論 文 の 内 容 の 要 旨

論文題目　The fibrinolytic pathway expands mesenchymal stem cells through a crosstalk with endothelial cells

(血液線維素溶解系による血管内皮細胞との相互作用を通じた間葉系幹細胞動態の制御機構)

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The bone marrow (BM) microenvironment, also called BM niche, consists of the extracellular matrix (ECM) and a plethora of stromal cells which include all non-hematopoietic cells such as endothelial cells, osteoblasts and fibroblasts. These cells orchestrate the quiescence of hematopoietic stem cells (HSCs) during homeostasis or their activation following a stress signal like chemotherapy. One crucial subset of stromal cells is the mesenchymal stem/stromal cells (MSCs), which are multipotent stromal cells characterized by their ability to adhere to plastic, to give rise to colony-forming unit-fibroblasts (CFU-Fs) and to differentiate into osteogenic, adipogenic and chondrogenic cells (Bianco et al., 2008). They are a rare population of cells forming only 0.001%- 0.01% of the human adult BM cells and can be localized primarily at perivascular spaces (Crisan et al., 2008). MSCs are defined by the lack of expression of endothelial and hematopoietic markers like CD45 and Ter119, and a positive expression of mesenchymal markers like platelet-derived growth factor α (PDGFRα) and stem cell antigen-1 (Sca-1) (Morikawa et al., 2009). These MSCs are known to have the potential of supporting hematopoiesis *in vivo* by generating the required stromal niche for the maintenance and regeneration of HSCs and their ablation from the BM impairs HSCs survival and homing (Mendez-Ferrer et al., 2010). Therefore, it is important to understand how BM cells communicate between each other under stressful conditions like myelosuppression in order to uncover new target cells to promote hematopoiesis and BM regeneration in patients.

Proteases like the fibrinolytic factor plasmin and the matrix metalloproteinase-9 (MMP-9) are upregulated within BM niche cells after myelosuppression (Heissig et al., 2002; Heissig et al., 2007). The serine protease plasmin is generated through cleavage of the proenzyme plasminogen (Plg) by urokinase-type plasminogen activator (uPA) or tissue-type plasminogen

activator (tPA) which are inhibited by the plasminogen activator inhibitors (PAI-1 and PAI-2). Activation of plasmin or MMPs not only can accelerate local ECM degradation, but also can lead to the release of ECM-bound signaling molecules, or to the activation/inactivation of cytokines, chemokines, cell surface receptors, growth factors and other proteases (Cauwe et al., 2007; Heissig et al., 2012). **Although plasmin or MMPs can play a role in the regeneration of HSCs, their effect on other stromal cells of the BM niche is not yet well characterized.**

To better understand the role of the fibrinolytic system in regulating stromal cells in the BM niche, we examined the $CD45^-$ $TER119^-$ $Sca\text{-}1^+$ PDGF-$R\alpha^+$ MSCs numbers in the BM of mice with homozygously disrupted genes of the plasminogen-plasmin cascade. We determined a 4 fold increase in MSCs numbers in the bone marrow (BM) of PAI-1 deficient mice compared to the wild type mice. Therefore, we investigated the role of tPA in regulating MSCs fate.

In this study, we demonstrate that tPA induces MSCs expansion through a cytokine crosstalk with endothelial cells that results in the activation and upregulation of PDGFRα on MSCs leading to their expansion.

More specifically, we administered tPA intraperitoneally (31250 IU) in WT mice for 2 days and observed an increase in the total number of MSCs. The isolated MSCs from tPA-injected mice showed a higher proliferation rate and colony forming unit capacity compared to MSCs isolated from PBS-injected mice. Moreover, when put in differentiation medium, both PBS and tPA-MSCs differentiated into adipocytes, chondrocytes and osteoblasts as shown by immunostaining and gene expression.

In order to determine the mechanism by which tPA expands the MSCs, we took advantage of mice deficient in proteolytic factor genes like Plg KO and MMP-9 KO mice. The absence of these genes abolished tPA effects on MSC proving that the activation of these proteases is required for tPA-mediated MSC expansion. Both Plg and MMP-9 had been shown to induce, either directly or indirectly, the shedding of the hematopoietic factor KitL. Therefore, we investigated if KitL was a critical downstream target of tPA in MSCs. In fact, a daily injection of 2.5μg/ mouse of KitL resulted in MSCs expansion after 2 days in Plg and MMP-9 KO mice. Of importance, although MSCs do not express the KitL receptor c-kit (confirmed by us), blockade of the KitL receptor c-kit by administrating a neutralizing antibody prevented tPA-mediated MSCs expansion. These data indicated the existence of a c-kit+ recipient cell.

To identify potential MSC-activating factors which were induced after tPA treatment, we looked for differential expression of various known growth factors after tPA administration both in the serum and in BM mononuclear cells. We showed that FGF-2 and PDGF-BB are increased both at the gene and protein level. Immunostaining of BM sections for these factors showed their localization at perivascular sites. Based upon the nature and the localization of these factors, we suspected the endothelial cells (ECs) to be the c-kit+ tPA responder cells. Indeed, we found an increase in PDGF-BB and FGF-2 in isolated primary ECs after tPA injection as well as in cultured ECs stimulated with tPA or KitL.

Moreover, increased expression and phosphorylation of PDGFRα but not PDGFRß was observed in tPA-MSCs compared to PBS-MSCs lysates using an RTK array. In addition, the *in vitro* stimulation of MSCs with FGF2 and PDGF-BB resulted in a

synergistic increase in PDGFRα expression.

In order to study MSC-EC crosstalk in more detail, we created a coculture system whereby both cell types are separated by a transwell chamber of 0.4μm pore diameter allowing only a cytokine interaction. We demonstrated that the addition of tPA to the coculture increased MSCs number and that the addition of the general RTK inhibitor Gleevec or specific inhibitors for PDGFRα (Crenolanib) and c-kit (ACK2) abolished tPA-mediated MSCs expansion, suggesting that both receptors are critical for tPA-mediated MSC expansion. Supporting this conclusion, *in vivo* co-administration of Gleevec equally inhibited tPA effect thus proving that the MSCs expansion relies on cytokine crosstalk/ receptor activation between MSCs and ECs.

MSCs exist in virtually all adult connective and vascularized tissues as pericytes, helping to restore the damaged tissue following injury. We next asked whether systemic administration of tPA *in vivo* expanded MSCs in other MSC niches. Indeed, higher numbers of MSCs within adipose and skeletal muscle tissues were found in tPA treated compared to control tissues.

To determine if endogenous tPA levels can equally expand MSCs, we focused on 5-FU myelosuppression model since it has already been shown that tPA is upregulated in the BM stroma following hematopoietic stress (Heissig et al., 2007). Indeed, we found that MSCs enter cell cycle after 5-FU treatment and that the number of MSCs in the BM increases in both WT and tPA deficient mice but in a significantly lower extent in the latter.

MSCs have long been suggested to contribute to cancer progression through their paracrine signals inducing angiogenesis and metastasis. We therefore speculated that cancer cells can secrete tPA in order to expand MSCs locally within the cancer niche. We found that the B16F10 melanoma cell line expresses high levels of tPA which is enough to induce MSCs expansion *in vitro* coculture. Moreover, the genetic modification of B16F10 to express lower levels of tPA resulted in impaired MSCs expansion in coculture and in tumor site.

Collectively, our study introduces a novel paradigm in MSCs biology by which fibrinolytic enzymes can regulate the MSCs content within multiple MSCs niches by catalyzing a cytokine crosstalk between EC and MSCs. We summarize our findings in the following model: