using dopamine.**

We i.p. injected groups of mice during four days with catecholamines (dopamine and epinephrine) and G-CSF, as a positive control. We collected their PB and cultured their cells in a CFU-F assay. We obtained an increase of CFU-F numbers in the G-CSF-treated group compared to those found with PBS. Interestingly, in the dopamine and epinephrine-treated groups, we obtained a higher number of CFU-F (Figure 4A). The differences were statistically significant only when comparing the mice mobilized with DA versus those mobilized with PBS, indicating the ability of dopamine to mobilize MPCs to PB. In the case of the G-CSF group, no CFU-F mobilization was detected in some experiments under the same conditions. Then, we checked the CFU-F numbers in BM and spleen. In all experiments, we found a low decrease (no statistically significant) of CFU-F numbers in the BM of the G-CSF-treated, but not in the dopamine- and epinephrine-treated groups (Figure 4B). This decrease in CFU-F in BM of mice injected with G-CSF was also previously reported by other group [23]. Moreover, we found a low increase of CFU-F numbers (no statistically significant) in the spleens (Figure 4C) in all cases in which no increase was found in PB. We observed no effect in CFU-F numbers in BM and spleen in the catecholamine-treated groups. These data indicated a more consistent mobilization of MPCs using dopamine than G-CSF, without depletion of CFU-F in BM, and without aberrant CFU-F accumulation in spleen of mobilized subjects.

In order to test the direct implication of dopamine receptors in MPCs mobilization, we used D1R-class and D2R-class antagonists. In agreement with *in vitro* data, both

eticlopride and U-99194, D2R-class antagonists, prevented the dopamine mobilization of MPCs. In contrast, the D1R-class antagonist SCH23390 did not affect MPCs mobilization (Figure 4D). These data indicate that also *in vivo* MPCs mobilization by dopamine is induced specifically through dopamine D2-class receptors.

Analysis and detection of circulating human MPCs.

MPCs do not have a specific surface marker which enables their *in vivo* detection. Aiming to phenotypically characterize MPCs in fresh PB apheresis, we studied diverse combinations of markers. Others groups have published that CD105 can be used to identify MPCs in bone marrow and peripheral blood [24-30], including a most primitive human MPCs subpopulation [29]. We defined a cell subpopulation characterized by the profile CD45-CD31-CD34-CD105+ (Figure 5A). In fresh, without a cell culture process, this subpopulation was negative for other hematopoietic and endothelial markers. We also studied the expression of classical markers of human MPCs in culture, and all of them were negative (supplementary Figure 2). However, these cells upregulated specific hMPCs markers in culture, while maintaining a negative expression of CD45 (Figure 5B). We isolated this subpopulation by immunomagnetic methods and seeded the cells into culture flasks. All attempts for *in vitro* expansion of this subpopulation were unsuccessful. In this sense, others authors have previously published the same negative results, using $CD105^{+}$ cells from human PB as the starting cell population [28]. A possible explanation could be that the MPC-like population in PB could be in G0, as it has been reported for the hematopoietic precursors in PB after G-CSF mobilization.[31, 32] We studied the cell cycle status of our MPC-like subpopulation and found that all CD45-CD31-CD34-CD105+ cells from PB were in G0/G1, with no cells in S or G2/M. Also, a small cell fraction was at the sub-G0-phase, indicating an apoptotic process (Figure 5C).

***In vivo* characterization of circulating human MPCs.**

Ectopic implantation of hMPCs has been recently reported as a bona fide method to assess *in vivo* differentiation potential and characterization of these cell populations [29, 33-35]. To identify PB-derived CD45-CD31-CD34-CD105+ cells as a real hMPCs population we isolated them, attached onto a ceramic compound, and subcutaneously implanted into immunodeficient mice. In Figure 6A is shown a gross morphology of harvested ossicles, where powders were clearly visible. Ossicles from the CD105+ subpopulation showed a harder consistency and denser vascularization when comparing with ossicles generated from the $CD105^-$ subpopulation. Using both anti-human β2 globulin and anti-human mitochondria antibodies, we found positive cells only in ectopic ossicles generated from the CD105+ subpopulation (Figure 6B). We detected positive human cells throughout the ossicles, forming chimeric tissues with host cells. Human cells could be observed in calcified areas, fibrous and bone marrow-like tissues, where positive cells either formed adipose tissue or appeared as interstitial cells. These results were confirmed using an anti-human vimentin antibody, showing the same results (Figure 6C). In addition, we used tissue-specific anti-human antibodies. Figure 6D shows representative images, indicating the capacity of these cells to engraft and differentiate into appropriate tissues. In sum, all data indicate that the PB-derived CD45-CD31-CD34-CD105+ subpopulation was highly enriched in human MPCs.

Catecholamines mobilize MPCs in humans.

In order to study the mobilization of MPC-like subpopulations by catecholamines in humans, we identified a group of patients treated with catecholamines or their agonists. These patients suffered of a parkinsonian syndrome after a cerebral stroke. As controls we studied either patients with stroke but without parkinsonism symptoms (not treated with catecholaminergic drugs), or healthy donors. We found a statistically significant increase of the CD45-CD31-CD34-CD105+ subpopulation in patients treated with catecholamines, compared with any other group (Figure 7), indicating a MPCs mobilization in humans treated with catecholamines.

In conclusion, our results suggest that catecholamines can mobilize MPCs into peripheral blood, in mice and in humans using their D2-class receptors. It might be possible to mobilize MPCs for clinical applications as it is the case with HSC.

DISCUSSION

The nervous system exerts direct and indirect effects on stem cells, the immune system, the bone, and the supportive stromal microenvironment, in order to maintain homeostasis [8]. Diverse neurotransmitters regulate retention, proliferation and recruitment of HSCs through noradrenergic sympathetic nerve fibers [36], which are closely associated with MPCs, forming the neuroreticular complex [9]. Recent data support the "brain-bone-blood" regulatory system hypothesis; since a G-CSF activation of peripheral noradrenergic neurons induces mobilization of HSCs [2], and also the neuropeptide substance-P is able to mobilize CD29+ stromal-like cells in mice and rabbits [37]. Stress, injury and inflammation induce the mobilization of stem cells as a steady-state homeostatic process [38]. Hematopoietic and endothelial progenitors increase transitorily their numbers in peripheral blood after a traumatic event. In addition, recent papers suggested that mesenchymal cells also would be mobilized in humans after injuries such as wound healing [39, 40], hip fracture[41] or cardiac and/or respiratory failure [42]. In this regard, we previously described a similar result in acute skeletal muscle injury in subjects running a long distance race [43]. We decided to study the effect of catecholamines on MPCs mobilization since it is well known that catecholamines significantly increase after an intensive exercise[44] and, also, catecholamines mobilize hematopoietic stem cells [2], while contradictory data exist about endothelial cells mobilization using catecholamines [6, 7]. A study showed that treatment with eticlopride, a D2 receptor antagonist, increased the numbers of circulating MPCs in PB blood of wound bearing mice [45], although treatment with other D1R-class and D2R-class antagonists had no effects on the mobilization of MPCs. Results from this paper do not define the precise role of dopamine over MPCs in steady state. However, our results indicate a clear *in vitro* MPCs migration and *in vivo* MPCs mobilization using dopamine and their D2-class receptors.

Most studies attempting to isolate MPCs from peripheral blood have used culture conditions similar to those defined for bone marrow-derived MPCs [46]. Nowadays the mechanisms underlying the migration of MPCs remain unclear but it would seem likely that MPCs transmigrate into tissues by a similar mechanism to that of leukocytes, employing some (but not all) of the same molecules [47]. In addition, it was published that adhesion of MPCs to fibronectin activates the mechanism that controls MPCs adhesion and migration [48]. This data are in agreement with our results where we found a high expression of CD49d (α4) in fresh circulating hMPCs, and it is well known that activated $\alpha4\beta1$ and $\alpha4\beta7$ integrins are receptors of fibronectin [49].

Some authors have claimed a word of caution about circulating MPCs because they indicate that although there is evidence for the existence of circulating MPCs, an unpersuasive fulfillment of the MPCs criteria is still lacking, as proof of *in vivo* function [50]. In this regard others authors have shown MPCs functions using *in vivo* models [37, 51-53], and here we demonstrate a plenty characterization of human PB-derived MPCs, including *in vitro* and *in vivo* immunological and differentiation studies.

Classical dopamine receptors signal by adenylyl cyclase (AC) and cAMP production. AC is stimulated by D1R-class, while D2R-class inhibits AC. However, dopamine receptors activation also could be mediated by transactivation of tyrosine kinase receptors [10]. These receptors are able to active the PI3k/Akt signaling pathway [11]. In this regard, Akt is phosphorylated by PI3K and thereby seems to be linked to migration and adhesion in several cell types [54, 55], including MPCs [48, 56, 57]. Tyrosine kinase receptors of bFGF and PDGF-BB also use this pathway to increase the migratory activity of human MPCs [13].

In conclusion, our results suggest that catecholamines can mobilize MPCs into peripheral blood, in mice and in humans. These findings reveal a new link between catecholamines and MPCs mobilization supporting the "brain-bone-blood" regulatory

system hypothesis and open the door to mobilize, in the future, human MPCs for clinical applications.

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Figure 1

Isolation and characterization of mesenchymal progenitor cells derived from human G-CSF-mobilized peripheral blood. (A) Cultures of human MPCs from fresh (black bar) or frozen (grey bar) mononuclear cells from complete mobilized apheresis or from their fresh (white bar) or frozen (striped bar) CD34- fraction, and MNCs from non-mobilized buffy coat (dotted bar). Data are shown as mean of eight experiments. (B) Adherent cell growth of primary cultures of hPB-MNCs and their differentiation capacities when cells were cultured in adipogenic (left) or osteogenic (right) specific mediums. (C) Histograms show immunophenotype of hPB-MPCs, using fluorescence intensity with specific antibodies (black area) and isotype as a negative control (white area). (D and E) Isolated CD3+ T-cells were cultured in the absence (c) or presence of allogeneic hPB-MNCs, at different ratios (1:1 and 1:10). Data are shown as mean standard error of three experiments, where the asterisk (*) indicates statistically significant difference with $p<0.05$. (F) In vivo effect of PBS (black line), hPB-MPCs (dotted line) and hBM-MPCs (striped line) upon the generation of GvHD produced after haploidentical hematopoietic transplantation in mice.

Figure 2

Immunocytochemical analysis of dopamine receptors expression in human MPCs derived from bone marrow (BM) or peripheral blood (PB). Staining of BM- and PB-derived hMPCs with specific monoclonal antibodies against dopamine receptors. Scale bar 100 μm. Data shown are from a single experiment that is representative of three independent experiments.

Figure 3.

Dopamine increases in vitro migration of human MPCs through D2-class receptors and PI3 kinase pathway. Human peripheral blood (PB) and bone marrow (BM) derived MPCs were grown in DMEM containing 10% fetal bovine serum. For serum-starved cells (ss), MPCs were grown in DMEM alone for 24 hours. The graph shows the average number of migrated MPCs in 10 high-power fields (HPFs) from three experiments combined (n=6) and bars represent the standard error of the mean. (A) Representative images showing the effect of 50 μM dopamine (DA) on in vitro migration of hMPCs in transwell chambers. (B) Graph showing the average number of migrated hMPCs per HPF after 50 μM DA treatment. (C) Effect of D1- and D2-class receptors agonists and antagonists on in vitro migration of hMPCs. DA, SKF-38393 (D1-class agonist), SCH-23390 (D1-class antagonist), bromocriptine (D2-class agonist) and eticlopride (D2-class agonist) were all assayed at 50 μM concentrations. Statistical analysis was performed according to the Wilcoxon test. *, $p<0.05$; **, $p<0.01$; ##, $p<0.01$, where the asterisk (*) indicates comparison of the sample vs. control group whereas the hash (#) indicates comparison of the sample vs. control and DA groups. (D) Effect of PI3K inhibition on in vitro migration of hMPCs in transwell chambers. (E) Determination of cAMP levels in hPB-MPCs in response to 50 μM DA. Intracellular levels of cAMp were measured as indicated in Material and Methods at the indicated times (min). (F) Western blot analysis of phospho-AKT in the absence (control) or presence of 50 μM DA from 1 to 40 minutes (min).

Figure 4.

Dopamine-induced mobilization in mice. Mice were inoculated during 4 days with PBS (control), G-CSF, dopamine or epinephrine. Mononuclear cells were obtained from peripheral blood (A), bone marrow (B) and spleen (C), cultured during seven days and then CFU-Fs were counted. (D) Identical experiments were performed with mice

treated with D2-class antagonist (eticlopride and U-99194) plus dopamine and the D1-class antagonist SCH23390. Data are shown as mean standard error of three experiments, where the asterisk (*) indicates statistically significant difference with $p<0.01$.

Figure 5.

Catecholamines induced mobilization in humans. (A) Representative flow cytometry analysis of human peripheral blood showing the CD45-CD31-CD34-CD105+ subpopulation. (B) Immunofluorescence of CD45-CD31-CD34-CD105+ cells after their adhesion in cell culture flask. Scale bar=25 mm (C) Cell cycle status of immunomagneticaly-isolated CD45-CD31-CD34-CD105+ subpopulation.

Figure 6

In vivo characterization of human peripheral blood-derived CD45-CD31-CD34-CD105+ subpopulation. (**A**) Representative ectopic ossicles formed from CD105- or CD105+ cell subpopulation.

(**B**) Immunohistochemistry, using anti-human b2 microglobulin, showing positive cells in ectopic BM from CD105+ subpopulation. Scale bar=50 mm.

(**C**) Immunohistochemistries using anti-human vimentin (left panel) and anti-human mitochondria (right panel). Vimentin stain showing positive cells in ectopic BM from CD105+ subpopulation. Scale bar=100 mm.

(**D**) Immunohistochemistry, using anti-human adipophilin (left panel) and anti-human oteonectin (right panel), showing positive cells in ectopic BM from CD105+ subpopulation. Scale bar= 50 mm.

Figure 7

Catecholamines induced mobilization in humans. Percentage of CD45-CD31-CD34-CD105+ subpopulation in 3 groups: normal donors (healthy), patients of a cerebral stroke treated with catecholamines (catecholamines) and patients of a cerebral stroke without catecholamines treatment (brain damage) (mean ±SD, n= 12 samples per group). The higher percentage of "Catecholamines" group is statistically significant respect the other groups ($p<0.005$).

Supplemental Figure 1

Isolation and characterization of human PB-MPCs. (A) Cultures of hMPCs from fresh (black bar) or frozen (grey bar) mononuclear cells from complete mobilized apheresis or from their fresh (white bar) or frozen (striped bar) CD34- fraction, and MNCs from non-mobilized buffy coat (dotted bar). Data are shown as mean of eight experiments. (B) A representative G-banded karyotype of hPB-MPCs at passages 15 (left) and 25 (right). (C) PB-MNC were cultured in the absence (c+ treated with PHA; c- without PHA) or presence of allogeneic hPB-MNCs, at different ratios (1:1; 1:5; 1:20 and 1:100).

Supplemental Figure 2

Phenotypical characterization of fresh MPC subpopulation in human peripheral blood. Each panel show the expression of CD105 and other markers in the CD45-CD34-CD31- subpopulation. Data shown are from a single experiment that is representative of three independent experiments.

Supplemental Table 1

List of antibodies used in flow cytometry experiments.

Antibody	Clone	Suppliers	Source
Anti-human CD105	2H6F11	Immunostep	Mouse IgG1
Anti-human CD105	SN6	eBioscience	Mouse IgG1
Anti-human CD45	HI30	eBioscience	Mouse IgG1
Anti-human CD31	AC128	Miltenyi Biotec	Mouse IgG1
Anti-human CD34	581/CD34	BD Pharmingen	Mouse IgG1
Anti-human CD14	M5EZ	BD Pharmingen	Mouse IgG2a
Anti-human CD19	HIB19	BD Pharmingen	Mouse IgG1
Anti-human CD71	M-A712	BD Pharmingen	Mouse IgG2a
Anti-human CD235a	GAR-2	BD Bioscience	Mouse IgG2b
Anti-human HLA DR	L243 (G46-6)	BD Bioscience	Mouse IgG1
Anti-human NG2	7.1	Beckman Coulter	Mouse IgG1
Anti-human CD13	WM-15	eBioscience	Mouse IgG1
Anti-human CD73	AD2	BD Pharmingen	Mouse IgG1
Anti-human CD90	eBio5E10	eBioscience	Mouse IgG1
Anti-human CD106	51-10C9	BD Pharmingen	Mouse IgG1
Anti-human CD146	P1H12	BD Pharmingen	Mouse IgG1
Anti-human CD166	3A6	BD Pharmingen	Mouse IgG1
Anti-human CD29	MAR4	BD Pharmingen	Mouse IgG1
Anti-humanCD49d	HP2/1	Beckman Coulter	Mouse IgG1
Anti-humanCD49e	SAM1	Beckman Coulter	Mouse IgG2b
Anti-human CD61	RUU-PL7F12	BD Bioscience	Mouse IgG1
Anti-human CD62P	AX4	BD Pharmingen	Mouse IgG1
Anti-human CD62E	68-5H11	BD Pharmingen	Mouse IgG1
Anti-human CD62L	DREG-56	BD Pharmingen	Mouse IgG1
Anti-human KDR	89106	R&D	Mouse IgG1
Anti-human GD2	8.5.77	US Biological	Mouse IgM

Supplemental Table 2

List of antibodies used in immunostaining experiments.

Antibody	Clone	Suppliers	Source
Dopamine D1 Receptor	---	Calbiochem	Rabbit polyclonal
Dopamine D2 Receptor	---	Calbiochem	Rabbit polyclona
Dopamine D2s Receptor	---	Calbiochem	Rabbit polyclonal
Dopamine D3 Receptor	---	Calbiochem	Rabbit polyclonal
Dopamine D4 Receptor	---	Calbiochem	Rabbit polyclonal
Dopamine D5 Receptor	---	Calbiochem	Rabbit polyclonal
Anti –human adipophilin	AP125	Fitzgerald	Mouse IgG1
Anti-human osteonectin	122511	R&D	Mouse IgG1
Anti-human vimentin	V9	AbCam	Mouse IgG1
Anti-human CD105		2H6F11	Immunostep
Anti-human CD45	F10-89-4	Millipore	Mouse IgG2a
Anti-human CD90	eBio5E10	eBioscience	Mouse IgG1
Anti-human CD106	B-N8	eBioscience	Mouse IgG1
Anti-human b2 microglobulin	P61769	Abcam	Rabbit IgG
Anti-human NG2	7.1	Beckman Coulter	Mouse IgG1
Anti-human CD29	JB1B	Abcam	Mouse IgG2a
Anti-human GD2	14.G2a	BD Pharmingen	Mouse IgG2a
Anti-human mitochondria	MTC02	Abcam	Mouse IgG1

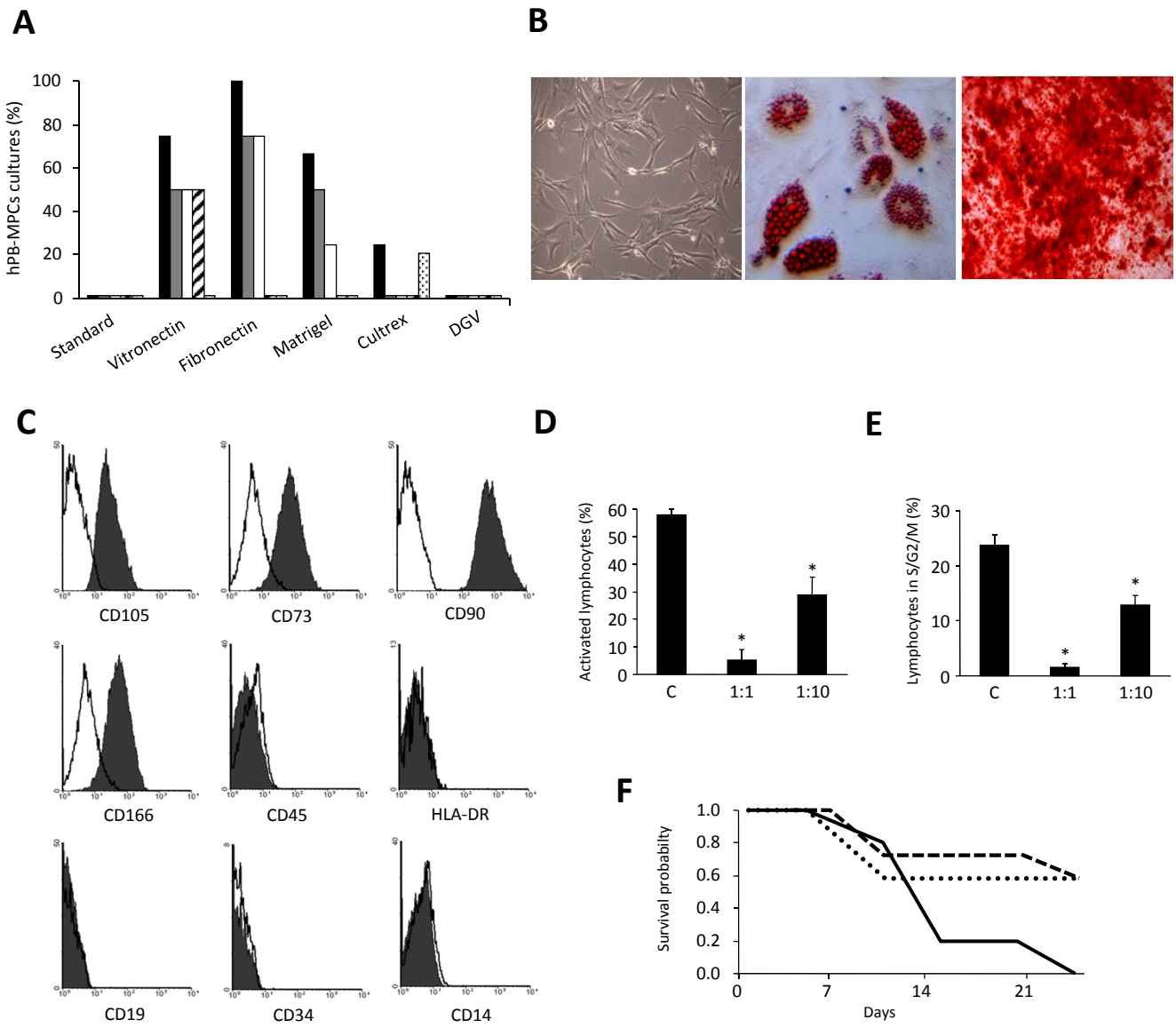

Figure 1

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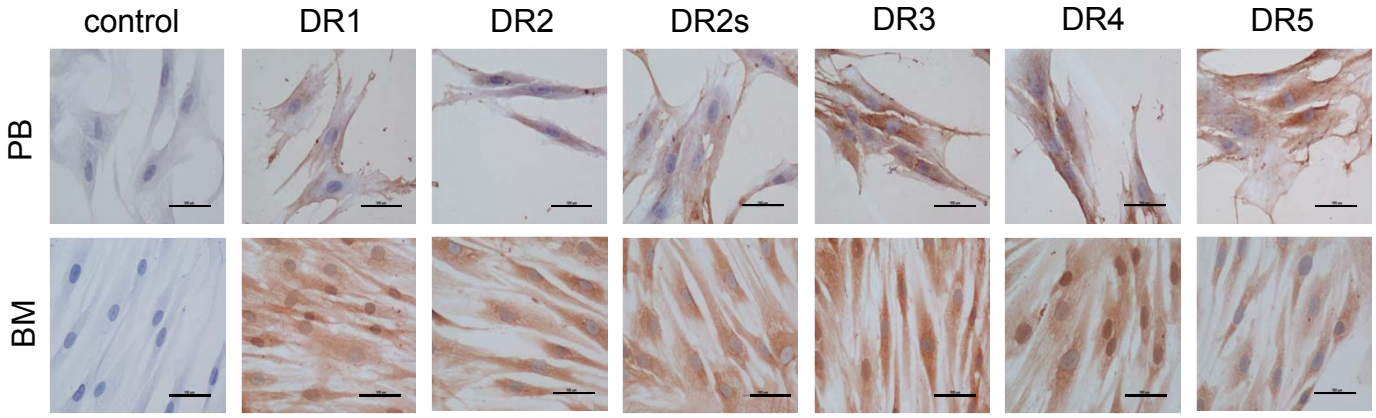

Figure 2
Immunocytochemical analysis of dopamine receptors expression in human MPCs derived from bone marrow (BM) or peripheral blood (PB). Staining of BM- and PB-derived hMPCs with specific monoclonal antibodies against dopamine receptors. Scale bar 100 μm. Data shown are from a single experiment that is representative of three independent experiments.

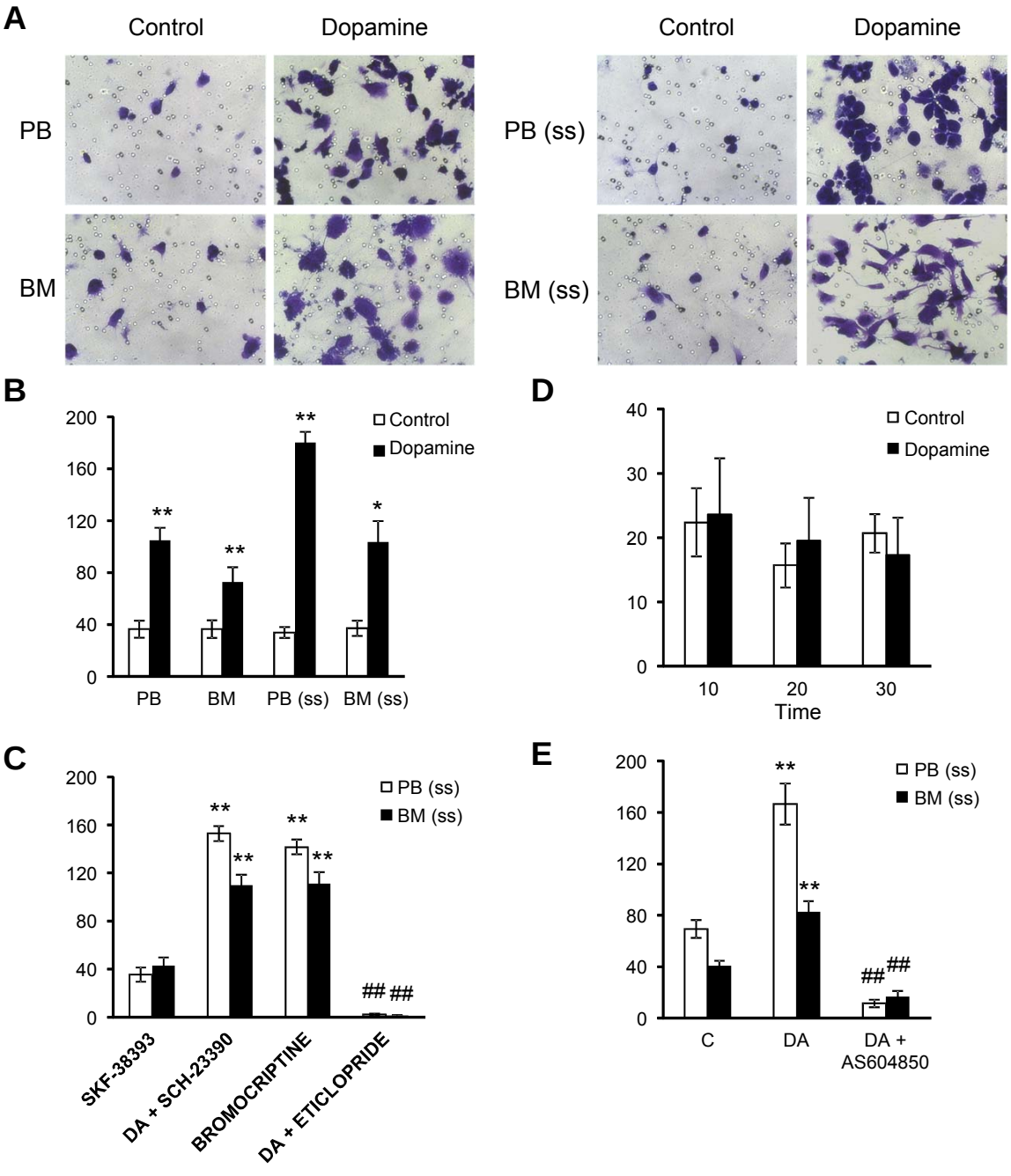
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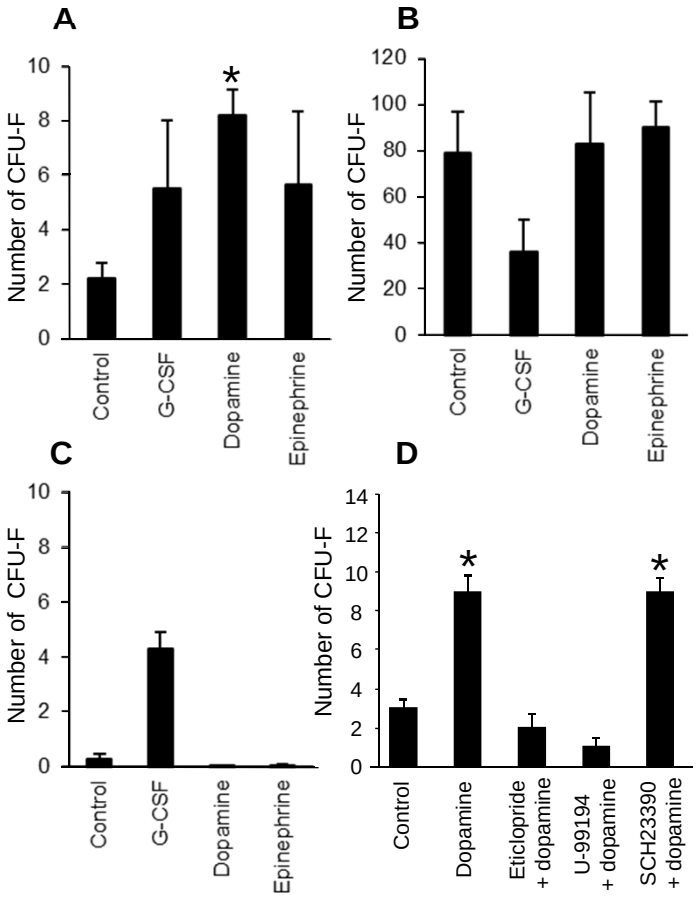
Figure 3.
Dopamine increases *in vitro* migration of human MPCs through D2-class receptors and PI3 kinase pathway. Human peripheral blood (PB) and bone marrow (BM) derived MPCs were grown in DMEM containing 10% fetal bovine serum. For serum-starved cells (ss), MPCs were grown in DMEM alone for 24 hours. The graph shows the average number of migrated MPCs in 10 high-power fields (HPFs) from three experiments combined (n=6) and bars represent the standard error of the mean. (**A**) Representative images showing the effect of 50 μM dopamine (DA) on *in vitro* migration of hMPCs in transwell chambers. (**B**) Graph showing the average number of migrated hMPCs per HPF after 50 μM DA treatment. (**C**) Effect of D1- and D2-class receptors agonists and antagonists on *in vitro* migration of hMPCs. DA, SKF-38393 (D1-class agonist), SCH-23390 (D1-class antagonist), bromocriptine (D2-class agonist) and eticlopride (D2-class agonist) were all assayed at 50 μM concentrations. Statistical analysis was performed according to the Wilcoxon test. *, $p<0.05$; **, $p<0.01$; ##, $p<0.01$, where the asterisk (*) indicates comparison of the sample vs. control group whereas the hash (#) indicates comparison of the sample vs. control and DA groups. (**D**) Effect of PI3K inhibition on *in vitro* migration of hMPCs in transwell chambers. (**E**) Determination of cAMP levels in hPB-MPCs in response to 50 μM DA. Intracellular levels of cAMp were measured as indicated in Material and Methods at the indicated times (min). (**F**) Western blot analysis of phospho-AKT in the absence (control) or presence of 50 μM DA from 1 to 40 minutes (min).



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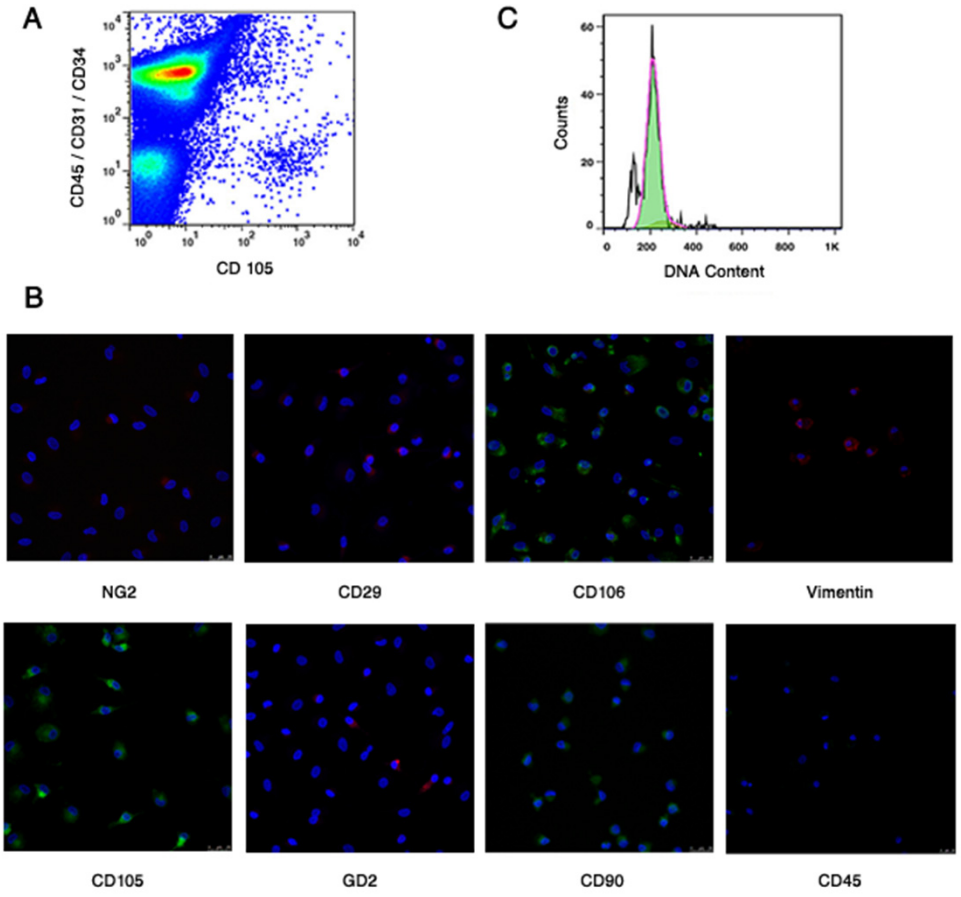

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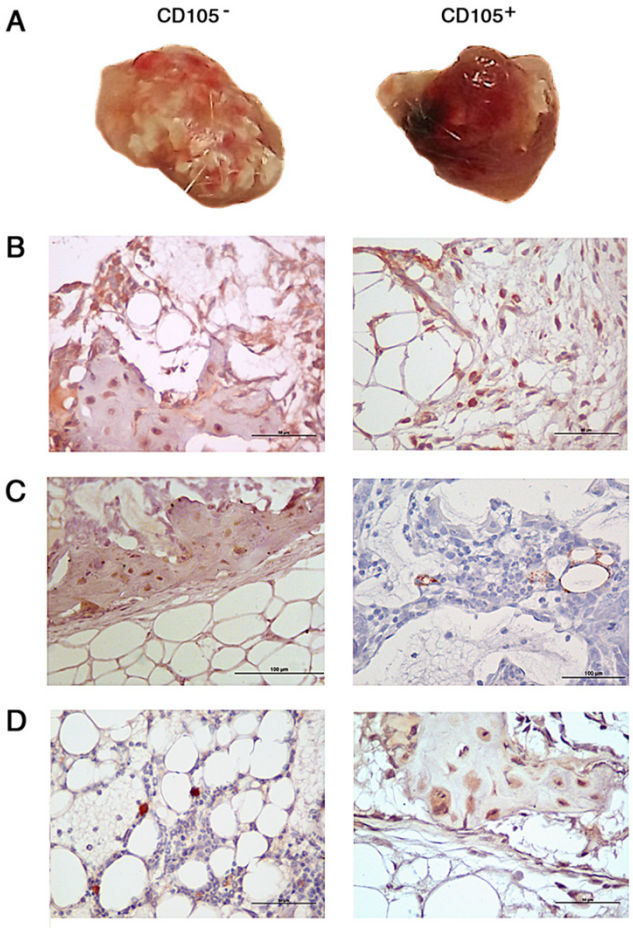

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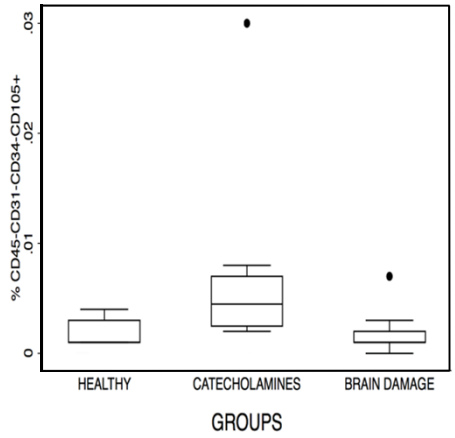

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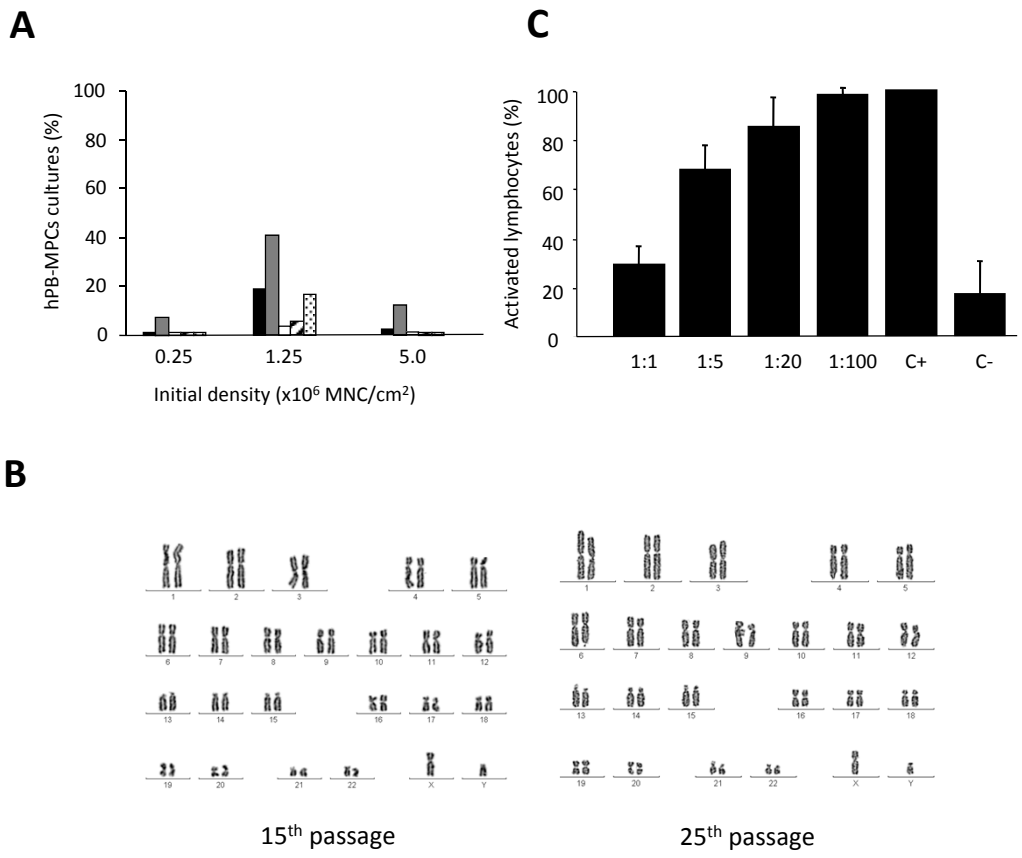

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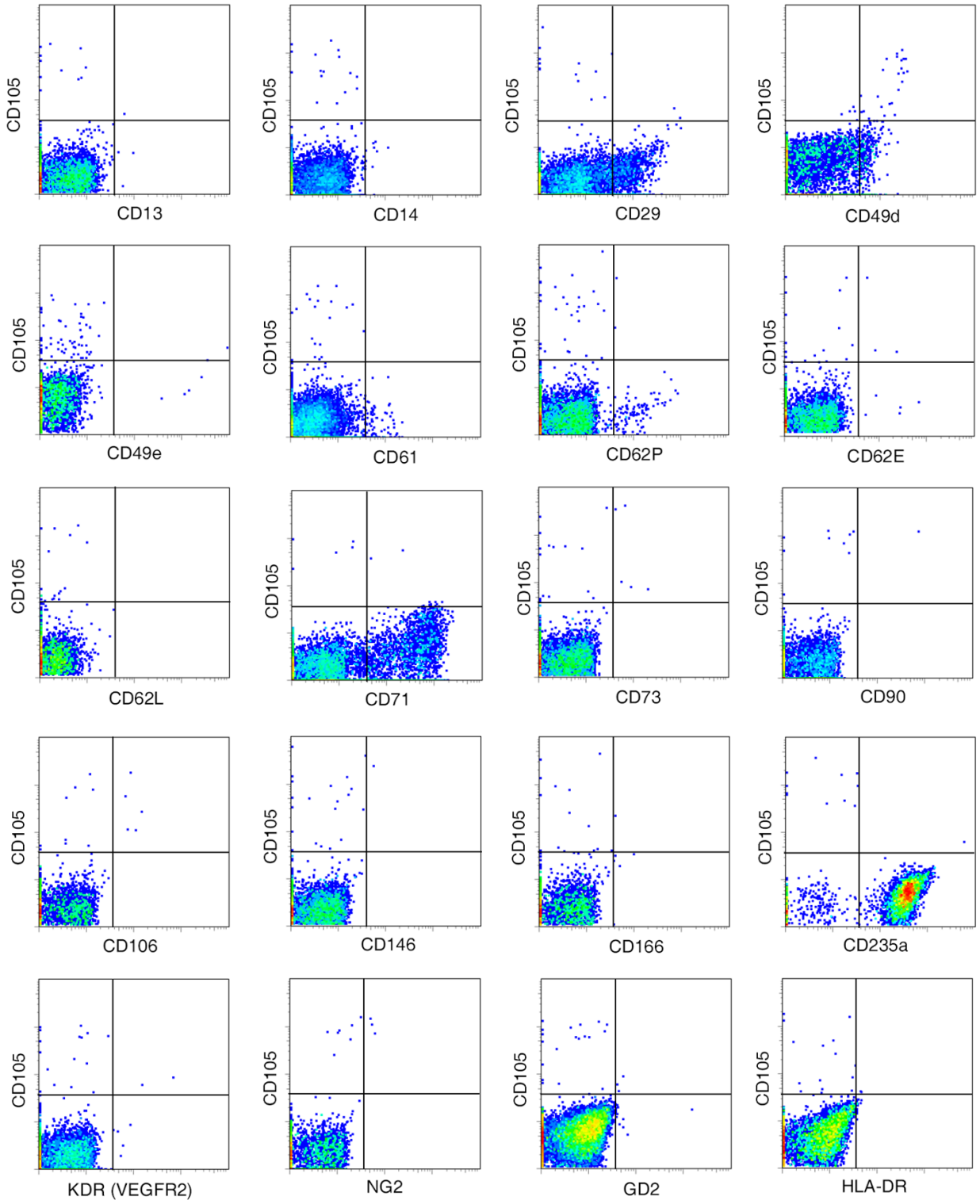

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Anti-human CD45	HI30	eBioscience	Mouse IgG1
Anti-human CD31	AC128	Miltenyi Biotec	Mouse IgG1
Anti-human CD34	581/CD34	BD Pharmingen	Mouse IgG1
Anti-human CD14	M5EZ	BD Pharmingen	Mouse IgG2a
Anti-human CD19	HIB19	BD Pharmingen	Mouse IgG1
Anti-human CD71	M-A712	BD Pharmingen	Mouse IgG2a
Anti-human CD235a	GAR-2	BD Bioscience	Mouse IgG2b
Anti-human HLA DR	L243 (G46-6)	BD Bioscience	Mouse IgG1
Anti-human NG2	7.1	Beckman Coulter	Mouse IgG1
Anti-human CD13	WM-15	eBioscience	Mouse IgG1
Anti-human CD73	AD2	BD Pharmingen	Mouse IgG1
Anti-human CD90	eBio5E10	eBioscience	Mouse IgG1
Anti-human CD106	51-10C9	BD Pharmingen	Mouse IgG1
Anti-human CD146	P1H12	BD Pharmingen	Mouse IgG1
Anti-human CD166	3A6	BD Pharmingen	Mouse IgG1
Anti-human CD29	MAR4	BD Pharmingen	Mouse IgG1
Anti-human CD49d	HP2/1	Beckman Coulter	Mouse IgG1
Anti-human CD49e	SAM1	Beckman Coulter	Mouse IgG2b
Anti-human CD61	RUU-PL7F12	BD Bioscience	Mouse IgG1
Anti-human CD62P	AX4	BD Pharmingen	Mouse IgG1
Anti-human CD62E	68-5H11	BD Pharmingen	Mouse IgG1
Anti-human CD62L	DREG-56	BD Pharmingen	Mouse IgG1
Anti-human KDR	89106	R&D	Mouse IgG1
Anti-human GD2	8.5.77	US Biological	Mouse IgM

Supplemental Table 2

List of antibodies used in immunostaining experiments.

Antibody	Clone	Suppliers	Source
Dopamine D1 Receptor	---	Calbiochem	Rabbit polyclonal
Dopamine D2 Receptor	---	Calbiochem	Rabbit polyclona
Dopamine D2s Receptor	---	Calbiochem	Rabbit polyclonal
Dopamine D3 Receptor	---	Calbiochem	Rabbit polyclonal
Dopamine D4 Receptor	---	Calbiochem	Rabbit polyclonal
Dopamine D5 Receptor	---	Calbiochem	Rabbit polyclonal
Anti –human adipophilin	AP125	Fitzgerald	Mouse IgG1
Anti-human osteonectin	122511	R&D	Mouse IgG1
Anti-human vimentin	V9	AbCam	Mouse IgG1
Anti-human CD105	2H6F11	Immunostep	Mouse IgG1
Anti-human CD45	F10-89-4	Millipore	Mouse IgG2a
Anti-human CD90	eBio5E10	eBioscience	Mouse IgG1
Anti-human CD106	B-N8	eBioscience	Mouse IgG1
Anti-human β2 microglobulin	P61769	Abcam	Rabbit IgG
Anti-human NG2	7.1	Beckman Coulter	Mouse IgG1
Anti-human CD29	JB1B	Abcam	Mouse IgG2a
Anti-human GD2	14.G2a	BD Pharmingen	Mouse IgG2a
Anti-human mitochondria	MTC02	Abcam	Mouse IgG1