Priming of human bone marrow-derived mesenchymal stromal cells with various down-stream effectors of the Transforming Growth Factor 1 pathway boosts their hematopoiesis-supportive ability.

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Abstract

Mesenchymal stromal cells (MSCs) isolated from various tissues are frequently used to expand hematopoietic stem/ progenitor cells (HSCs/HSPCs) in vitro. They are also co-infused with the HSCs to improve the HSC engraftment. However, the MSCs sourced from non-hematopoietic tissues could be less efficient in their hematopoiesis-supportive ability. Likewise, the hematopoiesis-supportive ability of the MSCs is known to decline after continuous in vitro culture – an unavoidable manipulation to get clinically relevant cell numbers. Hence, it may be necessary to boost the hematopoietic-supportive ability of the long-time cultured MSCs before their clinical applications in hematological disorders. In my earlier work, I showed that priming of MSCs isolated from human bone marrow (BMSC) with Transforming Growth Factor β1 (TGFβ1) boosts their hematopoiesis-supportive ability via activation of AKT-eNOS pathway. Accordingly, I also demonstrated that priming the BMSCs with nitric oxide (NO) donors mimics the effect of TGFβ1. Here I show that brief exposure of human BMSCs to pharmacological modulators of PKC and intracellular calcium – two downstream participants in the TGFβ1-eNOS signaling pathway – also boosts their hematopoiesis-supportive ability. Such an approach comprising priming the BMSCs with pharmacological compounds for a short duration and briefly exposing the HSCs to them can be used in clinical settings to improve the efficacy of stem cell transplantations. This concept would be helpful in other regenerative medicine protocols after identifying suitable pharmacological modulators giving desired effects on the target cells.

Introduction

Owing to their inherent hematopoiesis-supportive properties, mesenchymal stromal cells (MSCs) are frequently used for the expansion of hematopoietic stem cells (HSCs) in vitro [1,2]. In vitro expansion is needed for cord blood (CB) samples as the HSCs obtained from one CB unit are not sufficient for transplantation in an adult recipient [3,4]. Likewise, the MSCs are co-infused with the HSCs into the recipients to improve the success of stem cell transplantation [5-8]. However, MSCs are sourced from various hematopoietic and non-hematopoietic sources – the hematopoiesis-supportive properties of MSCs isolated from non-hematopoietic sources might not be at par with those isolated from the hematopoietic tissues. Donors' age is also an important factor governing the functionality of the MSCs [9,10]. Due to their low number in the tissues, MSCs need a long-term in vitro expansion – a process known to affect their regenerative potency [11]. Considering all these possibilities, priming the MSCs with various agents to boost their regenerative properties has become a focus of several studies [12-16]. However, such manipulation needs to be *time efficient and cost-effective* .

MSCs are typically characterized by their phenotypic and trilineage differentiation ability [17]; however, these parameters do not reflect their ability to support hematopoiesis. Long-term culture-initiating-cell assay (LTC-IC) is routinely used to determine the functionality of HSCs. Its readout not only helps estimate the number of primitive HSCs present in the sample but also indirectly reflects on the functionality of the

stromal cells used as the feeder layers. However, this assay takes about 10-12 weeks to complete, a timeline that may not be suitable for a clinical setup. Hence, there is a need to develop functional assays which can quickly judge the hematopoiesis-supportive ability of the MSCs [18,19].

I have earlier shown that priming bone marrow-derived MSCs (BMSCs) with Transforming Growth Factor β1 (TGFβ1) boosts their hematopoiesis-supportive ability via AKT-eNOS axis [18]. In the present study, I examined whether the BMSCs briefly primed with pharmacological activators of downstream effectors of TGFβ1 pathway could also boost their hematopoiesis-supportive ability. Indeed, I found that short-term treatments of BMSCs with activators of protein kinase C (PKC) and intracellular calcium [Ca^{2+}]i and various fibronectin (FN)- and integrin-specific bioactive peptides boost the potency of BMSCs as evidenced by the formation of a significantly higher number of colonies in semi-solid media and a rapid expansion of $CD34^+$ HSPCs from the BM-derived cells briefly interacted with them. Such an approach comprising priming the BMSCs with pharmacological compounds for a short duration and briefly exposing the HSCs to them can be used in clinical settings to improve the efficacy of stem cell transplantations. This concept would be helpful in other regenerative medicine protocols after identifying suitable pharmacological modulators giving desired effects on the target cells [14,20].

These data demonstrate that it is possible to boost the potency of BMSCs using various small molecule pharmacological agents targeting downstream effectors of the TGFβ1 pathway. Also, the assays used in the present study can be used as quick parameters to judge the functionality of BMSCs to be used in clinical applications, and to identify compounds having salutary effects on the hematopoiesis-supportive ability of BMSCs.

Materials and Methods

Ethical disclosure: The author states that she has obtained approval of the Institutional Ethics Committee (IEC) and Institutional Committee for Stem Cell Research (IC-SCR) of National Centre for Cell Science (NCCS), Pune. Informed consent was obtained from all individual participants included in the study.

Reagents:

Iscove's Modified Dulbecco's Medium(IMDM) and HiSep were from HiMedia Labs (Mumbai, India), Mesenchymal cell-specific fetal bovine serum (Mesen FBS) and 35 mm^2 low adhesion plates for colony formation assays were from Stem Cell Tech (Vancouver, Canada); Dynabeads® CD34 Positive Isolation Kit was from Invitrogen/Thermo Fisher Scientific (MA); SCF, Granulocyte Monocyte Colony Stimulating factor (GM-CSF) and Interleukin-3 (IL-3) were from Peprotech (NJ, USA), Erythropoietin (EPO), TGFβ1, non-enzymatic cell dissociation solution, Cyclopiazonic Acid (CPA), Indolactam (+), Indolactam (-), monensin, antibody to cellular fibronectin (mouse monoclonal), were from Sigma-Aldrich (MO, USA), BAPTA AM [1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester)], Thapsigargin, were from Molecular Probes/Invitrogen/Thermo Fisher Scientific (MA,USA), cyclic RGD peptide (AcPenRGDC-OH (Pen to C cyclised)), α4β1-specific peptide (1-adamantaneacetyl-Cys-Gly-Arg-Gly-Asp-Ser-Pro-Cys-OH), cell-permeant octapeptide PKC inhibitor (Myristoylated RKRTLRRL) and Fibronectin-adhesion-promoting peptide were from Bachem AG, Bubendorf, Switzerland, anti CD34 antibody (raised in mouse) was from BD Biosciences (CA, USA), and anti-Ki67 (raised in rabbit) antibody was from Abcam (Cambridge, UK) , FITC-conjugated anti-mouse and PE-conjugated anti-rabbit antibodies were from Chemicon (CA, USA), all tissue culture grade plastic ware was from Nunc/Thermo Fisher Scientific (MA, USA) and microfuge tubes were from Eppendorf (Hamburg, Germany). Dynabeads® CD34 Positive Isolation Kit was from Invitrogen/Thermo Fisher Scientific (MA, USA).

Peptide sequences:

α5β1: H-*CRRETAWAC*-NH2 [cyclised between C and C]

αIIbβ3: H-*CNPRGD(109)RC*-NH2 [cyclised between C and C]

Most of the pharmacological reagents including all peptides were dissolved in DMSO, unless otherwise

stated. Control BMSCs were treated with equal amounts of DMSO or its inactive control reagent dissolved in DMSO. The working concentration of all these reagents was determined by pre-screening them on BMSCs. The maximum tolerated non-toxic concentrations were used in the experiments.

Methods

Isolation of BM MNCs, $CD34^+$ cell isolation from the BM MNCs, isolation and culture of BMSCs, colony formation assay, co-culture, and immunofluorescence technique have been described earlier [18]. Briefly, MNCs were isolated from human bone marrow aspirates using density gradient centrifugation (HiSep, Hi-Media). The cells were further used to isolate $CD34^+$ HSCs [18,21], or plated in culture-grade Petri dishes of suitable size to obtain BMSCs. BMSCs were used in passage #3.

Interaction between BMSCs and MNCs: This protocol has been described in detail before [18]. Briefly, BMSCs ($2X10^5$/well) were seeded in the wells of a 24-well plate and were allowed to attach for 2 hours. They were then treated with TGFβ1 (10ng/ml) or other pharmacological agents, as mentioned in the results section. After incubation, the cells were washed 3X with plain IMDM to remove the treatments, and 150 µl of complete medium (IMDM+20% FBS) was added per well. $1X10^6$ MNCs suspended in 100 µl of complete medium were added per well, and the plates were incubated at 37°C for 1 hour. The MNCs and BMSCs were from different donors, and the samples were not matched for age/gender.

Non-adherent cells were removed by gentle washing with plain IMDM, and the cells adhering to the BMSCs were collected using non-enzymatic cell dissociation solution (Sigma). The cells were then subjected to colony-forming-unit (CFU) assay as described before. Colonies were scored after 14 days using morphological criteria as belonging to CFU-GM (Granulocyte Monocyte), BFU-E (Burst-forming Unit Erythroid), and CFU-GEMM (Granulocyte-Erythroid-macrophage-Megakaryocyte) using a phase contrast microscope (Zeiss).

Co-culture assay and immunofluorescence staining: Co-culture assay and immunostaining of cells were done as described before [18]. Briefly, BMSCs cultured on coverslips placed in 24-well plates were variously treated as described in the results section. After washing to remove the treatments, $1X10^5CD34^+$ HSCs were seeded on the BMSCs. After one hour of incubation, non-adherent cells were gently removed, and adherent cells along with the BMSCs were over-layered with 1% methylcellulose supplemented with growth factors (SCF, 50 ng/ml; IL6 and IL3, 20ng/ml). The cells were fixed after 48 hrs. and gently washed to remove the methylcellulose. Non-specific sites were blocked by incubating the cells in 1% BSA prepared in Phosphate buffered saline (PBS; pH 7.4), and the cells were immunostained with antibodies to CD34 and Ki67 and FITC- and PE-tagged secondary antibodies, respectively. The images were acquired on a laser-scanning confocal microscope (Zeiss).

Statistical analysis .

Data are expressed as mean ± SD. All CFU experiments were done in triplicate (n=3) using BM MNCs from different donors (N=3). Statistical significance of the data was analyzed by One-Way Repeated Measure Analysis of Variance (One-Way RM ANOVA) using Sigma Stat software version 3.5 (Jandel Scientific, CA, USA). * p[?]0.5, **[?] 0.01, ***[?] p0.001. The distribution of data was examined for normality using Shapiro-Wilk normality test. All the graphs were created using Sigma Plot software version 14.0.

Results and Discussion

Pharmacological inhibition of PKC and buffering of intracellular calcium affects the hematopoiesis-supportive ability of TGF-primed BMSCs.

TGFβ1 is known to activate eNOS via activation of PKC [22]. An increase intracellular calcium $[Ca^{2+}]i$ is also known to activate eNOS [23]. In my earlier study, I have shown that treating BMSCs with TGFβ1 boosts their hematopoiesis-supportive ability [18]. To determine whether a pharmacological inhibition of these two effectors could interfere with the action of TGFβ1on the BMSCs, I treated BMSCs with a cell-permeant octapeptide PKC inhibitor (Bachem, 10µg/ml) [24]and a cell-permeant chelator of $[Ca^{2+}]i$ BAPTA-AM (Molecular Probes, 10µg/ml) 1 hr. before applying TGFβ1 (Sigma, 10ng/ml; BMSC*TGF) to them.

The plates were incubated overnight at 37°C/5%CO_2. After gently washing the treated BMSCs with plain medium, 1 X 10^6human BM-derived MNCs were interacted with them for 1 hr., and then the nonadherent cells were gently washed. The cells closely interacting with the stromal layer were subjected to CFU assay as described [18]. This protocol has been used in all subsequent CFU-related experiments.

I found that the MNCs interacting with BMSC*TGF pretreated with the PKC inhibitor or BAPTA-AM formed a significantly lower number of colonies than those interacting with BMSC*TGF (Fig. 1a, 1b; $2^{nd}$and 3^{rd} bars). As seen in my earlier study [18], the MNCs interacting with BMSC*TGF formed significantly more colonies than those interacting with control BMSCs (Fig. 1a,1b; 1^{st} and 2^{nd} bars).

Next, I also examined the effect of such inhibition on the types of CFU formed. A differential count of colonies belonging to BFU-E, GM, and GEMM revealed that the effect was across all colonies formed (Fig. 1c). There was no bias towards any particular type of colony.

I further examined the effect of these BMSCs on $CD34^+$HSCs isolated from the BM MNCs. As described earlier [18], I co-cultured human BM-derived $CD34^+$ cells with these variously treated BMSCs, and after 48 hrs., immuno-stained them with an antibody to CD34. As seen in Fig. 1d, the pre-treatment of BMSC*TGF with the PKC inhibitor and BAPTA-AM completely inhibited the expansion of CD34 HSCs. As expected, $CD34^+$ cells co-cultured with BMSC*TGF proliferated luxuriously compared to those co-cultured with control BMSCs [18].

These data show that the proliferation of $CD34^+$ HSCs in response to the interaction with TGF-primed BMSCs critically involves activated PKC and increased intracellular calcium levels.

Pharmacological activation of PKC and increase in intracellular Calcium levels boost the hematopoiesis-supportive ability of BMSCs.

Since inhibition of PKC and buffering of $[Ca^{2+}]$i significantly affected the potency of TGF-primed BMSCs, I examined whether pharmacological activation of PKC and increase in $[Ca^{2+}]$i can boost the hematopoiesis-supportive ability of BMSCs. In this set of experiments, I used Indo (-) (Sigma) to activate PKC in the BMSCs [25]. BMSCs were treated with Indo (-)/Indo (+) for 1 hr. (both 5 μM for 30 minutes). Indo (+) (Sigma) was used as a negative control for Indo (-). As seen in Fig. 2a, the BMSCs primed with Indo (-) but not with Indo (+) robustly enhanced the colony formation from BM MNCs that interacted with them.

In another set of experiments, I used CPA (Sigma; 5μM for 30 minutes;) [26]and Thapsigargin (Tsg; Molecular Probes; 0.5μM for 30 minutes) [27]to increase the $[Ca^{2+}]$i in BMSCs. I found that treating BMSCs with CPA and Tsg also resulted in a significantly higher colony formation from the BM MNCs that interacted with them (Fig. 2b). Then I co-cultured $CD34^+$ HSCs with the BMSCs grown on coverslips and treated or not with CPA (BMSC*CPA) and Tsg (BMSC*Tsg), and after 48 hrs., subjected them to immunostaining with an antibody to CD34. I observed that the $CD34^+$ HSCs co-cultured with BMSC*CPA or BMSC*Tsg proliferated extensively compared to those co-cultured with control BMSCs (Fig. 2c).

These data show that pharmacological activation of PKC and an increase in $[Ca^{2+}]$i in BMSCs boost their hematopoiesis-supportive ability. Notably, the effect of these modulators was rapid – less than 1 hr., making the process*time-efficient* . The use of such synthetic compounds could also make the process *cost-effective* .

In my earlier work, I have shown that treating BMSCs with bFGF also boosts their hematopoiesis-supportive ability [19]; however, a combination of TGFβ1 and bFGF was counterproductive. I plan to perform similar studies using downstream effectors of the bFGF pathway. These studies would increase the gamut of agents for priming the BMSCs and help in understanding the cause of inhibitory effects of the combined treatment of TGFβ1 and bFGF on BMSCs [19]. Such understanding is necessary to avoid the combined application of priming agents having antagonistic effects on the desired therapeutic activity of BMSCs.

Peptide-mediated enhanced adhesion to fibronectin boosts the functionality of MSCs.

Fibronectin (FN) is one of the extra-cellular matrix proteins critically involved in HSC-MSC communication [28]. First, I examined whether TGFβ1 enhances the secretion of cellular fibronectin in the BMSCs. When

the control and TGF-treated BMSCs were immuno-stained with an antibody to cellular fibronectin (Sigma), I found that the BMSC*TGF exhibited a denser meshwork of cellular fibronectin on them when compared to the control BMSCs (Fig. S1a). Use of monensin (Sigma, 2 μM, added 1 hr. before TGF) to block trans-Golgi transport to visualize intracellular proteins [29] before the addition of TGFβ1 facilitated the detection of intracellular FN secreted by the BMSCs secreted FN in response to TGFβ1(Fig. S1a, lower panels).

Then I examined the effect of applying a bioactive FN-adhesion-promoting peptide (Bachem) on the BMSCs' hematopoiesis-supportive ability. I found that the MNCs interacting with the BMSCs primed with FN-adhesion promoting peptide (BMSC*FN-pep; overnight treatment) produced a significantly higher number of CFU than those interacting with the control BMSCs (Fig. 3a).

These data suggested that promoting the adhesion of BMSCs to FN enhances their hematopoiesis-supportive ability significantly. The octapeptide used in this study contains sequences from the heparin-binding domain of fibronectin, and it is a potent inducer of focal adhesion formation. Focal adhesions connect the cells to the ECM molecules so that extra-cellular signals get transmitted inside cells. These adhesions also regulate the osteoblastic differentiation of the MSCs [30]. Since osteoblasts support hematopoiesis [31,32], it will be interesting to identify whether HSC-supportive osteoblastic genes get quickly activated by this peptide in the BMSCs ([?] 18 hrs.).

The binding of exogenous RGD peptides elevates intracellular calcium and initiates downstream integrin-mediated signaling [33]. These peptides also enhance the attachment of cells to various ECM components like fibronectin [34]. Since cyclic RGD is more stable in solution than the linear one [35], I treated the BMSCs with cyclic RGD peptide (Bachem; 10μg) overnight. As seen in Fig. 3b, the cells interacting with cyclo-RGD-primed BMSCs also gave a significantly higher number of colonies than their control counterparts. RGD is the principal integrin-binding domain present within several ECM proteins and thus can bind to multiple integrin species. The use of RGD, instead of the native ECM molecules, reduces the risk of immunological reactivity or pathogen transfer associated with the ECM proteins derived from animals or cadavers. Also, synthesizing RGD peptides is relatively inexpensive, which would be advantageous in clinical settings. Coupling of such bioactive peptides with various biomaterials might be more effective in priming the BMSCs.

My data underscore the importance of FN-mediated adhesions of BMSCs in their hematopoiesis-supportive ability.

Integrin-specific bioactive peptides increase the potency of BMSCs.

Since both FN-adhesion-promoting peptide- and cyclic RGD-primed BMSCs stimulated hematopoiesis, I examined whether the peptides specific to FN-interacting integrins also would do the same. Several integrins form the receptors for FN, out of which α5β1 and αIIbβ3 are known to play essential roles in hematopoiesis [36,37]. Hence, I examined whether priming the BMSCs with peptides specific to α5β1, and αIIbβ3 would have any effects on the hematopoiesis-supportive ability of BMSCs. Since α4β1-mediated interactions are essential for the interaction of HSCs/HSPCs with the niche cells [37], I used α4β1 [1-adamantaneacetyl-Cys-Gly-Arg-Gly-Asp-Ser-Pro-Cys (disulfide bridge between residues 1-8); α4β1-ada] to examine whether α4β1-mediated interactions are involved in the BMSC-HSC/HSPC crosstalk.

I found that the BMSCs primed with cyclized peptides to α5β1 and αIIbβ3 stimulated significantly higher colony formation from the MNCs briefly interacted with them (Fig. 3c, 2nd and 3rd bars). On the other hand, α4β1-ada peptide-primed BMSCs exerted a potent dose-dependent inhibitory effect on colony formation (Fig. 3c, last bar; Fig. 3d). These data underscore the importance of integrin-mediated interactions in the BMSC-mediated regulation of hematopoiesis. In the future, I propose to examine the effect of other FN-specific integrin peptides, such as αvβ3, αvβ5, etc., on the hematopoiesis-supportive ability of BMSCs.

Considering the dose-dependent potent negative effect exerted by α4β1-ada peptide, here I examined whether its effect is dominant over the salutary effect of the α5β1 peptide by treating the BMSCs with α5β1peptide alone or in combination with α4β1-ada (both used at 10μg/ml, overnight). As seen in Fig. 3e, BMSCs treated with a combination of α5β1 and α4β1-ada peptides exerted an inhibitory effect on the colony formation from

the MNCs interacted with them. The CFU formed in this set were significantly fewer as compared to those obtained in BMSC*α5β1 and control BMSC sets, showing that α4β1-ada peptide acts dominantly. Since Ada-peptide inhibits the binding of the integrin α4β1to the FN connecting segment (CS-1) and to the vascular cell adhesion molecule 1(VCAM1) [38], the data also show that α4β1-CS-1 domain of FN-VCAM1 axis is crucial in the BMSC-HSC/HSPC interaction and subsequent development of hematopoiesis.

In my next set of experiments, I made differential scoring of the CFU formed by the MNCs to examine whether the effect of peptide-primed BMSCs was specific to any particular lineage. I observed that the peptide-primed BMSCs, both α5β1, and αIIbβ3, stimulated the formation of all types of colonies, including the GEMM ones formed by primitive HSCs (Fig. 3f). These data were further supported by the results obtained in the co-culture assays. As seen in the Fig. 3g, the $CD34^+$cells co-cultured with BMSCs primed with α5β1 or αIIbβ3 peptide proliferated luxuriantly, as compared to their control counterparts. Several $CD34^+$cells showed an expression of Ki67, indicating their proliferative state. Although the $CD34^+$ cells in the control sets were very few, they expressed Ki67, indicating they would divide eventually.

Direct interaction of integrin-specific peptides with the MNCs is not very effective.

Akin to BMSCs, HSC and HSPCs also express several types of integrins [36,37]. Integrins are known to have homophilic interactions [39]. Hence, I examined whether such homophilic interaction of bioactive cyclic peptides with the integrins expressed on the HSPCs would also simulate them to form more colonies. The MNCs were subjected to CFU assay in methylcellulose-based media supplemented with α5β1, αIIbβ3 and α4β1-ada peptides (10μg/ml). I found that the MNCs incubated with α5β1peptide did not yield a higher number of CFU, while those incubated with αIIbβ3 and α4β1-ada peptides yielded only a marginally higher number of CFU, as compared to the untreated MNCs (Fig. S1b).

Direct actions of pharmacological compounds on HSCs could differ from those mediated via stromal cells primed with the same compounds. We have shown earlier that the nitric oxide (NO) donors exert an age-specific effect on HSCs by differential induction of various transcription factors involved in commitment vis-à-vis self-renewal in adult and juvenile HSCs, respectively [40]. On the other hand, NO-primed BMSCs stimulate the expansion of HSCs without showing any age-specific differences [41]. The data obtained in the experiments involving the direct action of integrin-specific compounds also show such a difference. The inhibitory effect of α4β1-ada peptide seen in earlier experiments was not seen when it directly interacted with the HSCs/HSPCs. In fact, an increase in CFU, albeit marginal, was seen. Nonetheless, these data confirm that the inhibitory effect of α4β1-ada peptide seen in the earlier experiments was primarily due to its interference in FN-VCAM-mediated stromal cell-HSC interaction, and the peptide itself did not have any inhibitory activity on HSPCs.

The milder effect shown by the direct addition of αIIbβ3 peptide seen in this set of experiments also indicates that its action on BMSCs evoked more robust signaling, leading to the extensive proliferative response in the HSCs, perhaps due to its interaction with its ligand rather than homophilic interaction with its counterpart on the BMSCs. In the future, I plan to examine the direct effect of various ECM-specific bioactive peptides on the expansion of HSC/HSPCs. Such ligand-integrin interactions could be perhaps more effective in expanding HSCs.

In this study, I have not examined the in vivo functionality of $CD34^+$ cells co-cultured with the primed BMSCs. Although at 48-hr. co-culture, extensive proliferation of these HSCs was seen, I need to examine their engraftment potential using an immuno-compromised mouse model formally.

Our previous studies [41-43] showed that the extracellular vesicles (EVs) secreted by variously primed BMSCs are very effective in improving the functionality of the HSCs. It will be worthwhile to study the composition of the EVs secreted by the BMSCs primed with these pharmacological modulators and examine their effect on the HSCs' functionality. Since the EVs can be effectively cryopreserved without losing their functionality [44], they could serve as "ready-to-use" biologics for priming HSCs.

Conclusion: My data show that priming the BMSCs with various pharmacological reagents involved in

the TGFβ1 pathway boosts their hematopoietic supportive ability. This *cost-effective and time-efficient* approach can be used in clinical settings to improve the outcome of HSC transplantation.

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Competing of interest

The author declares no competing interest.

Author's contribution

Vaijayanti Kale: Conceptualization, Methodology, Investigation, Validation, Formal Analysis and investigation, Resources, Writing – original draft preparation and Reviewing & Editing, Project Administration, Funding Acquisition.

Availability of Data and materials

The article and its supplementary information files contain all data generated or analyzed during this study.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by *the Institutional* Ethics Committee (IEC) and Institutional Committee for Stem Cell Research (IC-SCR) of National Centre for Cell Science (NCCS), Pune. Informed consent was obtained from all individual participants included in the study.

Figure legends

Φιγ 1 ΤΓΦβ1 βοοστς τηε ηεματοποιεσισ-συππορτιε αβιλιτψ οφ ΒΜΣ˜ς ια αςτιατιον οφ ΠΚ˜ ανδ ρισε ιν ιντραςελλυλαρ ςαλςιυμ λεελς (a, b) 1 X 10^6 BM MNCs were interacted with BMSCs treated or not with TGFβ1 and PKC inhibitor, Myristoylated RKRTLRRL, or a calcium chelator, BAPTA-AM. After 1 hr., the non-adherent cells were gently washed, and the cells adhering to the BMSCs were subjected to CFU assay. The graphs depict that the increase in the number of CFU formed from the BM MNCs interacting with TGF-primed BMSCs gets significantly reduced in the presence of the PKC inhibitor (a) and BAPTA-AM (b). (c) Differential scoring of the colonies formed in various sets indicates that inhibition of PKC and buffering of intracellular calcium affected the formation of all types of colonies. Colonies belonging to BFU-E, GM, and GEMM were manually scored using a phase contrast microscope (Zeiss). All experiments were done on at least 3 independent BM samples (N=3); each set had triplicate plates (n=3). The data are represented as mean ± S.D. *** and ### p [?] 0.001. (d)**Inhibition of PKC and buffering of intracellular calcium in TGF-primed BMSCs abrogates their HSC-supportive ability** 1 x 10^5 $CD34^+$ HSCs isolated from human bone marrow were co-cultured with variously treated BMSCs grown on coverslips. After 3 days of incubation, the cells were fixed and immuno-stained with an antibody to human CD34 (raised in mouse; green) followed by anti-mouse-FITC antibody. DAPI was used to demarcate the nuclei (Blue.) Images show that $CD34^+$ cells grew luxuriantly on TGF-primed BMSCs (BMSC*TGF). This effect was abolished by applying a PKC inhibitor and a calcium chelator to them before the addition of TGFβ1. The images were acquired on a confocal laser-scanning microscope (Zeiss), and represent experiments done on 3 independent BM samples (N=3). Bar represents 10 μM.

Fig 2 Pharmacological activation of PKC and an increase in intracellular calcium boost the hematopoiesis-supportive ability of BMSCs (a, b, c) BMSCs were treated or not with Indo (-), its inactive counterpart, Indo (+) (both 5 μM for 30 minutes), CPA, or Thapsigargin (both 5μM for 30 minutes). After removing the treatments, $1X10^6$ BM MNCs were interacted with them for 1 hr. After incubation, the non-adherent cells were gently removed, and the cells closely interacting with the stromal cells were subjected to CFU assay. The graphs show that the MNCs interacting with the BMSCs treated with a PKC activator Indo (-), but not with its inactive counterpart Indo (+), (a), and agonists of intracellular calcium, cyclopiazonic acid (CPA) and Thapsigargin (Tsg) form a significantly higher number of CFU (b). All experiments were done on at least 3 independent BM samples (N=3); each set had triplicate plates (n=3). The data are represented as mean ± S.D. *** p [?] 0.001. **(c) A pharmacological activation of intracellular calcium in BMSCs boosts their HSC-supportive ability**BMSCs grown on coverslips were treated with CPA or Tsg (both 5μM for 30 minutes). After removing the treatments, bone marrow-derived $CD34^+$ HSCs ($1x10^5$) were co-cultured with them. After 3 days of co-culture, the cells were fixed and immuno-stained with an antibody to CD34 (raised in mouse, green) followed by anti-mouse-FITC antibody. DAPI was used to demarcate the nuclei (blue). The images show that BMSCs having increased intracellular calcium support an extensive proliferation of $CD34^+$HSCs. The images were acquired on a confocal laser-

scanning microscope (Zeiss) and represent 3 experiments done on independent BM samples (N=3). Bar represents 10 μM.

Fig 3 Peptide-mediated enhanced adhesion to fibronectin boosts the functionality of BMSCs (a, b) BMSCs were primed with an FN-adhesion-promoting peptide or cyclic RGD (both 10μM, overnight). 1x10^6 BM MNCs were seeded on them and incubated for 1 hr. Non-adherent cells were removed by gentle washing, and the cells closely associated with the stromal cells were subjected to CFU assay. The graphs show that the BMSCs treated with FN-adhesion-promoting peptide (a) or cyclic RGD (b) support a significantly higher colony formation from the BM MNCs interacting with them.**Integrin-specific bioactive peptides increase the potency of BMSCs** (c-f) BMSCs grown in a 24-well plate were primed with peptides specific to α5β1, αIIbβ3, and α4β1 integrins (all 10μM, overnight). After removing the treatment, 1X10^6 BM MNCs were seeded on them and incubated for 1 hr. Non-adherent cells were gently washed, and the cells closely interacting with the stromal cells were subjected to CFU assay. The graphs show that both α5β1- and αIIbβ3-primed BMSCs gave a significantly higher output of CFU from the BM MNCs interacting with them, but those primed with α4β1-ada peptide suppressed the colony formation (c). (d, e) The graphs show that the α4β1-ada peptide exerts a dose-dependent inhibitory effect on the hematopoiesis-supportive ability of BMSCs (d) and exerts a dominant effect over that of α5β1-specific peptide (e). (f) A differential scoring of colonies into BFU-E, GM, and GEMM shows that the effect of both α5β1-and αIIbβ3-primed BMSCs is across all types of colonies formed. All experiments were done on 3 independent BM samples (N=3); each set had triplicate plates (n=3). The data are represented as mean ± S.D. * p[?] 0.05, ** p[?]0.01; *** p [?] 0.001.**(γ) BMΣ"ς πριμεδ ωιτη α5β1 ανδ αIIββ3 εξπανδ "Δ34$^+$ HΣ"ς** CD34$^+$ HSCs isolated from human bone marrow were co-cultured with α5β1-and αIIbβ3-primed BMSCs for 3 days. The cells were fixed and immuno-stained with antibodies to CD34 (raised mouse; green) and Ki67 (raised in rabbit, red) followed by anti-mouse-FITC and anti-rabbit-PE antibodies, respectively. DAPI was used to demarcate the nuclei (Blue). The images were acquired on a confocal laser-scanning microscope (Zeiss), and represent experiments done on 3 independent BM samples (N=3). The images show that both α5β1-and αIIbβ3-primed BMSCs support an extensive expansion of CD34$^+$ HSCs co-cultured with them. The presence of Ki67 in several CD34$^+$cells showed that they are still in a proliferative state. The images were acquired on a confocal laser-scanning microscope (Zeiss). Bar represents 10μM.

Φιγ Σ1 (α) BMΣ"ς σεςρετε ςοπιους αμουντς οφ φιβρονεςτιν ιν ρεσπονσε το ΤΓΦβ1 BMSCs were treated with TGFβ1 (10 ng/ml) overnight, and then the cells were fixed using freshly prepared buffered paraformaldehyde (pH 7,4). The cells were immuno-stained with an antibody to cellular fibronectin (raised in mouse, green) followed by anti-mouse-FITC antibody. The images were acquired on a confocal laser-scanning microscope. Bar represents 10 μM. The images depict that the TGF-treated BMSCs showed a dense network of cellular fibronectin compared to the control BMSCs (upper panels). Use of monensin (2 μM, added 1 hr. before TGF) to block trans-Golgi transport to visualize intracellular proteins before the addition of TGFβ1 facilitated the detection of intracellular FN (lower panels). **(b)Direct interaction of integrin-specific peptides with the MNCs is ineffective** The BM MNCs were subjected to CFU assay in methylcellulose-based media supplemented with α5β1, αIIbβ3 and α4β1-ada peptides (10μg/ml). The graph shows that MNCs treated with the α5β1-specific peptide did not yield a higher number of CFU, while those treated with αIIbβ3- and α4β1-ada-specific peptides yielded only a marginally higher number of CFU, as compared to the untreated MNCs. The data are represented as mean ± S.D. (N=3) * p [?] 0.05.

Graphical abstract Hematopoiesis-supportive ability of BMSCs can be boosted by pharmacological means (a, b) Priming BMSCs with TGFβ1 boosts their hematopoiesis-supportive ability resulting in an extensive proliferation of CD34$^+$ HSCs interacting with them. (c) Inhibition of PKC and buffering intracellular calcium abrogates the hematopoiesis-supportive ability of TGFβ1-primed BMSCs. (e, f) Priming of naïve BMSCs with various pharmacological compounds such as PKC activators, boosters of intracellular calcium levels, Fn-adhesion-promoting peptides, Cyclic RGD peptide, and integrin-specific bioactive peptides also boost the hematopoiesis-supportive ability of BMSCs leading to expansion of CD34$^+$ HSCs interacting with them.

Figure 1

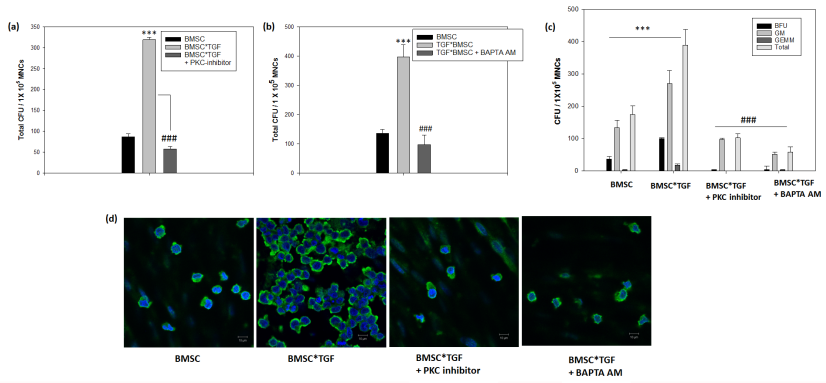

Figure 2

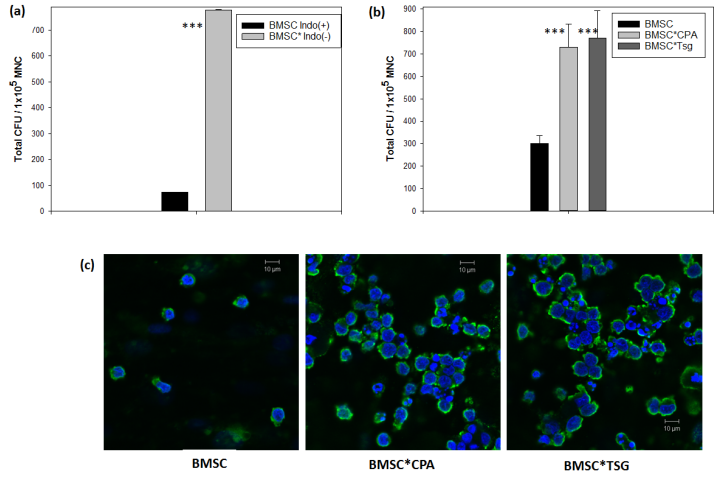

Figure 3