

The contribution of CD44v6 and CD44v7 to the crosstalk between
hematopoietic stem cells and the bone marrow niche

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Prabhu THIRUPATHI

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Dekan: Prof. Dr. Peter Roesky

Referent: Prof. Dr. Andrew Cato

Korreferent: Prof. Dr. Margot Zöller

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The deeper a well is dug, the more the water that springs;
the more one learns, the more the wisdom it brings.

Thirukkural

To my loving parents

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I hereby declare that this thesis is the result of my own work, that I used no other than the indicated references and resources, that all the information that has been taken directly or indirectly from other sources is indicated as such, and that I have regarded the statute of the Karlsruhe Institute of Technology on securing good scientific practice in its currently applicable version.

Prabhu THIRUPATHI

List of abbreviations

ADAM10	A Disintegrin and metalloproteinase domain-containing protein 10
bFGF	basic fibroblast growth factor
BSA	Bovine serum albumin
BMC	Bone marrow cells
BMP4	bone morphogenetic protein
BM-Str	bone marrow stroma
°C	Degree Celsius
CD	Cluster of differentiation
CD44wt	CD44 wild type containing all the exons
CD44v7 ^{-/-}	Knock out coding for the variant exon 7
CD44v6/7 ^{-/-}	Knock out coding for the variant exons 6 and 7
CFSE	Carboxyfluorescein succinimidyl ester
Coll IV	Collagen IV
CXCL12 (SDF1)	C-X-C motif chemokine 12/ Stromal cell-derived factor 1
CXCR4	C-X-C chemokine receptor type 4
DMSO	Dimethyl sulfoxide
EPO	Erythropoietin
ECL	Enhanced chemiluminiscence
ECM	Extracellular matrix
ERM	Ezrin, Moesin, Radixin
FACS	Fluorescence-activated cell sorting
FN	Fibronectin
FLT3	FMS-like tyrosine kinase-3
FLT3L	Fms-related tyrosine kinase 3 ligand

GCSF	Granulocyte colony stimulating factor
GMCSF	Granulocyte-macrophage colony stimulating factor
h	hours
HA	Hyaluronic acid
HSP	Heat shock protein
IGF	Insulin-like growth factor
IP	immunoprecipitation
IL1 α	Interleukin 1 α
IL1 β	Interleukin 1 β
IL3	Interleukin 3
IL6	Interleukin 6
IL7	Interleukin 7
IL9	Interleukin 9
IP10	Interferon gamma-induced protein 10
Lef1	lymphoid enhancing factor-1
LIF	leukemia inhibitory factor
LN111	Laminin 111
LN332	Laminin 332
μ	micro
MMP9	Matrix metalloproteinase 9
OPN	Osteopontin
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline
RANTES	regulated on activation, normal T cell expressed and secreted

rpm	Revolutions per minute
SCF	Stem cell factor
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TEMED	Tetramethylethylenediamine
TGF- β	Transforming growth factor beta
TNF α	Tumor necrosis factor α
TNFR1	tumor necrosis factor receptor 1
TNFR2	tumor necrosis factor receptor 2
TPO	Thrombopoietin
VN	Vitronectin
Wnt	wingless

Die Expression von CD44 auf hämatopoetischen Stammzellen (HSZ) als auch auf Leukämie-induzierenden Zellen (LIZ) ist für *Homing* in die osteogene Nische und Adhäsion, die für das Überleben von HSZ und LIZ erforderlich sind, notwendig. Dies konnte für LIZ mittels einer Therapie mit anti-CD44 gezeigt werden, die LIZ in die Differenzierung treiben. Es muss jedoch berücksichtigt werden, dass eine CD44-Blockade auch die Adhäsion von HSZ in der osteogenen Nische stören könnte. Um LIZ selektiv aus der Nische zu eliminieren ohne HSZ dabei zu beeinträchtigen, könnten möglicherweise variante Isoformen von CD44 als Target genutzt werden, die auf LIZ hoch, aber niedrig oder nicht auf HSZ exprimiert werden. Dazu zählen unter anderem die Isoformen CD44v6 und CD44v7, die in verschiedenen Leukämien hoch exprimiert werden.

Um die Möglichkeit einer auf anti-CD44v6 oder anti-CD44v7 basierenden Therapie zu überprüfen, habe ich kontrolliert, in wieweit diese beiden Isoformen an der Hämatopoese beteiligt sind. Überprüft wurde insbesondere Adhäsion, *Homing*, Migration, Quieszenz und Apoptoseresistenz der HSZ im Kontext mit dem Knochenmarksstroma (KM-Str), sowie die Einflussnahme von Standard-CD44 (CD44s), CD44v6 und CD44v7 von KM-Str-Zellen auf HSZ. Wildtyp- (wt-) Mäuse, CD44v7-Knockout- (KO-) oder CD44v6/v7-KO-Mäuse wurden als Modellsystem genutzt, um HSZ und KM-Str zu isolieren und die Interaktion zwischen HSZ und KM-Str zu untersuchen.

HSZ adhären überwiegend mittels CD44s an Matrixproteine. CD44v6 trägt zur Adhäsion an Hyaluronsäure (HA), Fibronectin und Kollagen I bei. Die Adhäsion von CD44wt HSZ an CD44wt KM-Str wird durch CD44v6/v7 auf den HSZ gesteigert. Hingegen wird die Adhäsion von CD44v6/v7^{-/-} HSZ an CD44wt KM-Str stark reduziert und es erfolgt fast keine Adhäsion an CD44v6/v7^{-/-} KM-Str. Untersuchungen mit blockierenden Antikörpern belegen, dass der Expression von CD44v7 auf KM-Str bei der Einbettung der HSZ eine wesentliche Rolle zukommt.

Migrationsstudien belegen, dass die Expression von CD44v6 auf HSZ eine wichtige Rolle bei deren Migration auf HA spielt. CD44v6/v7^{-/-} HSZ zeigen auch eine geringere Migration in Richtung Interleukin 6, Osteopontin, *Stem cell derived factor 1*. Die Migration der HSZ auf KM-Str belegt darüber hinaus, dass die Migration auch durch CD44v6/v7 auf KM-Str gefördert wird.

Die reduzierte Adhäsion von CD44v7^{-/-} und CD44v6/v7^{-/-} HSZ an Chemokine und KM-Str beeinflusst die Quieszenz der HSZ und die Resistenz gegenüber Zytostatika. CD44wt HSZ teilen sich weniger häufig als CD44v6/v7^{-/-} HSZ, die sich vermehrt in der Mitose befinden. CD44v6/v7-kompetentes KM-Str unterstützt den Ruhezustand der HSZ. Erste Untersuchungen unterstützen die Annahme, dass CD44v6/v7 über TGF-β an der Expression von CD117 und β-Catenin beteiligt ist.

Die Apoptoseresistenz der HSZ gegenüber *TNF-related apoptosis-inducing ligand* (TRAIL) wird durch das KM-Str unterstützt, das die Expression von Galektin3 fördert, wodurch eine verstärkte Aktivierung des PI3K/Akt-Signalweges in CD44wt HSZ initiiert wird.

Zusammengenommen lässt sich sagen, dass die Expression von CD44 sowohl auf HSZ als auch auf KM-Str zur Adhäsion, Migration, Quieszenz und Apoptoseresistenz der HSZ beiträgt. Die HSZ Migration wird maßgeblich durch die Expression von CD44v7 auf KM-Str unterstützt. HSZ Quieszenz und Apoptoseresistenz wird durch die Expression von CD44v6 auf HSZ und durch eine CD44v6-vermittelte Interaktion mit dem KM-Str begünstigt.

Das Wissen um den Einfluss von CD44v6, CD44v7 und assoziierter Moleküle auf HSZ, wird helfen LIZ selektiv anzugreifen, ohne die Wechselwirkung der HSZ mit der osteogenen Nische zu beeinträchtigen.

1. Introduction

1.1 Hematopoiesis and Hematopoietic stem cells

Hematopoiesis is the step-wise generation of all cellular components of the blood from a single type of cell – the hematopoietic stem cell (HSC). HSC have the ability to undergo asymmetric cell division to maintain (give rise to a daughter cell) its stem cell pool and also generate lymphoid and myeloid progenitor cells (Illustration 1). The progenitor cells are committed and differentiate to other types of mature cells like erythrocytes, macrophages, T cells, B cells and natural killer cells.

1.1.1 HSC characterization

HSC are used in many medical applications/therapies. One of them is bone marrow transplantation in the treatment of hematological disorders. But the number of HSC available for therapies is a limiting factor. Initial efforts put into the isolation of HSC lead to the characterization of mouse HSC first performed in 1988 [1]. HSC was identified negative for lineage markers like TER119 for erythroid cells, Mac1/ CD11b for monocytes, Gr-1 for granulocytes, CD45R/B220 for B cells, CD3, CD4 for T cells, low expression of Thy-1, expressed on mouse thymocytes and positive for stem cell antigen (Sca-1) and c-kit, receptor for stem cell factor. It was reported [2] that mouse bone marrow (BM) cells which express c-kit expression has hematopoietic progenitor activity. HSC having Lin⁻Sca-1^{c-kit} (LSK) markers are heterogeneous and contain HSC along with lineage-committed progenitors. HSC also retain the fluorescent DNA-binding dye – Hoechst33342 and termed ‘Side population’ [3] according to flow cytometry analysis. Recently, HSC are characterized based on the expression of signaling lymphocyte activation molecule (SLAM) family receptor proteins [4]: CD150, CD244, and CD48. The differential expression of SLAM family receptors correlates

with stemness of cells. Multipotent hematopoietic progenitors (MPP) are non-self-renewing and express $CD150^+CD244^+CD48^-$. Mouse HSC express $CD150^+CD244^+CD48^-$ [4].

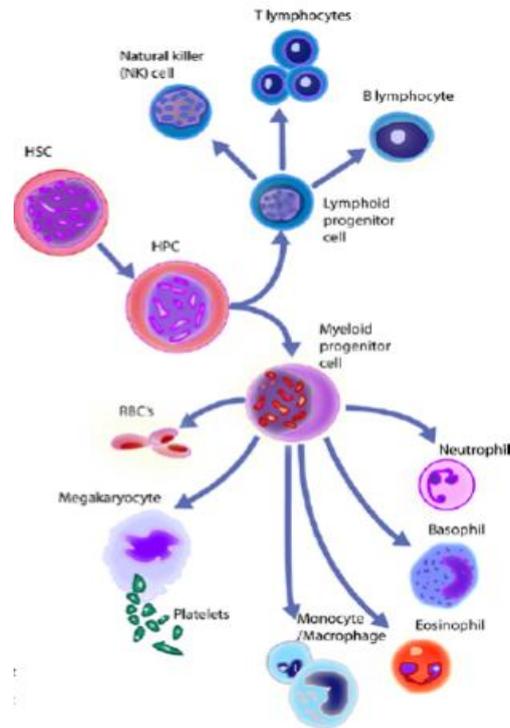


Illustration 1: Hematopoiesis inside BM: The illustration shows the HSC differentiate to progenitor cells from which the lymphoid and myeloid lineage-committed progenitors arise to differentiate further to give rise to all mature cells of blood. *Adopted from [www.http://stemcells.nih.gov/info/scireport/pages/chapter4.aspx](http://stemcells.nih.gov/info/scireport/pages/chapter4.aspx)* © 2001 Terese Winslow.

1.2 Hematopoiesis

1.2.1 Cytokines and chemokines involved in hematopoiesis

The pluripotential HSC is characterized by complex processes like self-renewal, survival, proliferation, lineage commitment and differentiation. These processes are managed by intrinsic cellular programming and extracellular cues such as interactions between the HSC and the BM-Str and cytokines. Most cytokines act on HSC with redundant actions on hematopoietic lineages and also multiple cytokines act on each hematopoietic lineage [5]. Information on the cytokines that are involved in the stem cell survival, maintenance and expansion are obtained for stem cell factor (SCF) [6], Fms-like tyrosine kinase 3 (Flt3) ligand

[7] and thrombopoietin (TPO) [8]. They act in association with interleukin (IL)-3 and granulocyte colony-stimulating factor (G-CSF) [9].

Stem cell factor (SCF) is encoded by steel locus which when mutated produces reduced HSC colonies in BM. The number of hematopoietic progenitor cells of many hematopoietic lineages is also reduced with SCF deficiency. Similarly, the mature cells of erythroid and mast cells are also dependent on SCF indicating the role of SCF on mature cells of these lineages [10]. The ability of HSC to regenerate hematopoiesis in transplanted myeloablated hosts and long-term maintenance of hematopoiesis is also influenced by SCF [11].

Flt3 ligand is specific for Flt3 receptor tyrosine kinase 3. Flt3 expressing HSC have long-term reconstituting capacity [12]. Flt3 ligand contributes to the myeloid, T- and B-lymphoid lineages formation. Flt3 mutants were deficient in the B-lymphoid lineage with smaller pro-B and preB cells formed in the bone marrow. The formation of dendritic and natural killer cell populations is also dependent on Flt3 ligand [13].

Thrombopoietin (TPO) is involved in the production of platelets in the liver. TPO interacts with its receptor c-Mpl in the maturation and production of megakaryocyte progenitors and megakaryocytes to produce platelets [14]. There was not only a decrease in the number of platelets and megakaryocytes [15] found in TPO-deficient mice but also a reduction in the progenitors of erythroid and myeloid lineages [16]. TPO is also required for colony formation of HSC.

The glycoprotein granulocyte colony-stimulating factor (GCSF) is secreted by activated macrophages, bone marrow stromal cells (BM-Str), fibroblasts and endothelial cells. It is important for the granulocyte colony formation in vitro and granulopoiesis in vivo. The blood neutrophil count in the blood is dependent on GCSF and it can be administered in vivo to increase the neutrophil count. GCSF receptor is expressed on neutrophils, monocytes,

multipotential and myeloid progenitor cells [17] indicating the importance of GCSF in hematopoiesis. In an experiment with irradiated mice where wild type and GCSF receptor deficient cells were transplanted to create hematopoietic chimeras, there was a multi-fold need in the number of mutant cells than wild type cells to ensure repopulation. Hence, GCSF signaling is important for the transplanted HSC to function in bone marrow.

Interleukin-6 (IL-6) and leukemia inhibitory factor (LIF) belong to the IL-6 family of cytokines. IL-6 deficiency causes a reduction in the number of colony formation by HSC as also seen in LIF deficiency. Lack of IL-6 on HSC contributes to a deficient reconstitution.

Apart from these, erythropoietin (EPO) increases the level of erythrocyte progenitors and IL-3 influences the growth of most lineages.

Stromal cell-derived factor 1 (SDF-1) also known as C-X-C motif chemokine 12 (CXCL12) secreted by BM-Str is important in increasing the number of B cell and myeloid progenitors in bone marrow [18]. Also, SDF-1, SCF and IL-7 together enhance the proliferation and survival of B cell precursors [19] and myeloid progenitor [20] with SDF-1 independently enhancing the myeloid progenitor survival.

1.2.2 Extracellular matrix, cells and associated signaling

Bone marrow is composed of extracellular matrix (ECM) components such as proteoglycans [21], fibronectin (FN) [22], laminins (LN) [23] and collagens (coll) [24]. They are secreted by BM-Str and along with cytokines and chemokines in the bone marrow help HSC to localize in the bone marrow by forming adhesive interactions. The secretion of FN, LN and coll by BM-Str coordinates with the hematopoiesis. The adhesive interactions with ECM help to form specific niches for HSC and to undergo erythroid and myeloid lineage-specific differentiation [25]. Apart from these, the HSC quiescence [26] and self survival [27] is achieved by their interactions with ECM components.

Proteoglycans such as hyaluronic acid (HA), heparan sulfate (HS), dermatan sulfate and chondroitin sulfate (CS) are distributed within the ECM. Long-term bone marrow cultures contain HA and CS which support HSC production [28]. The BM-Str-HSC interaction, HSC-FN binding [29], cytokine presentation [30] and HSC differentiation [31] are mediated by HS which interacts with LN and coll IV [32]. HS recruits hepatocyte growth factor [33] and beta fibroblast growth factor (bFGF) [34] and remodels the BM-Str and ECM [35]. HS modulates hematopoiesis by inducing erythroid differentiation in HSC [36].

Fibronectin favors the attachment of HSC to BM-Str [22] in vitro. FN is found at sites where the granulocytes and monocytes develop [37]. FN attaches to early erythroid and myeloid progenitors [38] with integrin CD49d assisting the process [39]. Similarly, CD49d expressed by HSC attach to FN on BM-Str [40]. The adhesive interactions of FN are promoted by integrins CD49d and CD49e and CD44 [41, 42] with the binding affinity of progenitor cells to FN is altered by the cytokines IL-3, TPO and SCF [43, 44]. Also the adhesion of mast cells [45], megakaryocytes [46], eosinophils and neutrophils [47] is mediated by FN.

Collagens (coll) are synthesized by the BM-Str and bone marrow-derived fibroblasts [48] and are localized in the endosteum of bone [49]. Coll I and coll IV are associated with endothelial cells. They help in reconstituting the bone marrow matrix and are a suitable environment for hematopoiesis. Adhesion of erythroid and granulocyte progenitors is seen with coll I [50]. The location of collagen within the bone and attachment to hematopoietic progenitors indicate that they are important for HSC homing [51].

Laminin is a major component of ECM [52]. It attaches to coll IV and proteoglycan and regulates leukocyte movement to chemokines [53]. HSC adhesiveness is mediated by laminin through the integrins [54]. The macrophage- colony stimulating factor mediated proliferation of macrophages is promoted by laminin [55].

HSC reside primarily in the BM but can also be found in extra-medullary sites in liver and spleen. Hematopoietic stem and progenitor cells can be found in small numbers in continuous

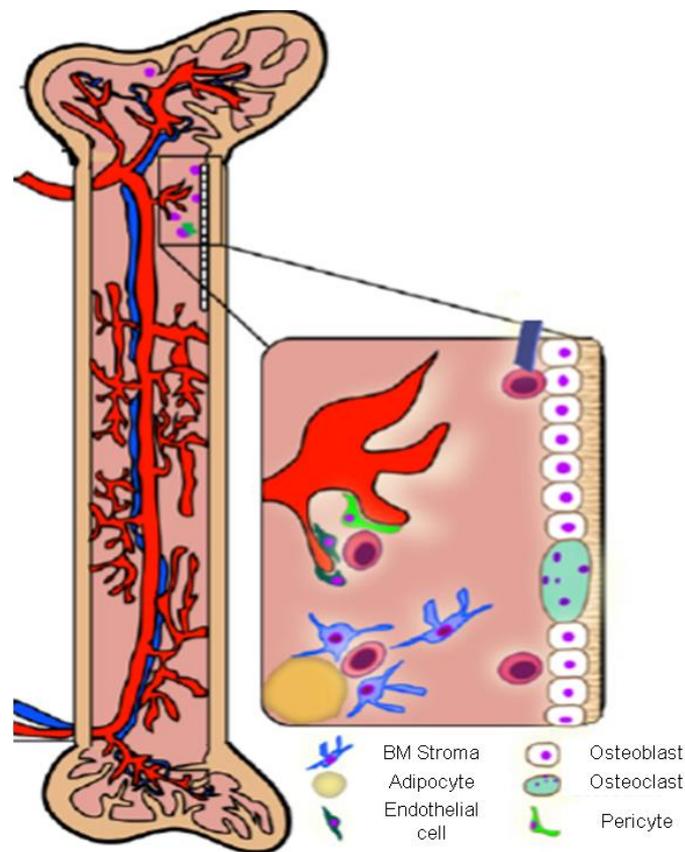


Illustration 2: BM composition: Cross-section of BM showing the different components like BM stroma, marrow sinuses, artery, osteoblasts, adipocytes and space for hematopoiesis where the different progenitors and precursors arise from hematopoietic stem cells. HSC is found associated with osteoblasts and BM-Str. *Adopted from* [www.
http://stemcells.nih.gov/info/scireport/pages/chapter4.aspx](http://stemcells.nih.gov/info/scireport/pages/chapter4.aspx) © 2001 Terese Winslow.

circulation in blood.

BM is composed of different cell populations (Illustration 2) like HSC and BM-Str differentiating to produce osteoblasts, osteoclasts, adipocytes and pericytes [56] and endothelial progenitor cells [57].

The specialized BM microenvironment that lodges the HSC and decides their fate is termed “niche” [58]. Niche functions to regulate the HSC location, adhesiveness, retention, homing, mobilization, quiescence / activation, symmetric and asymmetric differentiation, proliferation

and survival [58-61]. The existence of a niche was first proposed by Schofield for HSC [62]. The components of the niche are osteoblasts, osteoclasts, mesenchymal stem cells, reticular cells and endothelial cells [63]. The BM niche can be categorized into endosteal niche [64] and vascular niche.

The endosteal niche (Illustration 3) is defined in the endosteum in trabeculae to which HSC localize and associate with N-cadherin⁺ osteoblasts [65]. The illustration shows the various interactions between HSC and the osteoblasts that help in homing, retention and engraftment. Angiopoietin-1 (Ang-1) expressed by osteoblasts interacts with Tie2 –receptor tyrosine kinase expressed on HSC and regulate stem cell quiescence [66]. Ang-1 induces adhesion of HSC to bone giving protection to HSC from myelosuppressive stress [66]. HSC binding to osteoblasts of the endosteal niche is further enhanced by CD49d expressed on HSC binding to FN in the ECM of bone [67]. N-Cadherin mediates homophilic interactions between N-Cadherin expressed on both HSC and osteoblast through the extracellular domain [68]. SDF-1 secreted by osteoblasts bind to its receptor C-X-C chemokine receptor type 4 (CXCR-4) which is highly expressed by HSC and help in the recruitment of HSC to the bone marrow. Bone marrow is rich in calcium that helps in retaining the HSC in the niche through calcium receptor on HSC.

Osteoblasts secrete cytokines such as G-CSF, GM-CSF, IL-3 and leukemia inhibitory factor (LIF) to support HSC self-renewal [69, 70].

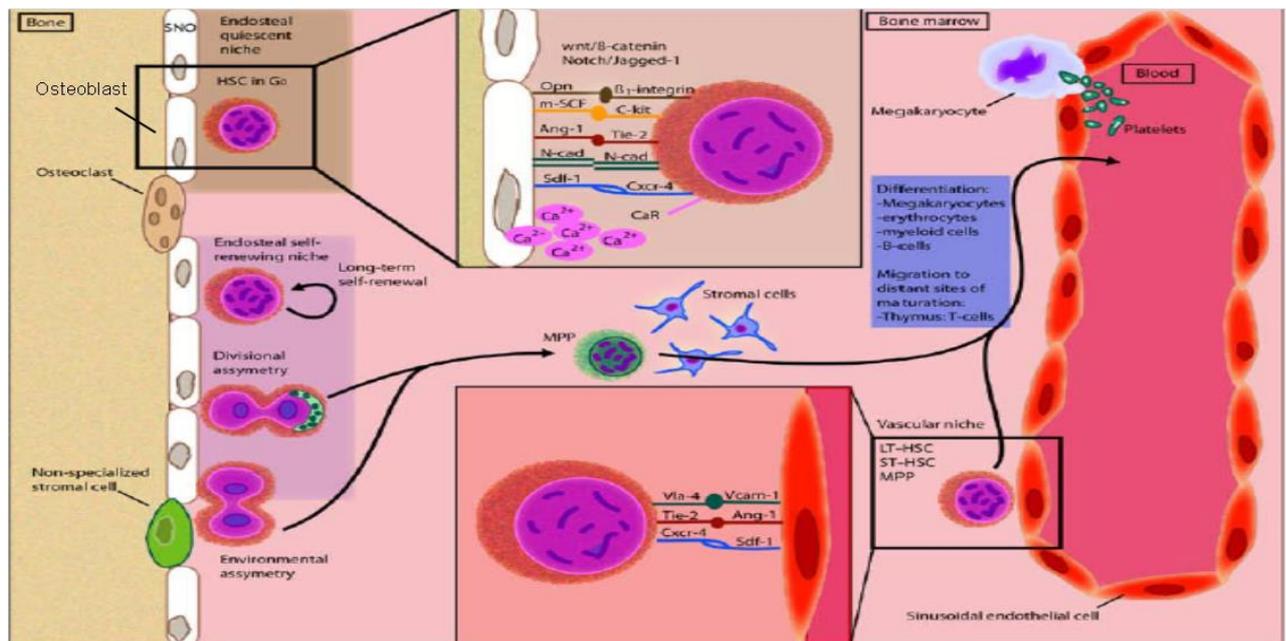


Illustration 3: Association of HSC with the endosteal niche. HSC are found in association with bone marrow osteoblasts. The fate of HSC is controlled by niches. Functions of HSC like cell maintenance, cell quiescence are controlled by the various interactions of HSC with bone lining osteoblasts are shown in the Illustration. *Adopted and modified from [71].*

Jagged 1, a ligand of Notch, expressed on osteoblasts and BM stromal cells activate Notch signaling in HSC resulting in an increase of HSC numbers in the BM [72]. There is a direct correlation between HSC numbers and number of osteoblasts [72]. Stem cell factor (SCF) through its receptor, c-kit, on HSC activates the signaling towards the proliferation of HSC [73]. HSC self-renewal is also attributed to canonical Wnt signaling [74, 75].

Wnt is a family of lipid-modified glycoproteins which contains 19 members. In the canonical Wnt pathway, in the absence of the Wnt ligand, β -catenin remain phosphorylated by a complex containing Glycogen synthase kinase 3 beta (GSK3 β), casein kinase 1 (CK1), adenomatous polyposis coli (APC) and axin. Phosphorylated β -catenin is ubiquitinated and proteosomally degraded. When Wnt ligand binds to seven-pass transmembrane receptor Frizzled (Illustration 4) and low-density lipoprotein receptor-related protein (LRP5/6) complex, axin and APC are recruited to the membrane after GSK3 β and CK1 binding sites are phosphorylated by LRP5/6 and axin gets degraded (and it is no longer attached with the cytoskeleton) [76, 77]. Activation of Disheveled protein by Wnt binding prevents the

phosphorylation of β -catenin. β -catenin accumulates in the cytoplasm and translocates to the nucleus and interact with transcription factors, T cell factor (TCF) and lymphocyte enhancer binding factor (LEF) targeting the gene expression of CD44 [78], cyclin D1 and c-Myc [77]. Recently, it is published that CD44 acts as a positive regulator of Wnt receptor complex and the signaling activity of Wnt/ β -catenin. Inactivation of canonical Wnt signaling causes loss of proliferation of progenitor cells affecting the stem cell maintenance [79].

A schematic representation of Wnt/ β -catenin signaling pathways in HSC is shown in Illustration 4.

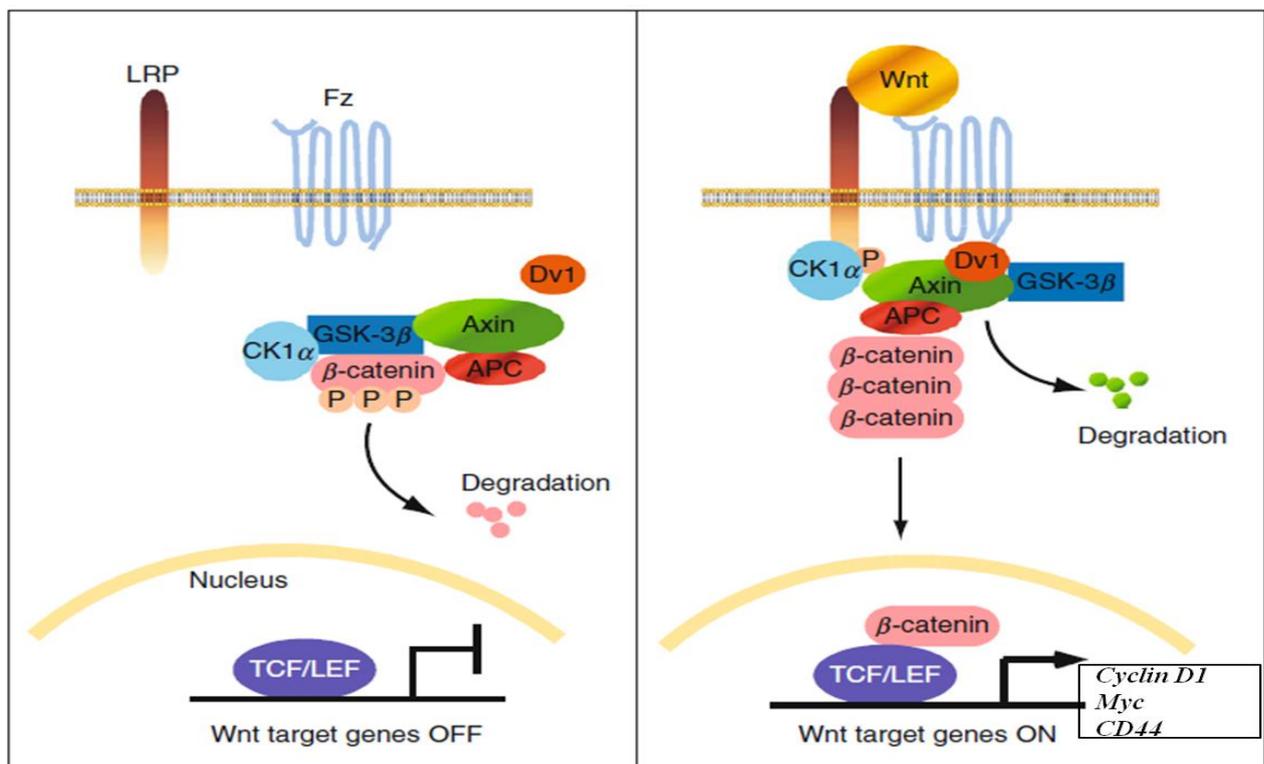


Illustration 4. Canonical Wnt pathway found in HSC: When Wnt is not in association with its receptor complex, Frizzled and LRP, β -catenin is continuously phosphorylated by a complex of GSK3 β , CK1, axin and APC and later degraded. When Wnt binds to its receptor complex, β -catenin gets dephosphorylated and gets translocated to the nucleus to bind to TCF/LEF complex of transcription factors to turn on Wnt target genes like *cyclin D1* and *Myc* and thereby the proliferation of HSC. *Adopted and modified from [80].*

The regulation of HSC maintenance is mediated by chemokines, growth factors and proteases.

Transforming growth factor beta, (TGF- β) is a negative regulator of HSC by blocking the expression of receptors like c-kit, Flt3 and IL-6R [81]. Under hematopoietic stress, matrix

metalloproteinase, MMP-9 is activated. MMP-9 cleaves membrane bound SCF to release HSC from the endosteal niche. The chemokine osteopontin (OPN) secreted by osteoblasts mobilizes HSC from the endosteal niche to the vascular niche [82].

1.2.3 Vascular niche

Apart from BM, the extramedullary sites of hematopoiesis like spleen and liver contain vascular niches [83] which lie proximal to vascular endothelium and comprise endothelial cells which provide higher oxygen concentration gradient and higher fibroblast growth factor-4 to support HSC [84]. The vasculature in this part of the niche contains arteries which pass through the bone and branch out into arterioles, and is supplied by arterioles and capillaries and they drain out into BM sinusoids [85]. The contents of BM sinusoids are finally drained into the BM central sinus [86]. HSC and endothelial cells arise from hemangioblast which is their common precursor cell and hence hematopoiesis and vascularization occur together during embryogenesis [87]. Contrary to the endosteal niche having more quiescent HSC, vascular niche contains more committed stem- and progenitor cells [88]. The molecules, SDF-1, E-selectin and vascular cell adhesion molecule (VCAM) are necessary for homing, engraftment and mobilization of HSC are provided by BM vasculature. Myeloablation by irradiation in bone marrow transplantation treatment causes collapse of BM vasculature [86]. Blocking of Tie-2 showed that HSC recovery and hematopoiesis can be restored only after the regeneration of BM vasculature [89].

When the cells are injured or under stress, they generate signals that are released into the blood and received by the BM sinusoids. The HSC or progenitor cells at the BM sinusoids respond to these signals and mount an immediate response by inducing HSC division and differentiation to restore normal homeostasis. In order to maintain sufficient response, quiescent HSC at the endosteal niche are also signaled to pull-out of dormancy and enter into proliferation and differentiation to meet the demand. So, physically the endosteal niche and

vascular niche are in proximity and maintain communication to balance HSC quiescence and self-renewal capacity.

1.3 CD44 and hematopoiesis

1.3.1 CD44

CD44 is a type I glycoprotein expressed on a variety of vertebrate cells. CD44 is involved in a variety of physiological processes like cell development, homing, adhesion, migration, proliferation and apoptosis.

The CD44 gene

CD44 is a single copy gene, with its genomic sequence highly conserved among the mammalian species of human and mouse between 85% to 87% in their N-terminal and C- and transmembrane regions respectively. The gene is 50 Kb, located on the short arm of chromosome 11 in humans and chromosome 2 in mouse. Of the 20 exons in CD44 gene [90], 10 are known as “standard” exons and the other exons are referred as variant v1 to v10. The variant exons correspond to exons 6 to 15. The different products of the same gene arise due to the insertion of alternatively spliced exon products of the same gene in the extracellular region of the molecule. The products vary in size due to difference in the N and O-linked glycosylations.

The CD44 molecule

The smallest form is the standard (CD44s) or hematopoietic form which is expressed on most vertebrate cells. The mature protein of the standard molecule has 341 aminoacids with a molecular weight of 37-38 KDa, out of which 248 constitute the extracellular domain of the molecule, 72 aminoacids towards the cytoplasmic domain and the rest as the transmembrane region [91, 92]. The exons 1-5, 16 and 17 form the extracellular domain. Between exons 5

and 16, are ten variant exons which produce variant exon products by alternative splicing. The exon 18 forms the transmembrane region and either exon 19 or 20 forms the cytoplasmic domain.

The extracellular domain

The first five non-variable exons of CD44 code for the amino-terminal globular domain. It contains motifs and docking sites for the various components of the extracellular matrix like HA, fibronectin, laminin and collagen which support migration. [93, 94]

The amino terminal region of the polypeptide has homology with cartilage link proteins. It comprises 90 aminoacids which contain the HA binding domain [91, 92] and also a glycosaminoglycan binding site [95]. The degree of binding of glycosaminoglycans to CD44 depends on the post-translational modifications of the protein and also the cell-type and growth conditions [96]. Six cysteine residues in this region form three disulfide bonds give the polypeptide a globular structure. Six N-linked glycosylation sites are found on the extracellular region.

The stem structure

Between the N-terminal globular domain and the transmembrane domain a stretch of 46 aminoacids forms the stalk-like structure. This stretch is heavily glycosylated and contains the putative proteolytic cleavage sites [97]. The stem is enlarged by sequences that are encoded by alternatively spliced variant exon products (v1-v10) (Illustration 5, 6). Serine-threonine residues close to the membrane proximal region constitute O-linked glycosylations sites [92, 98]. Chondroitin sulphate and heparin sulphate (HS) binding to serine-glycine motifs along with the glycosylations in this region add up to give the molecule 85-90 kDa, the molecular mass predominantly found in hematopoietic cells [99-101]. Different cell types have varying

glycosylations in the variant exon products, which make them different in their molecular weights [102, 103].

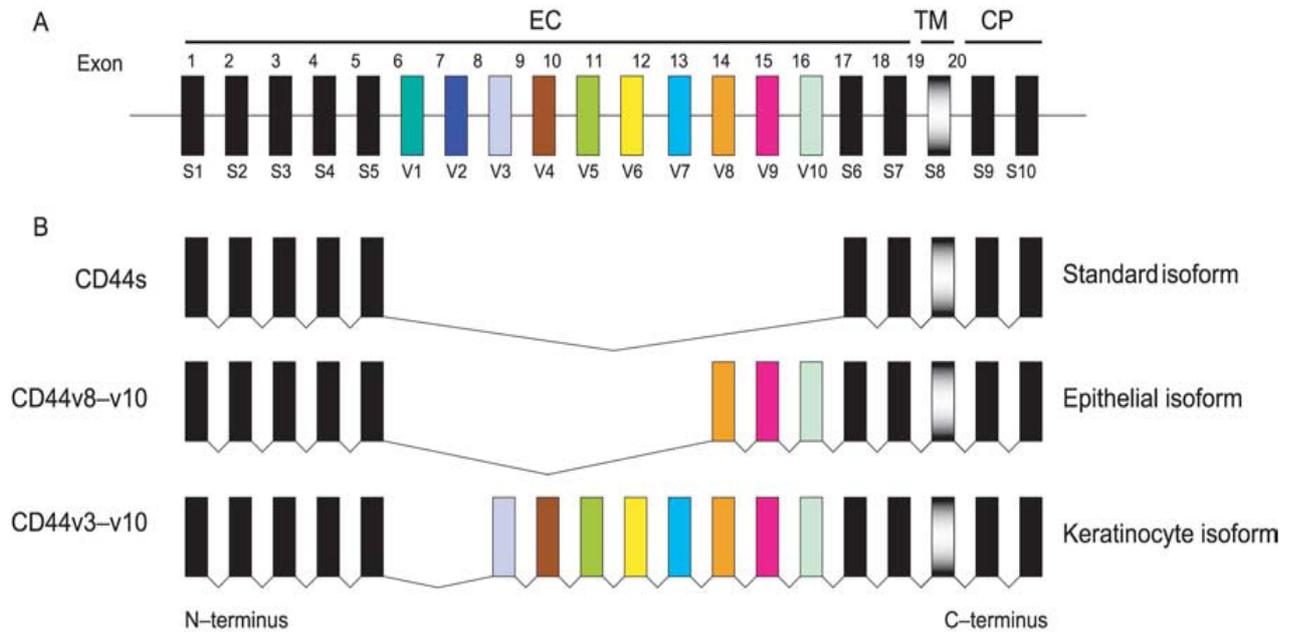


Illustration 5. The exon arrangement of *CD44* gene: **A, B:** The *CD44* gene has 20 exons of which 10 are variant exons which code for variant products of the CD44 molecule. The standard isoform is the shortest molecule with exons from 1-5 and 17-20 coding for it. Exons for the standard isoform if along with exons 14-16, code for the epithelial isoform and if along with exons 8-15, code for the keratinocyte isoform. *Adopted from [104]*

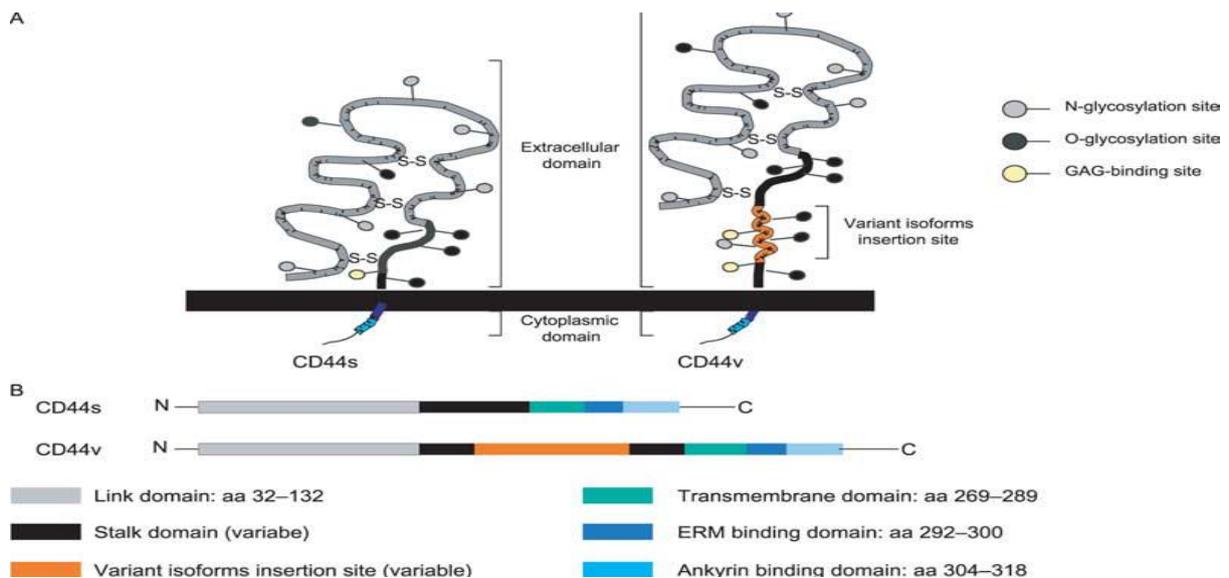


Illustration 6. Structure of CD44 molecules: (A) The CD44 molecule is composed of an extracellular domain, a stalk like region near to the transmembrane region. This region has the variant exon products inserted in it. This is followed by the transmembrane region and the cytoplasmic tail. There are multiple glycosylations which are N-linked (grey circles) and O-

linked (black circles) and two active GAG-binding sites (yellow circles). The link domain has the binding site for HA. The cytoplasmic tail contains the binding sites for cytoskeletal linker proteins ankyrin and ERM proteins. (B) The various domains of the CD44 standard and variant molecules shown in linear arrangement. *Adopted from [104]*

The transmembrane domain and the cytoplasmic domain

The transmembrane region of CD44 is made up of 23 amino acid residues. The lone cysteine residue is involved in the formation of CD44 oligomers. This domain is implicated in the recruitment of CD44 into lipid rafts.

The exons 19 and 20 form the cytoplasmic domain of CD44. This domain is involved in the binding of various intracellular proteins which participate in signaling, cytoskeletal reorganization and growth regulation [105, 106]. The cytoskeletal protein ankyrin binds to CD44 and mediates contact with spectrin which is implicated in HA-mediated cell adhesion and motility [107]. Ezrin, radixin and moesin (ERM) proteins bind to the basic-amino acid-motif of CD44, which is present between the transmembrane domain and the ankyrin-binding site with their N-terminal region while their C-terminal region binds to the filamentous actin (F actin) thus crosslinking CD44 with the actin cytoskeleton [108]. Another member of the ERM family of proteins, Merlin, a tumor suppressor protein, with its N-terminal domain also binds to the ERM-binding motif. Merlin is involved in the stabilization of the junctional-cortical actin interface [109]. ERM proteins are either phosphorylated by growth factors or protein kinase C. Activated ERM proteins then link CD44 to F-actin and the actin skeleton [110]. Merlin, when phosphorylated [111] does not bind to CD44 and at high cell density or with high-molecular hyaluronan gets dephosphorylated. At the active state it binds to CD44 and disrupts the link to the actin cytoskeleton and blocks Rac activation and signaling [112].

CD44 is involved in many functions and only those that are of interest with respect to CD44 in hematopoiesis are discussed here:

1.3.2 CD44 in hematopoiesis

CD44 is important in the interaction between HSC and bone marrow microenvironment for hematopoiesis. The development of hematopoietic cells can be mimicked in vitro in long-term bone marrow culture (LTBMC). They contain BM cellular constituents and promote the generation and differentiation of progenitor cells into mature cell lineages for several months [113]. Components of extracellular matrix like HA, HS, chondroitin sulfate and FN required by hematopoietic progenitors are produced during LTBMC culture by BM-Str [29, 41, 114, 115].

HSC and the interaction with the endosteal niche

a) Cell adhesion

The major receptor for HA, CD44 [116-118], is expressed on HSC and progenitor cells [117, 119] is essential for HSC binding [120]. Erythroid progenitors express high levels of CD44 make it the adhesion molecule for the HSC [119]. CD44 is required in myelopoiesis [121]. In murine long-term bone marrow cultures anti-CD44 prevented the formation of ‘cobblestone areas’ which are groups of early hematopoietic progenitor cells [122, 123]. The antibody bound HA binding site on CD44 which shows the HA-dependent HSC attachment to stromal cells through CD44. The proliferation and differentiation of precursor cells are dependent on the signals transduced by CD44 associated molecules after CD44 activation by HA [124]. Also, formation of BM-Str requires CD44. An antibody against the variant isoform CD44v6 delayed the stroma formation. Moreover, these variant specific antibodies cause the release of cytokines from macrophages and activate hematopoiesis [125].

b) Crosstalk via associated molecules

CD44-HA mediated adhesion of HSC

The expression of adhesion molecules is important for cell-cell and cell-matrix interactions. Osteoblasts are prime components of the osteogenic niche and express CD44. HA which is the principal component of the ECM and CD44 cross-linking on osteoblasts by HA induce the expression of CD54 and VCAM-1 and upregulates them [126]. CD54 expressed on hematopoietic progenitors of monocyte and macrophage lineage [127] and on endothelial cells for adhesion [128]. The expression of CD54 in bone marrow progenitors and its involvement in adhesion to stromal cells in the osteogenic niche indicates that it is an important molecule in hematopoiesis [127].

CD44 and CD49d in the homing of stem cells to the BM

CD49d, expressed on HSC, interacts with FN in the ECM and attaches HSC to the osteogenic niche [129]. CD44 cooperates with CD49d and mediates homing of HSC [41] to the osteogenic niche by adhering to matrix ligands such as HA, FN and coll [130]. Also, CD44 participates along with CD49d in the adhesion of hematopoietic progenitor cells to FN [41]. The homing of colony-forming units of spleen (CFU-S) to bone marrow was disrupted when anti-CD49d and anti-CD44 were incubated with stem cells [131]. Hence CD44 and CD49d are essential for the homing of stem cells to BM.

CD44 in TGF- β -mediated HSC quiescence

Growth factors act as messengers in the communication between the cells. They provide information on cell status and surroundings. The signals help the cells to decide upon cell migration, or to undergo proliferation or differentiation. TGF- β is secreted as a complex of peptides which maintain TGF- β in an inactive state [132]. The latent TGF- β is linked to the ECM and is unavailable for cell activation. In CD44-MMP9-mediated TGF- β activation of

cell migration [133], MMP9 which is proteolytically active cleaves the latent TGF- β into an active and mature peptide. TGF- β along with cyclin-dependent kinase inhibitor p27 has a synergistic effect [134] in maintaining quiescence in HSC.

HA-CD44 crosstalk in IQ motif containing GTPase activating protein 1-mediated cell migration

HA and CD44 are upregulated in remodeling tissues with CD44 modulating the cell-cell, cell-ECM interactions towards cell migration. HA activation of CD44 results in the association of ERM proteins with its cytoplasmic tail. The N-terminal region of ezrin binds to the cytoplasmic tail of CD44 while the c-terminal region binds to the F-actin thereby linking CD44 to the actin cytoskeleton [108]. HA-CD44 crosstalk is observed in the IQGAP1 supported actin cytoskeleton rearrangements and cell migration [135]. Proteomic analysis of CD44-binding proteins demonstrated interaction between the cytoplasmic tail of CD44 and IQ motif containing GTPase activating protein (IQGAP)1 [136]. IQGAP1 is a multidomain protein and interacts with F-actin, Rac1/Cdc42 and microtubules. IQGAP1 is a regulator of RhoGTPases Rac1 and Cdc42 [137]. The number of active Cdc42 correlates with the increase in IQGAP1 expression and promoting cell migration [138]. Though the binding partners, actin, calmodulin and adenomatous polyposis coli contribute to cell migration IQGAP1 knockdown reduces cell migration [138]. Downstream of Rac1, IQGAP1 alters the balance between β -catenin-E-cadherin complex at the cell adherens junctions and weakens the adherens junction [139]. Then it cross-links actin filaments and localizes to the leading edge of migrating cells assisting in cell migration [140].

Also, HA-mediated CD44 ligation serves as a matrix for assembling enzymes. HA-activated CD44 recruits MMP9 to the leading edge of the migrating cells [141] to cleave the ECM components and support directional cell migration.

CD44 and 14-3-3 proteins in the activation of PI3K/Akt pathway in apoptosis resistance

Most stem cells are found in the G0 phase of the cell cycle [142]. BM-Str often provides support to HSC in protecting them against apoptosis [143, 144]. HA-CD44v6 interaction promotes the assembly of a signaling complex at the cytoplasmic tail of CD44 which results in the activation of phosphatidylinositol 3-kinase (PI3K)/Akt signaling [145] accompanied by an increase in the expression of anti-apoptotic proteins such as Bcl2, Bcl-Xl and phosphorylation of BAD. The PI3K/Akt network plays an important role in hematopoiesis [146] by governing the important events of proliferation, differentiation and survival. PI3K is a lipid kinase. It catalyses the phosphorylation of phosphatidylinositol-3,4-bisphosphate, PI(3,4)P₂, to phosphatidylinositol-3,4,5-triphosphate, PI(3,4,5)P₃. PIP₃ binds to pleckstrin homology domain-containing proteins such as Akt and phosphoinositide-dependent protein kinase1 (PDK1) and recruits them to the plasma membrane. At the membrane PDK1 phosphorylates Akt and activates it. Flt3 ligand-activated Akt translocates into the nucleus and phosphorylates the Forkhead box protein FoxO3 (FOX3) transcription factor and inactivates it [147]. Entry of Akt into the nucleus leads to the expression of genes involved in oxidative phosphorylation and energy production, upregulation of chaperones and heat shock proteins, HSP70 [148] and HSP90 [149] and production of antioxidants. Akt also interacts with proapoptotic Bcl2-associated death promoter (BAD) protein, which is phosphorylated and bound by 14-3-3 proteins which inhibit the BAD-induced cell death [150].

c) CD44 in extravasation and homing

The mobilization of HSC from the BM niche occurs with the administration of G-CSF or with cytotoxic drug treatment [151]. As we move from endosteal niche to vascular niche the concentration of oxygen and FGF increases which promotes HSC mobilization. Also changes in the levels of SDF-1 in the BM due to stress [152] cause the mobilization of HSC from the endosteal to vascular niche and then into blood circulation. Administration of G-CSF releases

proteases like MMP2, MMP9, cathepsin-G and neutrophil elastase from neutrophils (Illustration 7) which cleave the HSC niche-retention signals like membrane bound SCF (mSCF) / membrane bound kit ligand (mkitL), SDF-1 and VCAM-1 [153-155]. The cleavage of mkitL to soluble form of kitL (skitL) induces HSC proliferation, differentiation and mobilization [155, 156]. The stress-induced release of MMP9 cleaves the N-terminal end of SDF-1 and abolishes the interaction between SDF-1 and CXCR4 and release localized HSC from the niche [153]. These released HSC then travel to distant sites. The small number of HSC found in the circulation could be due to the release of HSC when the bone remodeling happens continuously.

Migrating cells undergo extravasation in two steps of rolling and adhesion with CD44 used in adhesion/'tethering' (Illustration 7) [157]. Inflammatory cytokines stimulate the expression of CD44 on endothelial cells and increase their binding to HA [158]. Endothelial cells capture the rolling HSC by the interaction between CD44 and CD62P (P-selectin) and stalling them with CD44-HA binding. HSC binding to HA is mediated by CD44s. The variant exon products of CD44 and / or their glycosylations can vary the binding affinity and their migration on HA [159]. HA activation of CD44 allows ankyrin and phosphorylated ERM proteins bind to the cytoplasmic tail of CD44 and linking it with the cytoskeleton thereby CD44 is steered to the front edge of the migrating cells [110] for extravasation and releases them to circulation.

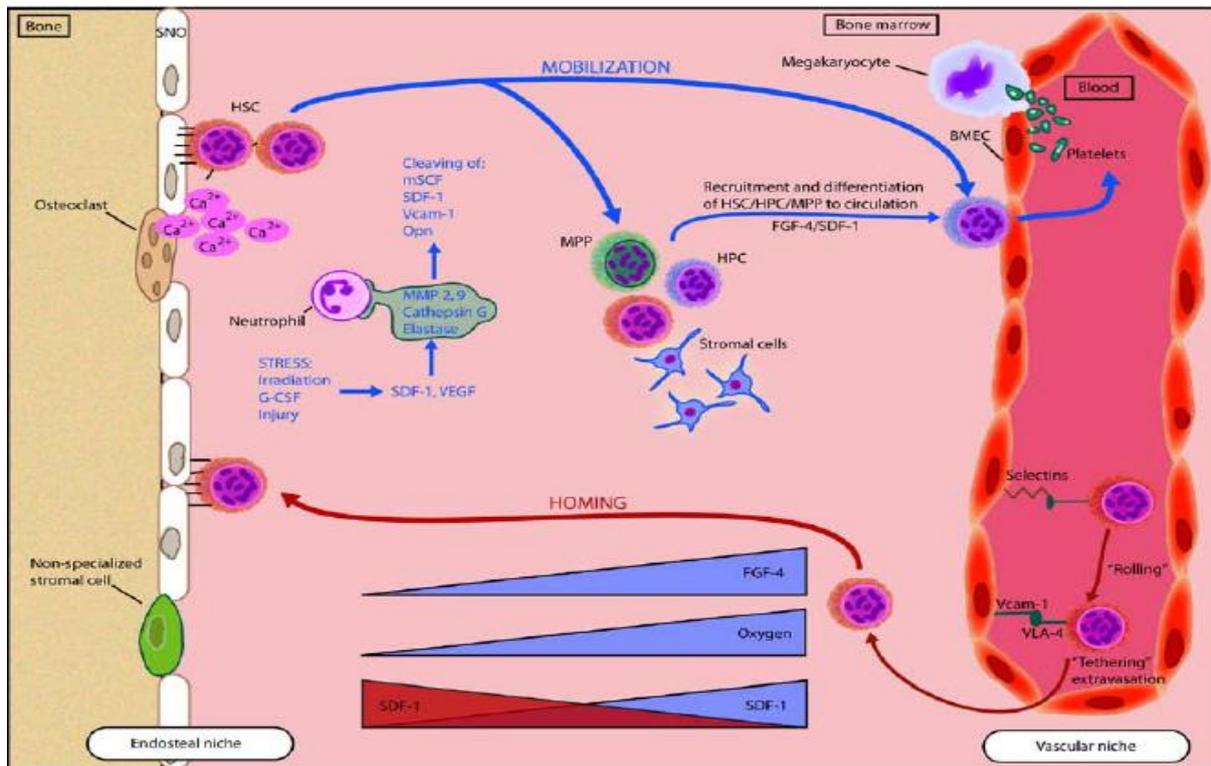


Illustration 7: HSC mobilization from endosteal niche to vascular niche. In response to stress or with administration of GCF, HSC can migrate from the endosteal niche to vascular niche and thereafter to the peripheral blood to distant sites of injury. Stress-induced signals cause the neutrophils to secrete a variety of proteases which inactivate SDF-1 and release HSC from the endosteal niche for migration. Later, they undergo rolling and adhesion to extravasate into circulation. *Adopted from [71].*

HSC synthesize HA and HA expression correlates with migration of HSC to the endosteal niche [160]. HSC homing can be blocked by anti-CD44 or soluble HA or hyaluronidase treatment [161]. SDF-1 stimulates the adhesion of HSC via CD44 that displays the mediation of CD44 in CXCR4 signaling with HA and CD44 having a prominent role in the SDF-1-dependent transendothelial migration of HSC and engrafting within endosteal niche [162]. Thus CD44 contributes to homing and engraftment of HSC in the osteogenic niche.

1.4 CD44 variant isoforms in hematopoiesis

The role of CD44v6 in homing, engraftment and maturation of HSC was analyzed in hematopoiesis of rat [163]. The functional activities of CD44v6 were assigned based on antibody inhibition studies. CD44 is widely expressed in many cell types but the expression

of CD44v6 is restricted. In LT BMC, the non-adherent cells containing large granulated cells of macrophages and dendritic cells have CD44v6 expression.

A small subpopulation of HSC expresses CD44v6 [163, 164]. Pre-treated stromal culture or the LT BMC-derived cells with anti-CD44v6 did not prevent the production of non-adherent progenitor cells. This indicates that the inhibition of CD44v6 did not prevent the progenitor cells from settling on bone marrow stroma culture.

LT BMC supports the maturation of hematopoietic progenitor cells. The influence of CD44v6 in the formation of stroma and in the expansion of progenitors was found when anti-CD44v6 was used on LT BMC of rat. It was found that the stroma formation does not require the support of CD44s but stroma formation was severely inhibited when anti-CD44v6 was used. This indicates the role played by CD44v6 in the formation of stroma. LT BMC cultures with anti-CD44v6 inhibition showed very late onset of stroma formation which was accompanied by lower numbers of cells with fibroblast morphology. Normal LT BMC supported stroma formation within the first week of seeding of BM cells and lasted till 4-6 weeks. Blocking of CD44v6 could not support the stroma formation which resulted in the death of most of the cells in the culture.

CD44v6 is important in the prothymocyte maturation. T-cell maturation was inhibited by anti-CD44v6 with the expression of CD4, CD8 and reduction in expression of Thy-1 specific for T-cells. It was hypothesized that the variant exon v6 could bind specific ligands which would trigger cytokine production as response and promoted stroma formation. When anti-CD44v6 was used the binding between the CD44v6-ligand was disturbed and prevented stroma formation and T-cell lineage differentiation.

CD44v6 influences myeloid differentiation. In experiments with human myeloid progenitor cell lines, myeloid cells express CD44v6 on maturation or after commitment and activation [165].

Towards understanding the contribution of CD44 variants v6 and v7 in lymphopoiesis and myelopoiesis, antibodies directed against exon-v6 and v7 specific epitopes on murine LTBMCM were used [125]. Antibody-treated LTBMCM showed increased nonadherent progenitors when compared to untreated LTBMCM. The increase in progenitor numbers was not due to detachment of adherent cells due to blocking of CD44v6. The number of cobblestone areas was not decreased. Macrophages in the adherent layer of LTBMCM, positive for CD11b also express CD44v6 epitope. Antibodies against v6 epitope mimicked as ligands and activated these macrophages to secrete GM-CSF and IL-6. This resulted in the increase in the number of lymphoid and myeloid progenitor cells. The use of exon v7-specific antibodies did not affect hematopoiesis. It is suggested that anti-CD44v6 acted as a stimulatory molecule or a ligand from the ECM towards binding and activating the macrophages to secrete lineage-specific cytokines.

In the treatment of hematological disorders after myeloablation and BM reconstitution, progenitor T cells must home into thymus and undergo T cell maturation. CD44s helps in thymocyte homing. CD44v6 has a role in the maturation of thymocytes to avoid graft-versus-host reaction in allogeneic transplantation. CD44v6 also promotes apoptosis resistance in thymocytes which is revealed when CD44v6 blocked-cells underwent apoptosis and affected the expansion of early thymocytes. The induction of apoptosis resistance by CD44v6 is through the activation of Akt [166].

Since expression of CD44v6 and CD44v7 were observed at particular stages of differentiation in embryo, they were expected to play significant role in hematopoiesis [167]. In CD44v7-competent and CD44v7-deficient mice, experiments were conducted to understand the role of

CD44v7 in the homing of HSC where CD44v7 is expressed in 20% BMC and 40% of BM-Str. Within the bone marrow 50% of CD117⁺ cells and 40% of SCA1⁺ cells express CD44v7. Except for the CD44v7 expression, BMC of CD44v7^{-/-} mice showed no difference in the expression of CD49d and CD44 as well as of CD44v6. Similarly, there was no difference in the adhesion molecule marker expression in LTBM. Since CD44v7 is expressed on HSC and BM-Str, experiments with antibody blocking against CD44v7 were performed to mobilize the progenitor cells. After CD44v7 blocking, there was a decrease in the CD49d expression observed in BM and spleen and there were more spleen cells found with increased colony forming units of granulocyte, granulocyte-macrophage and macrophage.

To check the BM repopulating ability, BM were transferred from CD44v7^{+/+} to CD44v7^{-/-} mice and vice versa. BMC from CD44v7^{+/+} mice did not home in CD44v7^{-/-} mice whereas BM from CD44v7^{-/-} mice homed better in CD44v7^{+/+} host with better BM repopulating ability. This indicated that CD44v7 expression on stromal cells is important for HSC homing [168].

Increase in mobilized progenitor cells obtained after CD44v7 blockade with anti-CD44v7 and sustained reconstitutive capacity of the same indicated that CD44v7-expressing stromal cells contribute to embedding of progenitor cells and homing.

Aim of the thesis

CD44 was identified as a leukemia initiating cell (LIC) marker, where it supports adhesion, migration, quiescence and proliferation. One of the therapeutic approaches involved blocking CD44 with antibodies to drive LIC out of the niche and to induce differentiation. But, anti-CD44 also affects HSC reconstitution even more severely than LIC embedding. CD44 is expressed on HSC and have the potential danger in targeting a common marker that is also expressed on LIC. Studies on variant forms of CD44 that are expressed on HSC but higher on LIC would be helpful to avoid HSC and improve LIC targeting. CD44v6/v7 are expressed in acute myeloid leukemia and CD44v6 is expressed in acute lymphocytic leukemia, chronic lymphocytic leukemia and multiple melanoma, suggesting alternative targets to the common marker CD44. A detailed understanding on the HSC crosstalk with BM-Str in the osteogenic niche could provide information on the contribution of CD44v6/v7.

To achieve this I evaluated

1. CD44v6 and CD44v7 expression and associating molecules on HSC and BM-Str.
2. The relevance of CD44v6 and CD44v7 in the adhesion and migration of HSC to matrix proteins and cytokines.
3. The role of HA and BM-Str in maintaining quiescence in HSC via CD44v6.
4. The effects of BM-Str CD44v6 and CD44v7 on HSC proliferation and signaling in apoptosis resistance.

2. Materials and Methods

2.1 Material

2.1.1 Instruments

Name of the instrument	Company
Bacterial shaker and incubator	Edmund Buehler GmbH, Hechingen
Camera system Spot CCD	Diagnostic Instruments, Sterling Heights, USA
Cell chamber Neubauer improved	Brand, Wertheim
Centrifuge Sorvall RC5B Plus	Kendro, USA
Centrifuge Sorvall GSA Rotor	Kendro, USA
Centrifuge Sorvall Ti 50 Rotor	Kendro, USA
Centrifuge Biofuge fresco	Heraeus, Hanau, Hanau
DNA-agarose gel electrophoresis chamber	Bio-Rad, Munich
Eagle eye (Mididoc)	Herolab, Wiesloch
ELISA plate reader	Anthos labtec, Wals, Austria
FACS Calibur	Becton-Dickinson, Heidelberg
FPLC	Merck Hitachi, Japan
Hyper processor (for processing films)	Amersham, Freiburg
Incubator for bacteria	Melag, Berlin
Cell culture incubator	Labotec, Goettingen
Invert microscope DM-IL	Leica, Bensheim
LSM710 (laser scanning microscope)	Zeiss, Goettingen
Master cycler (PCR cycler)	Eppendorf, Hamburg,
Magnetic stirrer 3000	Heidolph, Keilheim
Microscope DMBRE	Leica, Bensheim

Microwave	Phillips, Wiesbaden
Photocassette	Amersham, Freiburg
pH-Meter-761 Calimatic	Knick, Berlin
Photometer Ultraspec III	Amersham, Freiburg
Pipettus-Akku	Hirschmann, Eberstadt
Pipettes	Eppendorf, Hamburg
Powersupply PS 9009	GIBCO, Darmstadt
Rotor GSA	Kendro, USA
Rotor SW34	Kendro, USA
Rotor SW41 Ti	Beckman Coulter, Krefeld
Sterile hood	Heraeus, Hanau
Tabletop centrifuge	Heraeus, Hanau
Transfer apparatus Mini Trans-Blot®	Bio-Rad, Munich
Thermo-mixer	Eppendorf, Hamburg
Ultrasound homogenizer	Bandelin Elektronik
Video microscope	Carl Zeiss, Goettingen
Water-bath	Julabo, Seelbach
Weighing scale RC210 D	Sartorius, Goettingen
Whirlmixer Vortex Genie	Si Inc., New York, USA

2.1.2 Miscellaneous Material

Cell culture flasks 25cm ² , 75cm ²	Greiner Bio-one GmbH, Frickenhausen
Cell culture 96-well, 24-well, 6-well plates	Greiner Bio-one GmbH, Frickenhausen

Centrifugal concentrators Vivaspin 6ml, 20ml	Vivascience, Hannover
Centricon, 50,000 MWCO	vivaspin™
Cryovials	Greiner Bio-one GmbH, Frickenhausen
Coverglass	R. Langenbrinck, Emmendingen
ELISA plates	Greiner Bio-one GmbH, Frickenhausen
Falcon tubes 15ml, 50ml	Greiner Bio-one GmbH, Frickenhausen
Glass slides	R. Langenbrinck, Emmendingen
Hyperfilm ECL	Amersham, Freiburg
Needles	BD Biosciences, Heidelberg,
Nitrocellulose membrane Hybond ECL	Amersham, Freiburg
Parafilm	American Nat. Can., Greenwich, Great Britain
Petriplates	Greiner Bio-one GmbH, Frickenhausen
Pipette tips	Sarstedt, Numbrecht
Sterile filter 0,2µm	Renner, Darmstadt
Syringes	BD Biosciences, Heidelberg
Trans-well migration (Boyden) chambers 48 well	Neuroprobe, Gaithersburg, USA
Whatman™ 3MM paper	Schleicher & Schüll, Dassel
2.1.3 Chemicals and Reagents	
Acetic acid	Ridel-de Haen, Seelze
Acetone	Fluka, Buchs, Switzerland
Agarose	Sigma, Steinheim
Ammonium persulphate (APS)	GIBCO, Darmstadt
Ampicillin sulphate	Calbiochem, Darmstadt

Annexin FITC V	Becton Dickinson, Heidelberg
Bactoagar	Fluka, Buchs, Switzerland
Bradford reagent	Bio-Rad, Munich
Biotin-X-NHS	Calbiochem, Darmstadt
Bovine Serum Albumin (BSA)	PAA, Pasching, Austria
Bromo phenol blue	Merck, Darmstadt
Calcium chloride	Merck, Darmstadt
Carboxyfluorescein succinimidyl ester (CFSE)	Invitrogen, Darmstadt
Chloroform	Riedel-de Haen, Seelze
Cisplatin (cis-Diamine platinum(II)dichloride)	Sigma, Seelze
CNBr-Activated sepharose 4B	GE Healthcare, Freiburg, Germany
Coomassie R-250	Merck, Darmstadt
Crystal violet	Sigma, Steinheim
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
Ethanol	Carl Roth, Karlsruhe
Ethidium bromide	Merck, Darmstadt
Ethylenediamine tetraacetic acid (EDTA)	Sigma, Steinheim
Foetal Calf Serum (FCS)	Gibco/Thermo Scientific, Germany
Formaldehyde (37%)	Merck, Darmstadt
Glucose	Merck, Darmstadt
L-Glutamine	AppliChem, Darmstadt
Glycerine	Carl Roth, Karlsruhe
Glycine	GERBU, Gaiberg
HEPES	GERBU, Gaiberg
HiPerfect-Reagent for transfection	Quiagen, Hilden

Hydrochloric acid (HCl)	Riedel-de Haen, Seelze
Hydrocortisone	Sigma, Seelze
Immersion oil	Zeiss, Goettingen
Isopropanol	Fluka, Buchs, Switzerland
Lipofectamine TM 2000	Invitrogen, Darmstadt
Lubrol MX (17A17)	Serva, Heidelberg
Magnesium carbonate	Merck, Darmstadt
Magnesium chloride	Merck, Darmstadt
Magnesium sulphate	Merck, Darmstadt
Milk powder	Carl Roth, Karlsruhe
Methanol	Carl Roth, Karlsruhe
N,N,N',N''-Tetramethylethylenediamine (TEMED)	Sigma, Steinheim
Paraformaldehyde	Sigma, Steinheim
Penicillin	Sigma, Steinheim
Phenylmethylsulphonylfluoride (PMSF)	Sigma, Steinheim
p-nitrophenyl phosphate, disodium salt	Sigma, Steinheim
Potassium carbonate	Carl Roth, Karlsruhe
Potassium chloride	Merck, Darmstadt
Potassium dihydrogenphosphate	Merck, Darmstadt
Propidium iodide	Immunotools, Friesoythe, Germany
Protease Inhibitor Cocktail Tablets	Roche Diagnostics, Mannheim
Protein G Sepharose 4 Fast Flow	Amersham Biosciences, Freiburg
Rotiphorese Gel 30 (Acrylamide-mix)	Carl Roth, Karlsruhe
Rhodamine DHPE	Invitrogen, Darmstadt
RPMI 1640	GIBCO, Darmstadt

Sodium acetate	Merck, Darmstadt
Sodium azide	AppliChem, Darmstadt
Sodium carbonate	AppliChem, Darmstadt
Sodium chloride	Fluka, Buchs, Switzerland
Sodium hydrogen phosphate	Merck, Darmstadt
Sodium dodecyl sulphate (SDS)	GERBU, Gaiberg
Sodium hydrogen carbonate	AppliChem, Darmstadt
Sodium hydroxide	Riedel-de Haen, Seelze
Sodium pyruvate	Merck, Darmstadt
Tris(hydroxymethyl)aminomethane (Tris)	Carl Roth, Karlsruhe
Triton-X-100	Sigma, Seelze
Trypan bue	Serva, Heidelberg
Trypsin	Sigma, Steinheim
Trypton	AppliChem, Darmstadt
Tween 20	Serva, Heidelberg
Yeast Extract	GIBCO, Darmstadt
β -Mercaptoethanol	Sigma, Seelze

2.1.4 Standard buffers and solutions

Annexin FITC/PI binding buffer	10mM HEPES pH 7.4. 140nM NaCl, 25mM CaCl ₂
Bicarbonate buffer	15mM Na ₂ CO ₃ , 35mM NaHCO ₃ , pH 9.6
Blotting buffer	25mM Tris, 192mM Glycine, 0.1% SDS, 20% Methanol
Diethanolamine buffer	97ml diethanolamine, 100mg MgCl ₂ .6H ₂ O, pH9.8
Ethidium bromide	0.01% (w/v) in water. Stored in dark

Glycine buffer	0.2M glycine, pH2.7
Lysis buffer	25mM HEPES, 150mM NaCl, 5mM MgCl ₂ , 1mM PMSF, protease inhibitors
6x Laemmli-buffer	350mM Tris, pH6.8, 10% (w/v) SDS, 36% (w/v) Glycerin, 0.01% (w/v) Bromophenol blue
LB medium	10g peptone, 5g yeast extract, 10g NaCl. Volume made up to 1L. Add 15g bacterial agar for LB plates.
Running buffer (10x) for SDS-PAGE	1% SDS (w/v), 144g Glycine, 30g Tris. Make volume to 1L with double distilled water
PBS	137mM NaCl, 8.1mM Na ₂ HPO ₄ , 2.7mM KCl, 1.5mM KH ₂ PO ₄ , pH 7.4
Phosphate buffer (0.02M)	0.2M NaH ₂ PO ₄ , 0.2M Na ₂ HPO ₄ , pH 7.2
Stripping buffer for western blots	62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.1 M 2-Mercaptoethanol
Subcellular fractionation buffer	250 mM Sucrose, 20 mM HEPES (7.4), 10 mM KCl, 1.5 mM MgCl ₂ , 1 mM EDTA, 1 mM EGTA, 1 mM DTT, PI Cocktail
TAE buffer	242g Tris base, 57.1ml Glacial acetic acid, 100ml 0.5M EDTA pH 8.0. Make volume to 1l and adjust pH to 8.5

2.1.5 Kits

Qiaquick Midiprep kit	Hilden, Germany
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ECL Western Blotting Detection	GE Healthcare, Freiburg, Germany
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2.1.6 Size markers

Pageruler™ Prestained Protein Ladder MBI Fermentas, St. Leon-Rot

2.1.7 Antibodies

2.1.7.1 Primary Antibodies

Antibody (anti-mouse)	Company
9E10 (anti-cMyc)	ATCC CRL-1729
14-3-3	Cell Signalling, Frankfurt , Germany
β-Catenin	Becton Dickinson, Heidelberg, Germany
Actin	Becton Dickinson, Heidelberg, Germany
ADAM10	Becton Dickinson, Heidelberg, Germany
Akt	Becton Dickinson, Heidelberg, Germany
BAX	Becton Dickinson, Heidelberg, Germany
Bcl2	Becton Dickinson, Heidelberg, Germany
Bcl-xl	Cell Signalling, Frankfurt , Germany
BMP4	Santa Cruz, Heidelberg, Germany
act. Caspase 3	Becton Dickinson, Heidelberg, Germany
Caspase 8	Becton Dickinson, Heidelberg, Germany
Caspase 9 cleaved	Becton Dickinson, Heidelberg, Germany
CathepsinG	Becton Dickinson, Heidelberg, Germany
CD4	Becton Dickinson, Heidelberg, Germany
CD8	Becton Dickinson, Heidelberg, Germany
CD11a (M17/5.2)	European Association of Animal Cell Cultures, Porton Down, UK
CD11b/YBM 6.6.10	European Association of Animal Cell Cultures, Porton Down, UK
CD11c	European Association of Animal Cell Cultures, Porton Down, UK

CD16	Immunotools, Friesoythe, Germany
CD18	Becton Dickinson, Heidelberg, Germany
CD19	Becton Dickinson, Heidelberg, Germany
CD44 (IM7)	American Type Culture Collection, Virginia, USA
CD44v6	Bender Medsystems GmbH, Vienna, Austria
CD44v7 (LN7.1)	[169]
CD45	European Association of Animal Cell Cultures, Porton Down, UK
CD45R	European Association of Animal Cell Cultures, Porton Down, UK
CD49d (PS2)	[170]
CD54 (YN1/1.7.4)	European Association of Animal Cell Cultures, Porton Down, UK
CD95	Becton Dickinson, Heidelberg, Germany
CD95L	Becton Dickinson, Heidelberg, Germany
CD105	Abcam, Germany
CD117	Becton Dickinson, Heidelberg, Germany
CD126	Becton Dickinson, Heidelberg, Germany
Cyclin D1	Becton Dickinson, Heidelberg, Germany
Ezrin	Becton Dickinson, Heidelberg, Germany
Flt3	Santa Cruz, Heidelberg, Germany
GM CSF	Becton Dickinson, Heidelberg, Germany
Gr1	Immunotools, Friesoythe, Germany
HSP70	Becton Dickinson, Heidelberg, Germany
HSP90	Becton Dickinson, Heidelberg, Germany
IL-6	Becton Dickinson, Heidelberg, Germany
Inhibin	Santa Cruz, Heidelberg, Germany
LEF1	Becton Dickinson, Heidelberg, Germany
LIF	Becton Dickinson, Heidelberg, Germany
Ly6	Becton Dickinson, Heidelberg, Germany
MMP9	Becton Dickinson, Heidelberg, Germany
Moesin	Becton Dickinson, Heidelberg, Germany

OPN	Becton Dickinson, Heidelberg, Germany
pAkt	Becton Dickinson, Heidelberg, Germany
pBAD	Cell Signalling, Frankfurt , Germany
PLC γ	Cell Signalling, Frankfurt , Germany
PGK1	Santa Cruz, Heidelberg, Germany
Poly ADP	Becton Dickinson, Heidelberg, Germany
PP1a	Cell Signalling, Frankfurt , Germany
RhoGD1	Santa Cruz, Heidelberg, Germany
SDF1	Becton Dickinson, Heidelberg, Germany
SOD2	Santa Cruz, Heidelberg, Germany
Talin	Becton Dickinson, Heidelberg, Germany
TGF β	Santa Cruz, Heidelberg, Germany
Tubulin	Becton Dickinson, Heidelberg, Germany
Ter-119	Becton Dickinson, Heidelberg, Germany
TNFR1	Becton Dickinson, Heidelberg, Germany
TNFR2	Becton Dickinson, Heidelberg, Germany
Trail	Becton Dickinson, Heidelberg, Germany
Tubulin	Santa Cruz, Heidelberg, Germany
Wnt	Santa Cruz, Heidelberg, Germany

2.1.7.2 Secondary Antibodies

Name	Company
Anti-mouse IgG HRP	Amersham, Freiburg, Germany
Anti-rabbit IgG HRP	Amersham, Freiburg, Germany
Anti-rat IgG HRP	Amersham, Freiburg, Germany
Anti-mouse IgG PE	Jackson Laboratories, Bar Harbor, USA
Anti-hamster IgG FITC	Jackson Laboratories, Bar Harbor, USA
Anti-mouse IgG FITC	Jackson Laboratories, Bar Harbor, USA

Anti-mouse IgG APC	Jackson Laboratories, Bar Harbor, USA
Anti-rat IgG FITC	Jackson Laboratories, Bar Harbor, USA
Anti-rat IgG PE	Jackson Laboratories, Bar Harbor, USA
Anti-rat IgG APC	Becton Dickinson, Heidelberg, Germany
Streptavidin FITC	Jackson Laboratories, Bar Harbor, USA
Streptavidin PE	Jackson Laboratories, Bar Harbor, USA
Streptavidin APC	Jackson Laboratories, Bar Harbor, USA
Streptavidin HRP	Rockland, PA, USA

2.1.8 Matrix proteins, Cytokines / Chemokines

Substance	Supplier	Dose
bFGF	Immunotools, Friesoythe, Germany	1:1000
BSA	Sigma, Munich, Germany	100µg/ml
Collagen I	Sigma, Munich, Germany	10µg/ml
Collagen IV	Sigma, Munich, Germany	10µg/ml
CXCL12 (SDF1)	Immunotools, Friesoythe, Germany	50ng/ml
FN	Sigma, Munich, Germany	2µg/ml
GCSF	Immunotools, Friesoythe, Germany	100ng/ml
GM CSF	Immunotools, Friesoythe, Germany	100ng/ml
Hyaluronic acid	Sigma, Munich, Germany	100µg/ml
IL-3	Sigma, Munich, Germany	100ng/ml
IL-6	Immunotools, Friesoythe, Germany	100ng/ml
IL-7	Immunotools, Friesoythe, Germany	100ng/ml
LN111	Sigma, Munich, Germany	2µg/ml

LN332	K.Miyazaki, Yokohoma, Japan	50µg/ml
OPN	Biotrend Chemikalien GmbH, Köln	2µg/ml
SCF	Biotrend Chemikalien GmbH, Köln	100ng/ml

2.1.9 Animals

BALB/c (H-2^d), SVEV (H-2^b), CD44v7^{-/-} and CD44v6/7^{-/-} (back-crossed to SVEV) mice [171] [172], kindly provided by U. Günthert, Department of Microbiology, University of Basel, Basel, Switzerland, were bred at the central animal facilities of the University of Heidelberg. 8-10wk old mice were used for experiments.

2.1.10 Cells and Cell lines

BMC, bone marrow stroma, HSC were obtained from mice. The hybridoma cell lines used for producing monoclonal antibodies are IM7 (anti-CD44), PS2 (anti-CD49d), YN1 (anti-CD54), 9E10 (anti-cMyc), LN7.1 (anti-CD44v7). HEK-293-Ebna1 is used for overexpression of recombinant proteins of CD44s, CD44v6 and CD44v7.

Cells	Origin
HEK293-Ebna1	ATCC CRL-1573 derived; transfected with the EBNA1 viral gene.
IM7.8.1, expressing anti-panCD44	ATCC TIB-235
9E10, expressing anti-cMyc	ATCC CRL-1729
YN1/1.7.4, expressing anti-CD54	European Animal Cell Culture Collection, Porton Down, UK
PS/2, expressing anti-CD49d	[170]

LN 7.1, expressing anti-CD44v7	[169]
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2.1.11 Expression vector

The recombinant proteins were expressed from constructs made from pCEP-Pu vector with puromycin resistance. The constructs were available.

2.1.12 Bacterial strain

<i>E.coli DH5α</i>	Genotype: F-, Φ80dlacZΔM15, Δ(<i>lacZYA-argF</i>)U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (rk-,mk+), <i>phoA</i> , <i>supE44</i> , <i>thi-1</i> , <i>gyrA96</i> , <i>rel A1</i> , λ- (Invitrogen, Darmstadt, Karlsruhe)
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2.2 Methods

2.2.1 Protein Biochemistry

2.2.1.1 Immunoprecipitation (IP)

10x10⁶ cells or 1mg/ml of precleared cell lysate was precipitated with the indicated antibody against the antigen (2μg/ml) and allowed to bind overnight at 4°C. After overnight incubation, 5% of Protein G sepharose was added and allowed to bind for 1 hr at 4°C. The beads are washed with lysis buffer three times and then boiled with laemmli buffer for 10 min at 95°C and loaded to the gel.

2.2.1.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Analysis of protein samples by western blot or coomassie blue are performed using “Mini Protean II” with discontinuous buffer system. The stacking gel was cast over the resolving gel

(10%) (with the constituents of them given) After complete polymerization of the gel, the protein samples boiled with laemmli buffer are loaded on to the gel and run at 110V with 1x running buffer. After the run is complete, the gel is either transferred in a wet-transfer system onto a nitrocellulose membrane or dyed with coomassie blue for further analysis.

2.2.1.3 Coomassie blue staining of protein gels

After the protein samples were separated on SDS-PAGE gels, the gels were washed once with bidest water and then incubated in fixing solution containing (50% methanol and 10% acetic acid) for 1hr with gentle agitation at room temperature. Then the gels were stained in coomassie blue staining solution containing (0.1% Coomassie Brilliant blue R-250, 50% methanol and 10% glacial acetic acid) for a period of 2-3 hr with gentle agitation. The stained gels were washed first with bidest water and then destained with destaining solution containing 40% methanol and 10% glacial acetic acid with gentle agitation and periodic exchange with fresh destainer. The destained gels were stored in bidest water for further analysis.

2.2.1.4 Western blotting

The SDS-PAGE protein gels, prepared for transfer, were laid on 3 layers of 3MM whatman paper equilibrated with transfer buffer. Over the gel the nitrocellulose membrane cut to the size of the gel was overlaid and then 2 layers of whatman paper were placed. The whole arrangement was kept in a cassette and wet transfer was carried out at 30V overnight at 4°C. After the transfer is complete, the membrane was blocked in (5% w/v) fat-free milk in PBST (PBS, 0.1% (v/v) tween-20) at room temperature. Primary antibody incubation in PBST-milk was carried out at 2h at RT followed by washing thrice with PBST and later incubating with secondary antibody conjugated with horse radish peroxidase (HRP) (diluted 1:10000 in PBST) for 1h at RT and washing again thrice with PBST. The subsequent detection of

proteins was performed by chemiluminescence using the 'ECL Western blotting detection reagents' and 'ECL radiography films' from GE.

2.2.1.5 Antibody purification

The supernatants from hybridoma cell lines were centrifuged at 10,000 rpm for 20min and removed off their cell debris using the Sorvall centrifuge (rotor GSA). Antibodies from hybridoma supernatant and recombinant proteins were purified by affinity chromatography using either sepharose protein G-4B column or CNBr-coupled with IM7 or anti-cMyc antibody. Filtered supernatant were mixed with 0.01% of NaN_3 and passed over column using gravity flow. The column was washed with 0.1M phosphate buffer, pH7.2 and eluted with 0.2M glycine solution, pH2.7 and neutralized with 200 μ l of 1M Tris pH 8.0. The whole operation was performed using FPLC apparatus. The eluted fractions were pooled, concentrated and dialyzed against PBS using Vivaspin tubes (50kDa cut off). The concentration of the purified protein was measured using Biorad assay, filtered and stored at -20°C.

2.2.1.6 CNBr-sepharose coupling

5 mg of purified IM7 antibody or recombinant proteins were dialyzed against coupling buffer pH8.3 overnight at 4°C. 0.286g of CNBr-sepharose was swollen in 1mM HCl solution for 30min at 4°C and slurry is prepared. It is then washed with 200ml of ice cold sterile filtered 1mM HCl solution for 15min. The next two steps were performed faster to enhance the coupling. The slurry was washed once with 5ml of coupling buffer and removed. The dialyzed protein was then immediately added to the slurry and incubated in a rotating wheel at 4°C overnight. Next day, the beads were spun at 2000rpm for 1min and the supernatant was collected and the concentration was checked for efficiency of binding. If the concentration was 10 fold lower than the starting concentration coupling was proceeded to the next step.

The coupled beads were blocked off the vacant sites with 1M Tris pH 8.0 for overnight at 4°C. Finally, the beads were washed 2x alternatively with low pH buffer and coupling buffer. The first and the fourth washes were checked of its OD_{595nm}. The processed beads was poured into a chromatography column and washed with 5 volumes of PBS-azide pH 7.4 and stored at 4°C.

2.2.1.7 Membrane protein preparation

The cells were washed and lysed using fractionation buffer. The lysate is passed through a 25G needle for 10 times using a 1 ml syringe. This is then left on ice for 20 min. The lysate is then centrifuged at 3000 rpm for 5 min. The supernatant is taken in a fresh tube and centrifuged at 8000 rpm for 15min using tabletop centrifuge. The supernatant thus obtained is the cytosolic and membrane fraction. For the membrane fraction, the supernatant is centrifuged at 30000 rpm for 2 hrs. The pellet thus obtained is dissolved in fractionation buffer and used for pulldown assays.

2.2.1.8 Pulldown assay

Membrane protein preparations of bone marrow stroma and HSC were allowed to bind over the CNBr-Sepharose bound CD44s, v6 and v7 columns overnight at 4°C with gentle mixing. The bound supernatants were removed and the columns were washed with 30 volumes of 0.02M Phosphate buffer, pH 7.2 and then eluted with 0.1M glycine buffer, pH 2.7. The eluted supernatants were neutralized using 1M tris, pH9.0, concentrated using viva spin columns (10,000 kDa cutoff) and then boiled with 6x laemmli buffer and loaded to SDS-PAGE gels for further analysis.

2.2.1.9 Tryptic digestion and mass spectrometry

The tryptic digestion and mass spectrometry of the 1D SDS-PAGE resolved proteins of IP and pulldown assays were kindly performed by Dr. Tore Kempf and Dr. Martina Schnölzer,

Head, Functional Proteome Analysis, DKFZ, Heidelberg, Germany. The protocols used for the tryptic digestion of the proteins to be analyzed and subsequent mass spectrometry analysis are as follows:

2.2.1.9.1 Tryptic digestion

After 1D SDS-PAGE gel electrophoresis and Coomassie staining of the IP and pulldown samples, the stained area was cut out of the gel. Each gel piece was further chopped up into smaller gel plugs. They were incubated with 150 μ l water for 5 min at 37°C. After removing the solution from the gel plugs proteins were reduced with 150 μ l 10 mM DTT in 40 mM NH_4HCO_3 for 1h at 56°C, alkylated with 150 μ l 55 mM iodoacetamide in 40 mM NH_4HCO_3 for 30 min at 25°C in the dark, followed by three alternating washing steps each with 150 μ l of water and water/acetonitrile at 37°C. Gel pieces were dehydrated with 150 μ l neat acetonitrile for 1 min at room temperature, dried for 15 min. They were subsequently rehydrated with porcine trypsin (sequencing grade, Promega, Mannheim, Germany) with a minimal volume sufficient to cover the gel pieces after rehydration (100 ng trypsin in 40 mM NH_4HCO_3). Digestion was carried out at 37°C overnight.

After tryptic digestion of the proteins the supernatant containing the peptides was collected in PCR-tubes and the gel pieces were subjected to four further extraction steps. First, gel pieces were covered with acetonitrile/0.1% TFA (50:50, v/v) and sonicated for 5 min. After centrifugation the supernatant was collected and gel pieces were covered with neat acetonitrile and sonicated for 5 min. After collecting the supernatant gel pieces were sonicated for 5 min in 0.1% TFA followed by a final extraction with neat acetonitrile. The gel pieces were discarded and the combined solutions were evaporated to dryness in a speed-vac. Peptides were redissolved in 5-20 μ l 0.1% TFA/2.5% hexafluoroisopropanol by sonication for 5 min and subjected to ESI-MS/MS analysis.

2.2.1.9.2 Mass spectrometry

Tryptic peptides were separated using a nanoAcquity UPLC system (Waters GmbH, Eschborn, Germany). Peptides were loaded on a C18 trap column (180 μm x 20 mm) with a particle size of 5 μm (Waters GmbH, Eschborn, Germany). Liquid chromatography separation was performed on a BEH130 C18 main- column (100 μm x 100 mm) with a particle size of 1.7 μm (Waters GmbH, Eschborn, Germany) at a flow rate of 0.4 μl / min. For protein identification the following 2h gradient was applied: from 0 to 4% B in 1 min, from 4% to 30% B in 79 min, from 30 to 45% B in 10 min, from 45 to 90% B in 10 min followed by a washing step with 90% B for 10 min. After this step the concentration was stepped down to 0% B and equilibration was continued at 100% A for 15 min. Solvent A contained 98.9% water, 1% acetonitrile and 0.1 % formic acid, solvent B contained 99.9% acetonitrile and 0.1% formic acid. The nanoUPLC system was coupled online to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). Data were acquired by scan cycles of one FTMS scan with a resolution of 60000 at m/z 400 and a range from 300 to 2000 m/z in parallel with six MS/MS scans in the ion trap of the most abundant precursor ions. Instrument control, data acquisition and peak integration were performed using the Xcalibur software 2.1 (Thermo Scientific, Bremen, Germany).

Database searches were performed against the NCBI nr database with taxonomy mouse and mammals using the MASCOT search engine (Matrix Science, London, UK; version 2.4.0). Peptide mass tolerance for database searches was set to 5 or 10 ppm and fragment mass tolerance was set to 0.4 Da. Significance threshold was $p < 0.01$. Carbamidomethylation of cysteine was set as fixed modification. Variable modifications included oxidation of methionine and deamidation of asparagine and glutamine. One missed cleavage site in case of incomplete trypsin hydrolysis was allowed.

2.2.2 Molecular biology

2.2.2.1 Bacteria

Transformation of plasmid DNA was carried out with DH5 α using heat shock method. The competent cells stored at -80 °C were thawed on ice. 1 μ g of plasmid DNA was added to the DH5 α competent cells, mixed and incubated on ice for 5min. The mixture was incubated at 45°C for 45s and then placed on ice for 2min. 1ml of LB media was added to the incubated mixture and incubated and agitated for 30 min at 37°C. The agitated cells were then briefly centrifuged and the supernatant is removed. 200 μ l of fresh LB media was added and the cells were mixed and plated on LB-agar plates with ampicillin and incubated overnight to obtain transformed colonies.

2.2.2.2 Plasmid-DNA- Preparation

Plasmid DNA preparation were done with mini and midi-prep kits according to supplier's instructions.

2.2.3 Cell biology

2.2.3.1 Cell culture

The hybridoma cell lines were grown in RPMI 1640-medium with 10% heat inactivated fetal calf serum (FCS), 2mM glutamine, 100U/ml penicillin, 100 μ g/ml streptomycin, maintained at 37°C, 95% humidity and 5% CO₂. The HEK-Ebna cell lines having the recombinant protein constructs were grown in Iscove's minimal medium containing 10% heat inactivated fetal calf serum (FCS), 2mM glutamine, 100U/ml penicillin, 100 μ g/ml streptomycin and supplemented with 300 μ g/ml of neomycin and 2 μ g/ml of puromycin for selection. They were exchanged with new media every 3 days and HEK-Ebna cells were passaged every 3 days using 5mM EDTA.

2.2.3.2 Establishment of long-term bone marrow stroma cells (BM-Str)

Bone marrow cells were collected from femura and tibiae of 6-10 week old mice by flushing off the bones with PBS and 1% FCS using a 16 gauge needle, dispersed and washed and seeded in a 50ml cell culture flask at a density of 2×10^6 cells/ml Iscove's minimal medium/20% horse serum (HS)/25 μ M 2-ME/2mM L-glutamine/10 μ M hydrocortisone. The medium from the culture was exchanged every week with 50% of new medium. The resulting monolayer of cells obtained after 6 weeks was used as adherent bone marrow stroma (BM-Str).

2.2.3.3 Magnetic Beads separation of HSC

Bone marrow cells (BMC) from mice were stepwise depleted of CD4⁺, CD8⁺ and NK cells, CD19⁺, CD45⁺, CD11c⁺ and CD11b⁺, Ter119⁺ and Ly6C/G⁺ cells by magnetic beads coated with the respective antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany). The depleted cell population was incubated with anti-CD117-coated beads collecting the adherent fraction. The CD117⁺ cells mostly were further enriched for HSC by incubation with biotinylated anti-SCA1 and anti-biotin coated beads, again collecting the adherent population.

2.2.3.4 Cryopreservation of cells

1×10^7 cells were harvested, washed once with warm fresh medium and resuspended in cryotubes at 5×10^6 cells/ml in ice-cold FCS/10% DMSO. Cells were kept overnight at -80°C and then transferred to liquid nitrogen.

2.2.3.5 Transfection in mammalian cells

6×10^5 cells were seeded on each well of a 6-well plate and were allowed to grow till they were 80% confluent on the next day in the Iscove's medium with 10% FCS. For each well of cells in the plate, 2 μ g of pDNA was diluted in 500 μ l of minimal medium and 10 μ l of LipofectamineTM was diluted in 500 μ l of minimal medium. The pDNA and LipofectamineTM

was mixed and incubated at room temperature for 15 min. The old media from the 6-well plate was removed and supplemented with new medium. The incubated mixture was slowly added to the cells and the plate was gently rocked before incubating the cells at 37°C. After 6h of transfection, the media with LipofectamineTM was removed and selection media was added. The supernatant from the transfected cells as well as the cells used as control for transfection was analyzed on western blot for the presence of recombinant protein.

2.2.3.6 Cell-matrix Adhesion assay

BMC, HSC and BM-Str from CD44wt, CD44v7^{-/-} and CD44v6/v7^{-/-} mice were seeded on BSA, HA, FN and Coll IV and in another setting HSC from CD44wt, CD44v7^{-/-} and CD44v6/v7^{-/-} mice were seeded on BSA, IL3, IL6, IL7, SDF1, OPN, bFGF, SCF, GMCSF. CD44wt BMC, HSC, BM-Str were pretreated with anti-panCD44, anti-CD44v6 and anti-CD44v7 if indicated. The experiments were performed on precoated 96 well plates. Cells were incubated for 4h at 37°C, washed vigorously and then the adherent cells were stained with crystal violet and the absorbance was measured at OD_{595nm} after lyzing with methanol and acetic acid in a fluorescence ELISA reader. The percentage of adherent cells taking the total input of cells as 100% is shown.

2.2.3.7 Stimulation of HSC

HSC were stimulated for 2h or 24h by CD44 cross-linking via plastic-coated HA or by seeding on BM-Str.

2.2.3.8 Migration assay

Migration assay was performed using Boyden chamber. The lower part of the Boyden chamber contained 30µl of RPMI medium with 0, 10 and 20% FCS, HA (50µg/ml), fibronectin (10µg/ml), collagen I and collagen IV (10µg/ml). Over this laid the 8-µm pore size polycarbonate membrane and the cells in question were counted and seeded on to the upper

chamber of the set up. The Boyden chamber was incubated for 16 hours at 37°C. The migration of the cells was evaluated after 16 hours by staining the lower membrane with crystal violet measured at OD_{595nm} after lysing with methanol and acetic acid. The assay was evaluated by the percentage cells that migrated into the lower chamber.

2.2.3.9 Video microscopy

BM-Str cells of CD44wt, CD44v7^{-/-} and CD44v6/v7^{-/-} were seeded on 24-well plates coated with or without HA for overnight to attain monolayer of cells. HSC cells were stained with CFSE and seeded on the top of the stroma layer. The cells were either blocked with IM7, anti-v6 or anti-v7 antibodies or without blocking incubated in a chamber at 37°C, 5% CO₂ for 24 hrs using a Carl Zeiss LSM780 microscope with an Hg/Xe lamp. Two pictures (20-fold magnification) per chamber (2-millisecond exposure) were taken every 20 minutes for 12 hours. Migration was quantified according to Manual tracking plugin (F.P. Cordelière, Centre de Recherche de l'Institute Curie) running in the open-source software ImageJ. Path length of 20 individual cells in each setting was calculated for every 20 minutes by customized programs. The mean pathway length per 20min is presented.

2.2.3.10 Immunofluorescence

3x10⁵ cells were grown on cover slips for 48hrs. Prior to staining, cells were fixed with 4% paraformaldehyde for 15min at RT, permeabilized with 0.2% tween in PBS-1%BSA, washed, blocked with 0.2% gelatin (freshwater goldfish) in PBS-1%BSA 15min and incubated with primary antibodies (2-10µg/ml, 60min, 4°C). Cover slides were rinsed and incubated with a fluorochrome-conjugated secondary antibody (60min, 4°C). After blocking, and incubation with a second, dye-labeled antibody (60min, 4°C), cover slides were washed and mounted in elvanol. Digitized images were generated using a Leica DMRBE microscope or a Carl Zeiss LSM710 confocal microscope and software Carl Zeiss Axioview Rel. 4.6.

2.2.3.11 Flow cytometry

Cells at 1×10^5 cells per well were taken in a 96-well U-bottom plate. The cells were washed once with ice-cold PBS (pH 7.4, supplemented with 1% FCS) and then stained with primary antibody (1-5 μ g/ml) for 30min at 4°C, washed twice with PBS (pH 7.4, supplemented with 1% FCS) and thereafter incubated with secondary, fluorochrome-conjugated antibody (0.3-0.5 μ g/ml) for 30min, 4°C. After 3 washes, samples were acquired and analyzed with the FACS Calibur (BD, Heidelberg, Germany). For probing internalized markers, cells were fixed in 1% formalin (20min, 4°C), washed and permeabilized with 0.2% tween in PBS (pH 7.4, supplemented with 1% FCS) before staining with the antibodies.

2.2.3.12 Apoptosis Assay

Apoptosis or programmed cell death in cells activated with or without cisplatin is evaluated by using Annexin-FITC/ PI double staining and flow cytometry. BMC and HSC cells were seeded on to BSA, HA or BM-Str in the presence of absence of 5 μ g of cisplatin for 24h. 1×10^5 cells of these conditions were seeded in 96 well plates, washed once with 1xPBS with 1% FCS, stained with Annexin-FITC/PI according to the manufacturer's conditions, incubated in dark for 15min and evaluated by FL-1 for annexin-FITC and FL-3channel for PI on flow cytometry.

2.2.3.13 Cell proliferation

CFSE-labeled BMC and HSC from CD44wt and CD44v6/v7^{-/-} mice were grown on BSA, HA, CD44wt BM-Str and CD44v6/v7^{-/-} BM-Str. Cell division was evaluated by flow cytometry after 24-72h. Cell cycle progression was evaluated by PI staining after incubation for 15min in the dark. The cells were pretreated with anti-panCD44, anti-CD44v6 and anti-CD44v7 if indicated.

2.2.3.14 Statistical analysis

All assays, which were statistically evaluated, were repeated at least 3 times. P-values <0.05 (two tailed Student's t-test and Anova) were considered significant.

3. Results

3.1 BM-Str as surrogate for the osteogenic niche

Hematopoiesis takes place in the bone marrow within the osteogenic niche. An in vitro surrogate is long-term bone marrow culture stroma (LTBMC) that contains endothelial cells, fibroblasts, adipocytes, macrophages and mesenchymal stem cells [173]. I used LTBMC stroma mostly for evaluating the crosstalk with freshly isolated HSC.

3.2. HSC CD44 in the crosstalk with the surrounding

3.2.1 Matrix proteins

Matrix proteins in adhesion

Bone marrow-matrix contains besides others HA, FN and coll IV, which are known to bind to CD44 [174]. Based on this, I studied the adhesion of BMC, HSC and BM-Str from CD44wt, CD44v7^{-/-} and CD44v6/7^{-/-} mice and evaluated the impact of CD44v on matrix adhesion. BMC from all three mice strains did not vary in binding to these matrix proteins. HSC from CD44wt showed strong affinity towards HA and HSC from CD44v6/7^{-/-} mice bound less to HA when compared to CD44wt and HSC from CD44v7^{-/-} showed less binding to HA (Fig.1A). BM-Str from CD44v6/7^{-/-} mice showed reduced binding to HA when compared to BM-Str from CD44wt and CD44v7^{-/-} mice.

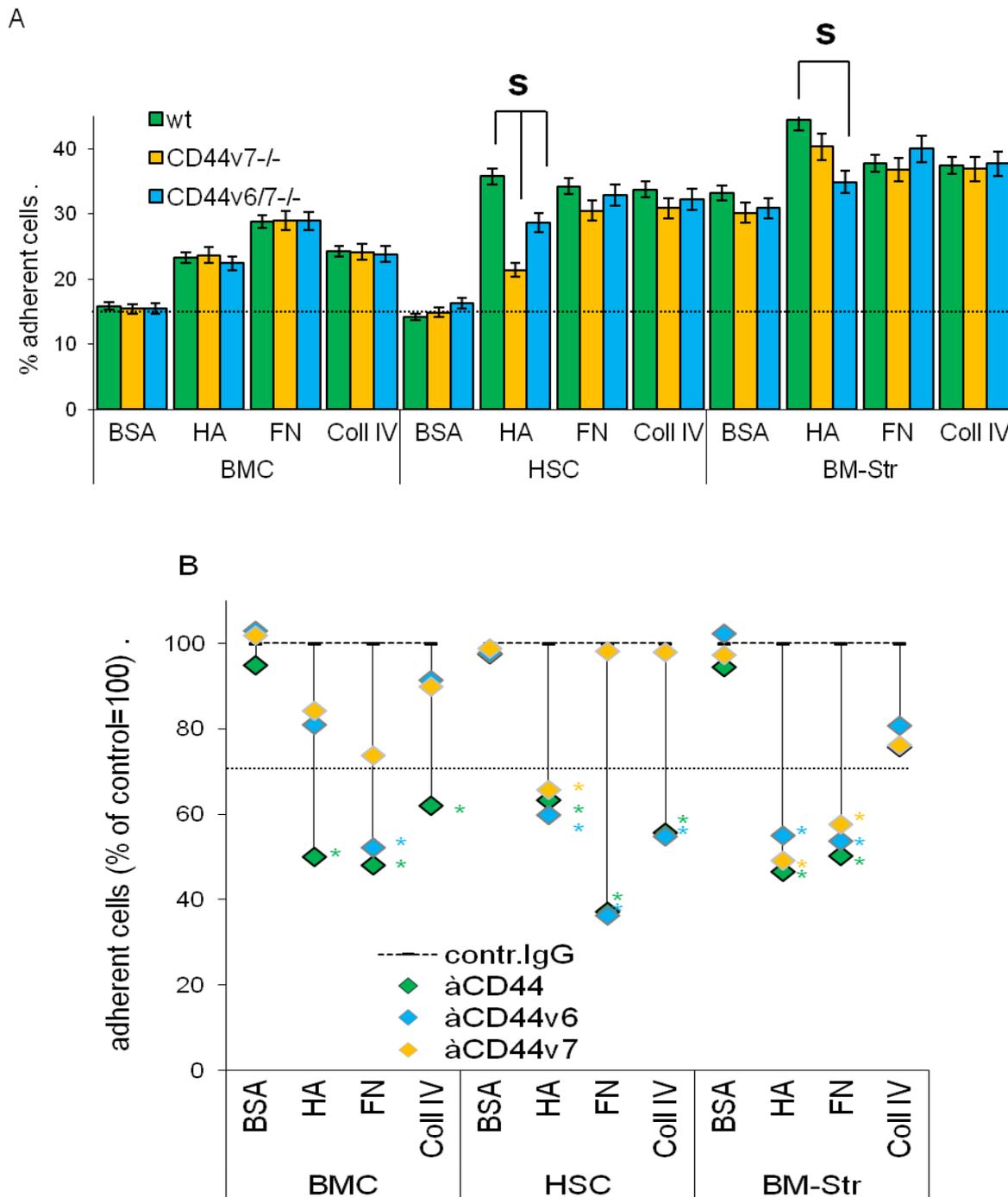


Figure 1: Impact of CD44 in the adhesion of BMC, HSC and BM-Str to matrix proteins: (A) The percentage adherent BMC, HSC and BM-Str from CD44wt, CD44v7^{-/-} and CD44v6/v7^{-/-} mice strains were evaluated by seeding them on BSA, HA, FN, and coll IV. The cells were allowed to adhere at 37°C for 4h. The cells were then washed and stained using crystal violet in methanol and the absorbance is read on ELISA reader at 595nm. Significant differences were observed depending on CD44v6 and/or CD44v7 expression are indicated by “S”. (B) Antibody blocking studies using control IgG, anti-panCD44, anti-CD44v6 and anti-CD44v7 to check the involvement of CD44 variants in the adhesion of BMC, HSC, BM-Str to matrix proteins. Percentage adhesion (mean of triplicates) in comparison to control IgG (=100%). Significant differences between anti-CD44s, anti-CD44v6 and anti-CD44v7 are

indicated by '*'. CD44 contributes to HA, FN and less pronounced, coll IV adhesion in HSC and CD44v6 is primarily engaged in HA adhesion.

Variations observed in the binding of HSC and BM-Str from CD44v7^{-/-} and CD44v6/7^{-/-} mice were due to absence of CD44 variant exons v6 and/or v7. The contribution of individual variants of CD44v6 and CD44v7 in BMC, HSC and BM-Str adhesion to matrix proteins was found by blocking the cells with anti-panCD44, anti-CD44v6 and anti-CD44v7 and observing the reduction in adhesion to matrix proteins. It was observed that CD44s contributes to BMC adhesion to matrix proteins with low efficiency of blocking CD44v6 and CD44v7. The use of anti-CD44v6 and anti-CD44v7 on HSC blocked the binding of HSC to HA while anti-CD44v6 blocked HSC adhesion to FN and coll IV. Similarly with anti-CD44v6 and anti-CD44v7 the adhesion of BM-Str to HA and FN (Fig.1B) was blocked thus showing the contribution of CD44v6 and CD44v7, though to a minor degree, on HSC and BM-Str to matrix adhesion. If the variations observed in binding of cells to matrix proteins are due to the variant exons of v6 and v7 then the adhesion profile of rCD44v6 and rCD44v7 to matrix proteins must be similar.

Recombinant proteins of CD44v6 and bind differentially to matrix proteins when compared to CD44s.

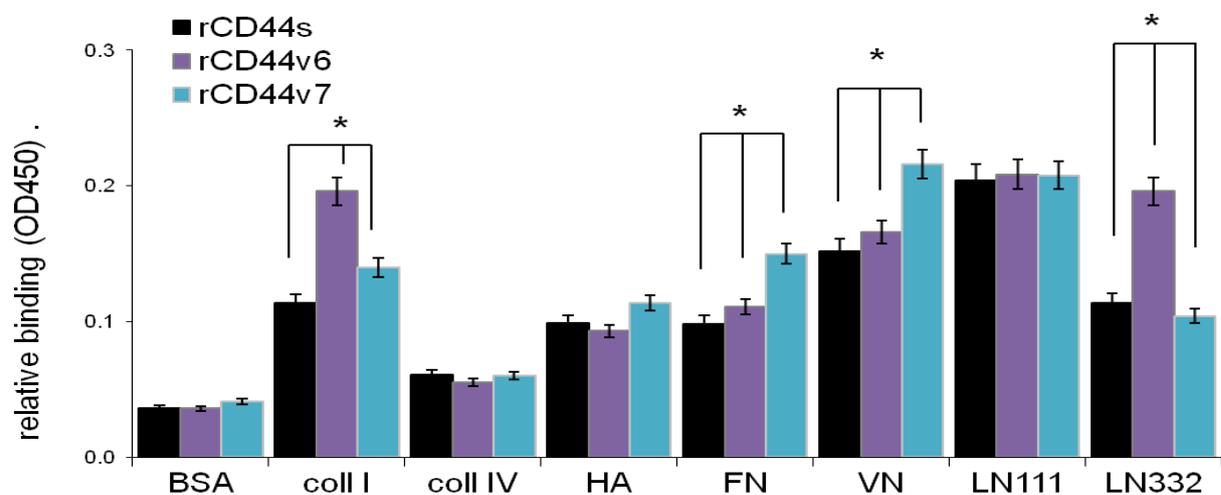
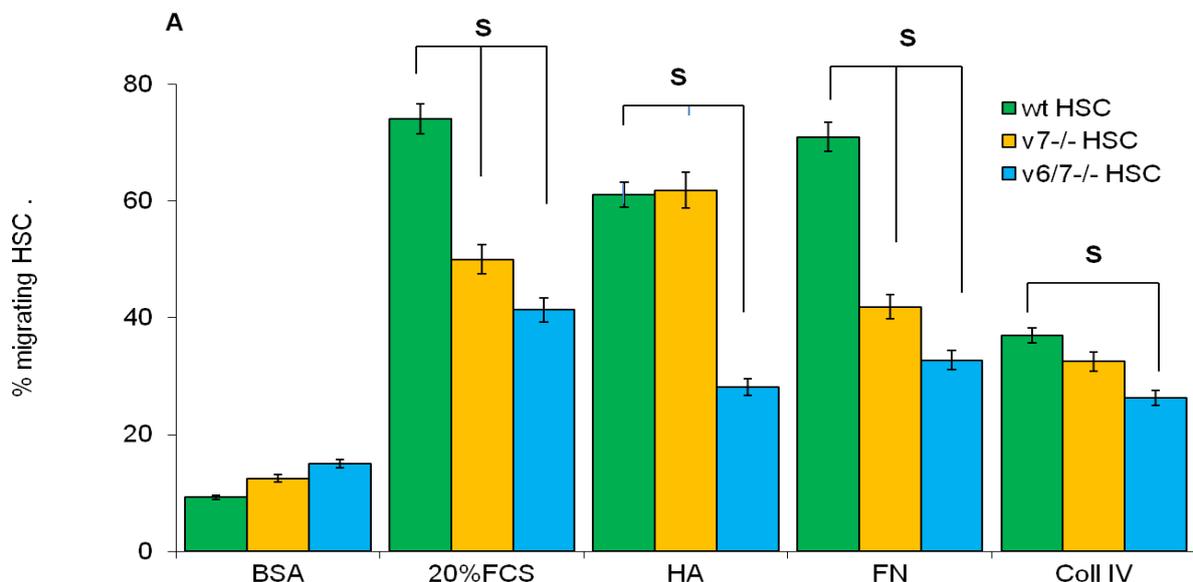


Figure 2: Binding of recombinant CD44s, CD44v6 and CD44v7 to matrix proteins: The binding of rCD44s, rCD44v6 and rCD44v7 to matrix proteins was evaluated by ELISA. The relative binding was evaluated by optical density with mean values of triplicates are shown. Significant differences between CD44s, CD44v6 and CD44v7 are indicated by ‘*’.

Here, rCD44v6 showed pronounced binding to coll I and LN332 and rCD44v7 showed stronger binding to FN and VN (Fig.2).

Matrix proteins in migration

Data from adhesion experiments indicated that HA-CD44 interaction was promoting HSC adhesion towards HSC migration [175]. CD44wt, CD44v7^{-/-} and CD44v6/7^{-/-} HSC migration was performed in Boyden chamber with matrix proteins like HA, FN and coll IV as stimulus. The migration of HSC from CD44v6/7^{-/-} mice towards HA was strongly reduced, when compared to CD44wt and CD44v7^{-/-} HSC, indicating that CD44v6 contributes to HSC migration towards HA. Migration towards FN was lesser in CD44v7^{-/-} and CD44v6/7^{-/-} HSC. CD44v6 and CD44v7 supported CD44wt HSC in migration to FN. The deletion of variant exon v7 did not influence the HSC migration towards coll IV (Fig.3A).



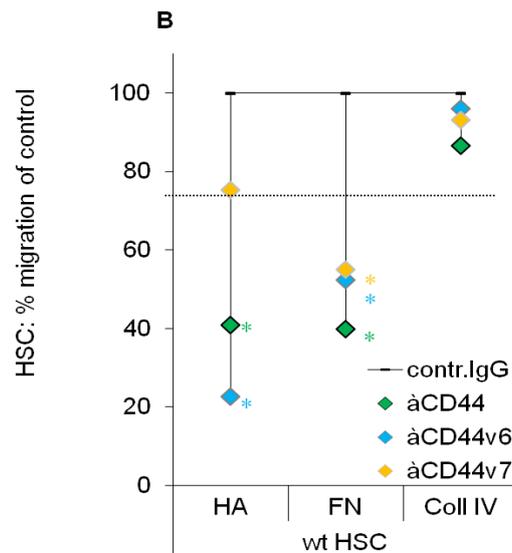


Figure 3: Migration of HSC towards matrix proteins: CD44wt, CD44v7^{-/-} and CD44v6/7^{-/-} HSC were seeded onto the top of the Boyden chamber which contains (A) HA, FN, coll IV and 20% FCS as stimuli at the bottom of the assembly. After 6h of migration, the cells at the bottom chamber are counted. The mean percentage +/- SD (triplicates) of migrating HSC with significant differences in the migration of cells between CD44wt, CD44v7^{-/-} and CD44v6/7^{-/-} HSC are indicated by ‘S’. (B) Antibody inhibition of migrating CD44wt HSC, towards these stimuli, against CD44s, v6 and v7 with IgG (=100%) as control indicates the importance of the variant isoforms of CD44v6 and v7 for migration. Significant antibody inhibition is denoted by ‘*’.

Migration of CD44wt HSC in the presence of anti-CD44s, CD44v6 and CD44v7 (Fig.3B) confirmed the contribution of CD44v6 in the migration of HSC towards HA and the support of CD44v6 and CD44v7 in the migration of HSC to FN.

HSC respond to cellular cues and undergo directional migration [176] of homing in repopulating the bone marrow (in bone marrow transplant to sustain hematopoiesis) and also mobilize themselves in response to stress signals in tissue injury. Boyden chamber migration assay measures chemotaxis in vitro but this approach cannot measure individual cell migration parameters [177]. In order to monitor the cells under real time, time lapse video microscopy was employed to monitor the HSC movement on HA under physiological conditions of CO₂ supply and temperature. HSC movement was tracked by capturing pictures at regular intervals and individual cell migration was calculated (data not shown) by the average displacement using ImageJ software [178].

CFSE labeled CD44wt and CD44v6/7^{-/-} HSC were seeded on surface coated with HA, with and without anti-panCD44, IM7 and observed for migration.

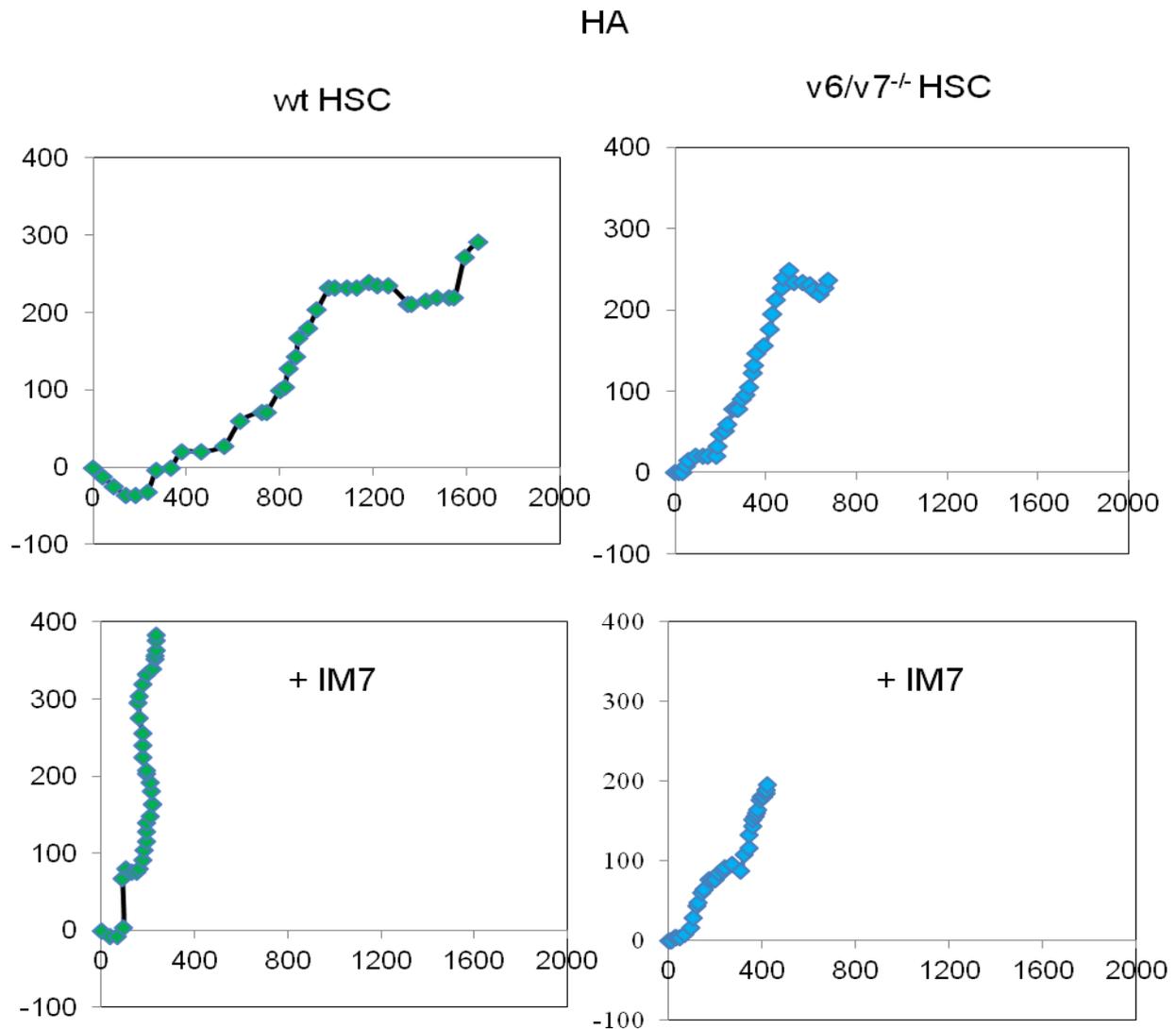
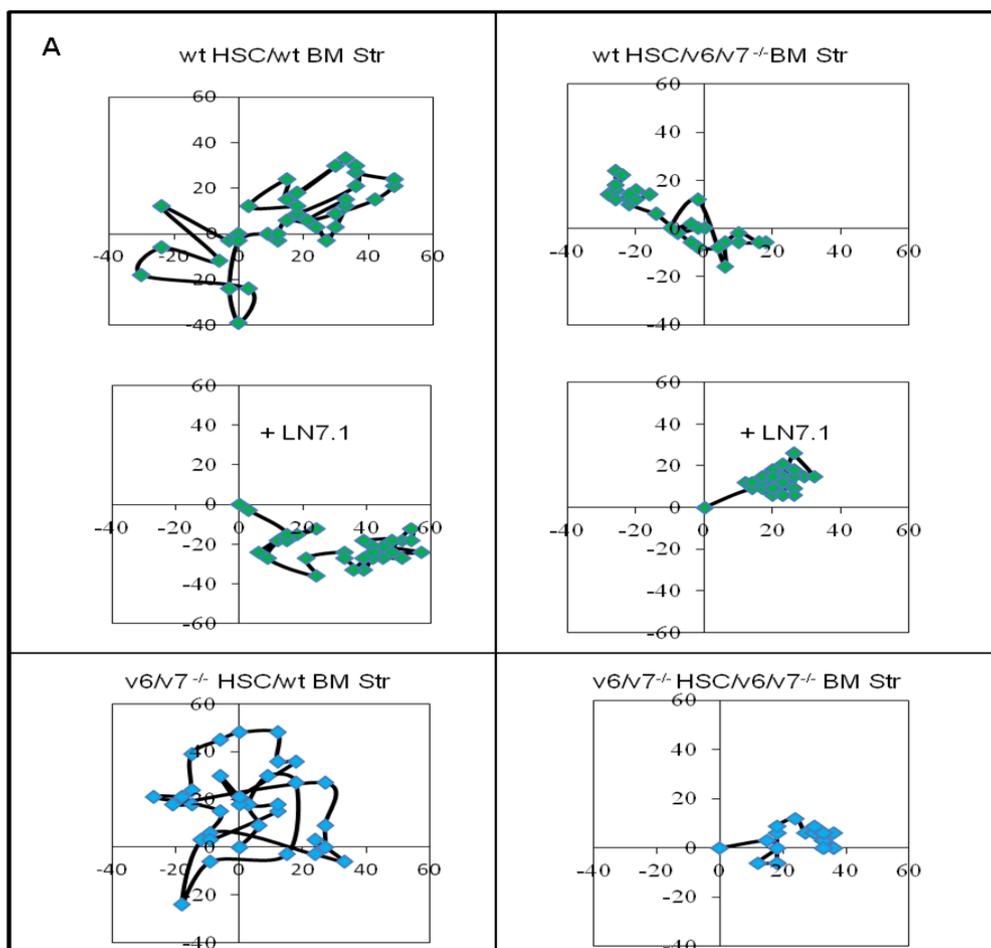


Figure 4: CD44 in the migration of HSC on HA: CFSE labeled CD44wt and CD44v6/7^{-/-} HSC were seeded on HA-coated plates. The real-time migration of HSC on HA with and without IM7 was monitored using video microscope under physiological conditions. CD44wt HSC migration on HA was reduced by IM7. Migration of CD44v6/7^{-/-} HSC was reduced when compared to uninhibited CD44wt HSC migration. The migration of CD44v6/7^{-/-} HSC was further retarded on antibody inhibition by IM7. The retarded movement of CD44v6/v7^{-/-} HSC on HA was due to the combined effect of antibody inhibition and absence of CD44v6/v7. Migration of HSC on HA was influenced by CD44v6

CD44wt HSC migrates freely on HA whereas CD44v6/v7^{-/-} HSC shows poor movement. The poor movement of CD44v6/v7^{-/-} HSC indicates that CD44v6 is important for HSC migration on HA (Fig.4).

CD44 in HSC migration on BM-Str

CD44 on HSC interacts with HA and firm up their adhesion for engraftment into osteogenic niche. The variant exon products of CD44v6 and CD44v7 have different affinities to HA attachment and hence to the BM-Str. Having tested the HSC migration on HA, I was interested in testing the HSC migration on BM-Str. Towards this, and to determine the contribution of variant(s) on HSC in their migration on BM-Str, a monolayer of BM-Str coated onto plates which mimic osteogenic niche inside the bone and over them CFSE labeled CD44wt and CD44v6/7^{-/-} HSC were seeded and were observed under the video microscope to track the real-time movements of HSC on the stroma. Antibody- LN7.1, against the variant CD44v7 was used as a part of the setup to inhibit the movement of HSC on BM-Str.



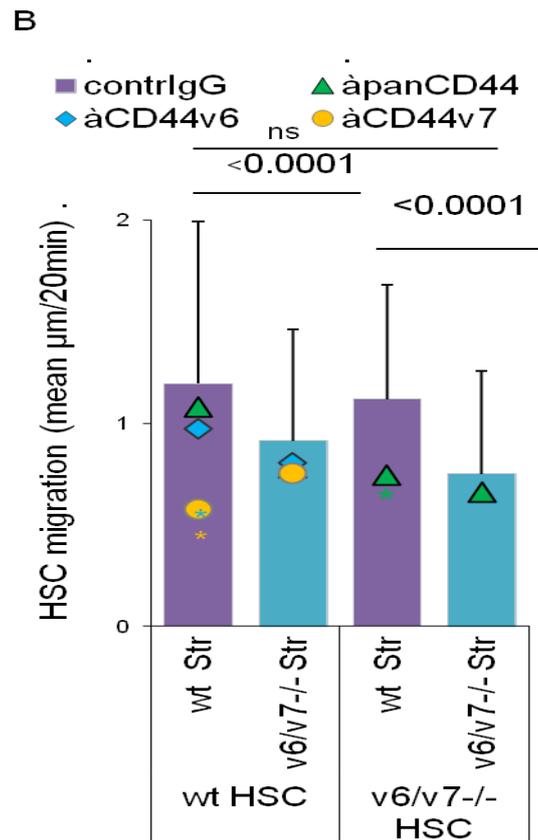


Figure 5: Contribution of CD44 to migration of HSC on BM-Str: (A) CFSE-labeled CD44wt HSC were seeded on CD44wt BM-Str and CD44v6/v7^{-/-} BM-Str with and without anti-CD44v7 (LN7.1) incubation. In another setup CFSE-labeled CD44v6/v7^{-/-} HSC were seeded on CD44wt BM-Str and CD44v6/v7^{-/-} BM-Str. The figure shows the trajectory of the cells in the videomicroscopy setup and the retarded movement on CD44v6/v7^{-/-} BM-Str that was more pronounced with the antibody inhibition and also the movement of CD44v6/v7^{-/-} HSC on CD44v6/v7^{-/-} BM-Str. (B) CFSE-labeled cells from CD44wt and CD44v6/v7^{-/-} HSC were seeded on CD44wt and CD44v6/v7^{-/-} BM-Str in the presence of antibodies against CD44, v6 and v7 and the mean migration (in μm) of 20 individual cells/20 min are shown. Significant differences between CD44wt and CD44v6/v7^{-/-} HSC and BM-Str are indicated with significant inhibition by antibodies by ‘*’. Migration of HSC on BM-Str is influenced by CD44v7 on both HSC and BM-Str.

Figure 5A shows the representative trajectories of HSC under different incubation conditions. Since the incubation conditions do not contain any chemoattractant gradient, I did not expect any directional motion of the incubated HSC. CD44wt HSC explored the local environment on CD44wt BM-Str and displayed various coordinates of movements. The blocking antibody, LN7.1 efficiently curbed HSC movements on BM-Str. CD44wt HSC moved on CD44v6/v7^{-/-} BM-Str but did not acquire relaxed motion due to deletion of CD44v6/v7 on BM-Str. The movement was further reduced to a limited area with antibody blocking of CD44v7 on HSC.

The movement of HSC from CD44v6/7^{-/-} mice was not restricted on CD44v7 expressing BM-Str. The combined unavailability of CD44v7 on both HSC and BM-Str diminished the motion of HSC indicating the contribution of CD44v7 in the migration of HSC on BM-Str.

The mean migration of CD44wt HSC on CD44v6/7^{-/-} BM-Str was lesser (Fig.5B) when compared to the migration on CD44wt BM-Str. The same observation was found with the migration of CD44v6/7^{-/-} HSC on CD44v6/7^{-/-} BM-Str.

3.2.2 CD44 associating molecules and cellular ligands on HSC and BM-Str

Variations in adhesion of HSC and BM-Str obtained from CD44wt, CD44v7^{-/-} and CD44v6/v7^{-/-} mice to matrix proteins may be due to the CD44 variant exons v6 and v7. But similar experiment with rCD44v6 and rCD44v7 adhesion on matrix proteins has shown otherwise. It was hypothesized that the variations observed could be due to the different associating molecules on CD44v6 and CD44v7. Hence I proceeded towards finding the associating molecules of CD44s, CD44v6 and CD44v7.

CD44 associating molecules

The associating molecules of CD44s, CD44v6 and CD44v7 were found by immunoprecipitation of lysates of HSC and BM-Str using anti-panCD44, anti-CD44v6 and anti-CD44v7. The stringency of washes after immunoprecipitation removed the chances of false positives in the results. The resulting precipitates were resolved on SDS-PAGE gels and the co-immunoprecipitating molecules were analyzed using mass spectroscopy. Proteins that are abundant with at least 2 unique peptides are taken for further analysis. The prominent partners are listed in Tables 1 and 2. The major molecules associated with HSC and BM-Str were similar and belong to glycolytic pathway/metabolism, proteins against oxidative damage and to alkylating agents, cytoskeleton-related proteins, peptidases and endoplasmic reticulum stress response proteins. Molecules such as 14-3-3 gamma, phosphogluconate dehydrogenase,

annexin A2, annexin III, ATP synthase, caspase3, moesin are found associated with both HSC CD44v6 and CD44v7 and annexin A5, calreticulin, complement C1q, galectin-3, gelsolin, radixin, thrombospondin, ras GTPase-activating-like protein IQGAP1 are found associated with HSC CD44v7. On BM-Str, annexin A2, calreticulin, cathepsin B, cathepsin D, cofilin-1 are found associated with CD44v6 and annexin A5, CD16, CD63, radixin, are found associated with both CD44v6 and CD44v7. The results of the mass spectrometry analysis are tabulated below.

Table 1: CD44, CD44v6 and CD44v7 associating molecules on HSC: HSC lysates of 1mg was precipitated with anti-panCD44, anti-CD44v6 and anti-CD44v7 and the immunoprecipitates were resolved on SDS-PAGE gels, coomassie blue stained, destained and analyzed by orbitrap mass spectrometry which gives the raw data searched on NCBI database with taxonomy mouse and mammals using the MASCOT search engine. Only protein hits identified with at least 2 unique peptides are listed in the table.

Protein Description	HSC - coIP					
	IP: panCD44		IP: CD44v6		IP: CD44v7	
	Match	Cov.[%]	Match	Cov.[%]	Match	Cov.[%]
14-3-3 protein gamma			2	7.3	9	18.8
6-phosphogluconate dehydrogenase			2	4.6	6	8.3
78 kDa glucose-regulated protein	5	11	8	13.1	11	12.2
actin, beta	59	67.3	17	47.6	11	16.6
actin, gamma	62	67.1	16	48.4	33	43.2
actin-related protein 2/3 complex subunit 3					4	7.3
adenylyl cyclase-associated protein 1					3	3.6
aldehyde dehydrogenase family 3 member A1	19	22.9	2	4.4	13	11.8
alpha-2-macroglobulin-P	9	2.3	3	1.1	20	63.9
alpha-actinin-1	8	5.8				
alpha-fetoprotein	9	6.2	19	4.5	2	6.2
annexin A1	8	17.1	17	31.5	35	57.2
annexin A2			3	9.1	6	18.3
annexin A5					11	21.9
annexin III			5	9	9	16.1
ATP synthase subunit beta			2	2.4	7	8.7
band 3 anion transport protein	6	4.1			3	2.3
beta-arrestin-1					2	4.1
beta-globin	35	76.9	27	87.8	32	83.7
bone marrow proteoglycan	3	8.5	11	24.2	4	13.9
calcium-binding and spermatid-specific protein 1			6	25.4	11	25.4
calreticulin					4	9.4
carbonic anhydrase 2	6	23.5	7	11.9	11	30.4
casein kinase II subunit alpha'			2	8.4		
caspase 3			2	5.1	5	14.2

cathepsin D					3	6.8
cathepsin G	11	26.1	46	50.2	21	33.3
CD11b	8	5.5	9	5.3	6	2.7
CD16	10	11.4			5	16.9
CD177	8	6			10	8.4
CD44	13	8			4	7.4
chitinase-like protein 3	12	24.6	6	13.3		
coactosin-like protein					4	17.6
complement C1q					4	7.7
complement C3	5	1.6			10	2.7
complement C4	3	1.8			5	3.7
copine-3	2	1.6			3	3.4
cyclophilinA	13	41.5	5	48.2	9	28.7
delta-aminolevulinic acid dehydratase	2	4.9	5	9.1	13	26.7
dihydrolipoamide S-acetyltransferase			2	5.9		
DJ-1	1	5.9	2	6.5	1	3.1
endoplasmin	13	9.1			7	6.5
eosinophil peroxidase	13	18.9	65	41.6	34	35.5
ezrin	10	8.7	9	8.5	34	20.3
ferritin	3	9.9	14	52.5	14	43.7
fibrinogen			2	1.4		
filamin-A1	11	4.1	11	3.3	15	5
fructose-bisphosphate aldolase A2					2	6.9
Galectin-3					3	6.4
GAPDH	10	28.5	8	33	5	11.7
gelatinase	4	3.8			3	1.6
gelsolin					5	3.3
glucose-6-phosphate 1-dehydrogenase X	6	7.8	2	3.5	9	16.3
hemoglobin alpha	31	76.8	15	43	4	15.6
hemoglobin beta-2	16	43	25	76.8	27	66.9
HSP70	8	13.9	18	16.7		
HSP84	31	18.6	7	5.4		
HSP90	24	16.9	7	5.9	23	16.4
Continued						
	HSC - coIP					
	IP: panCD44		IP: CD44v6		IP: CD44v7	
Protein Description	Match	Cov.[%]	Match	Cov.[%]	Match	Cov.[%]
hypoxanthine-guanine phosphoribosyltransferase					3	10.6
inosine-5'-monophosphate dehydrogenase 2			7	14.8		
lactotransferrin	3	4	31	31.6	80	60.4
leukotriene A-4 hydrolase	2	1.6			5	5.4
L-lactate dehydrogenase	3	4.5	4	14.8	6	11.7
lysozyme C-1 precursor			6	16.9	5	16.9
lysozyme C-2 precursor			8	35.8	11	39.9
M2-type pyruvate kinase	24	31.5	14	23	10	11.7
moesin			13	13.3	48	33.4
myeloperoxidase	30	20.3	83	49.3	53	36.2
myosin heavy chain			5	3.6	9	2.4

nascent polypeptide-associated complex alpha			1	0.6	2	0.6
neutrophil cytosol factor 4					2	2.9
neutrophil elastase	8	11.7	10	35.5	8	13.2
neutrophil gelatinase-associated lipocalin			8	25.5	7	25.5
nucleoside diphosphate kinase A			6	43.4	7	29.6
peptidoglycan recognition protein 1			5	32.4	2	6.6
peroxiredoxin 1	10	31.8	11	31.8	15	33.3
phosphoprotein associated with GEM	1	3.3	1	3.3	3	7.3
profilin-1	12	38.6	9	25.7	14	50.7
protease, serine, 1	9	12.2	16	12.2	12	12.2
protein broad-minded					2	0.6
protein CREG1					2	5
protein disulfide-isomerase A6					3	6.5
rab-10	5	16.5			5	16.5
rab7					6	15
rac	6	14.1	3	9.9	7	21.4
radixin					29	17.2
ras GTPase-activating-like protein IQGAP1					2	0.6
rho GDP-dissociation inhibitor 2					6	30.5
S100-A8	2	23.6	1	22.5	3	22.5
S100-A9	12	31	12	23.9	22	23.9
S-adenosyl-L-homocysteine hydrolase			2	2.3		
serotransferrin	2	6.1	2	6.1	12	15.8
succinate dehydrogenase			4	8.5		
talin	3	0.8	2	0.4	9	2.1
T-complex protein 1 subunit beta			4	7.3	3	3.8
thrombospondin-1					3	1.5
transaldolase					9	13.1
tropomyosin-1					3	7.4
tubulin	16	22.5	2	2.4	12	20.5
tyrosine-protein kinase CSK			5	8.4		
voltage-dependent anion-selective channel 3.2			4	19.4		

Table 2: CD44, CD44v6 and CD44v7 associating molecules on BM-Str: BM-Str lysates of 1mg was precipitated with anti-panCD44, anti-CD44v6 and anti-CD44v7 and the immunoprecipitates were resolved on SDS-PAGE gels, coomassie blue stained, destained and analyzed by orbitrap mass spectrometry which gives the raw data searched on NCBI database with taxonomy mouse and mammals using the MASCOT search engine. Only protein hits identified with at least 2 unique peptides are listed in the table.

Protein Description	BM-Str - coIP					
	panCD44		CD44v6		CD44v7	
	Match	Cov.[%]	Match	Cov.[%]	Match.	Cov.[%]
78 kDa glucose-regulated protein			16	18.2		
actin, beta	20	41	16	47.3		
actin, gamma	33	43.2	20	48.1		
alcohol dehydrogenase 1			3	5.5		
alpha-2-macroglobulin-P	7	1.8			4	1
alpha-fetoprotein	9	10.8	4	6.5	5	6.5
alpha-N-acetylgalactosaminidase			2	2.7		

annexin A1	8	17.1	6	21.1	2	8.7
annexin A2			4	8.8		
annexin A5			14	17.6	2	3.3
apolipoprotein E			5	15.4	4	18.8
aquaporin-1			2	3.3		
ATP synthase subunit beta			6	7.6		
beta-globin	10	49	8	29.9	3	44.2
bone marrow proteoglycan	2	5.4	1	5.3	2	8.5
calcium-binding and spermatid-specific protein 1			7	25.4		
calreticulin			5	8.2		
cathepsin B			4	11.2		
cathepsin D			2	6.8		
cathepsin S	11	14.6	12	23.5	11	24.1
cathepsin Z			10	15		
CD101					3	0.9
CD11b	8	5.5				
CD16			3	6.9	16	53.8
CD177	8	6				
CD18					6	8.4
CD44	13	8			2	2.6
CD63			2	8.8	2	8.8
CD64					2	3.3
cofilin-1			4	40.4		
complement C1q			2	11	4	15
complement C3	2	0.5			7	2
complement C4	2	1			6	4.4
cyclophilinA			17	23.6	3	7.3
cytoplasmic dynein 1	5	0.3			4	0.3
dual specificity protein kinase TTK			21	27.1	4	7.1
endoplasmic	13	9.1				
erythrocyte band 7 integral membrane protein	3	8.5	9	29.6	6	22.9
ezrin	10	8.7	28	15.7	2	1.5
fatty acid-binding protein, epidermal			4	21.5	2	6.7
ferritin	7	23.1	19	44.8	16	53.8
Galectin-3	10	20	10	13.6	4	6.4
GAPDH	10	28.5	2	4.2		
gelatinase	4	3.8				
glucosylceramidase			7	5.4		
glycosylation-dependent cell adhesion molecule 1			4	4.7		
glypican-6					2	1.1
granulins	6	12.2	11	16.6	6	7.1
hemoglobin alpha	2	6.3	5	16.9	4	15.6
heparanase precursor			4	5.8		
HSP70	2	1.9	5	5.6		
HSP84	31	18.6				
HSP90	24	16.9				
junction plakoglobin			2	3.5		
LAMP-1			8	9.6		

L-lactate dehydrogenase	3	8.4	6	21.1		
lysozyme C-1 precursor			8	16.9	6	16.9
lysozyme C-2 precursor			15	39.9	7	35.8
M2-type pyruvate kinase	24	31.5	2	2.1		
macrophage-capping protein			6	11.1		

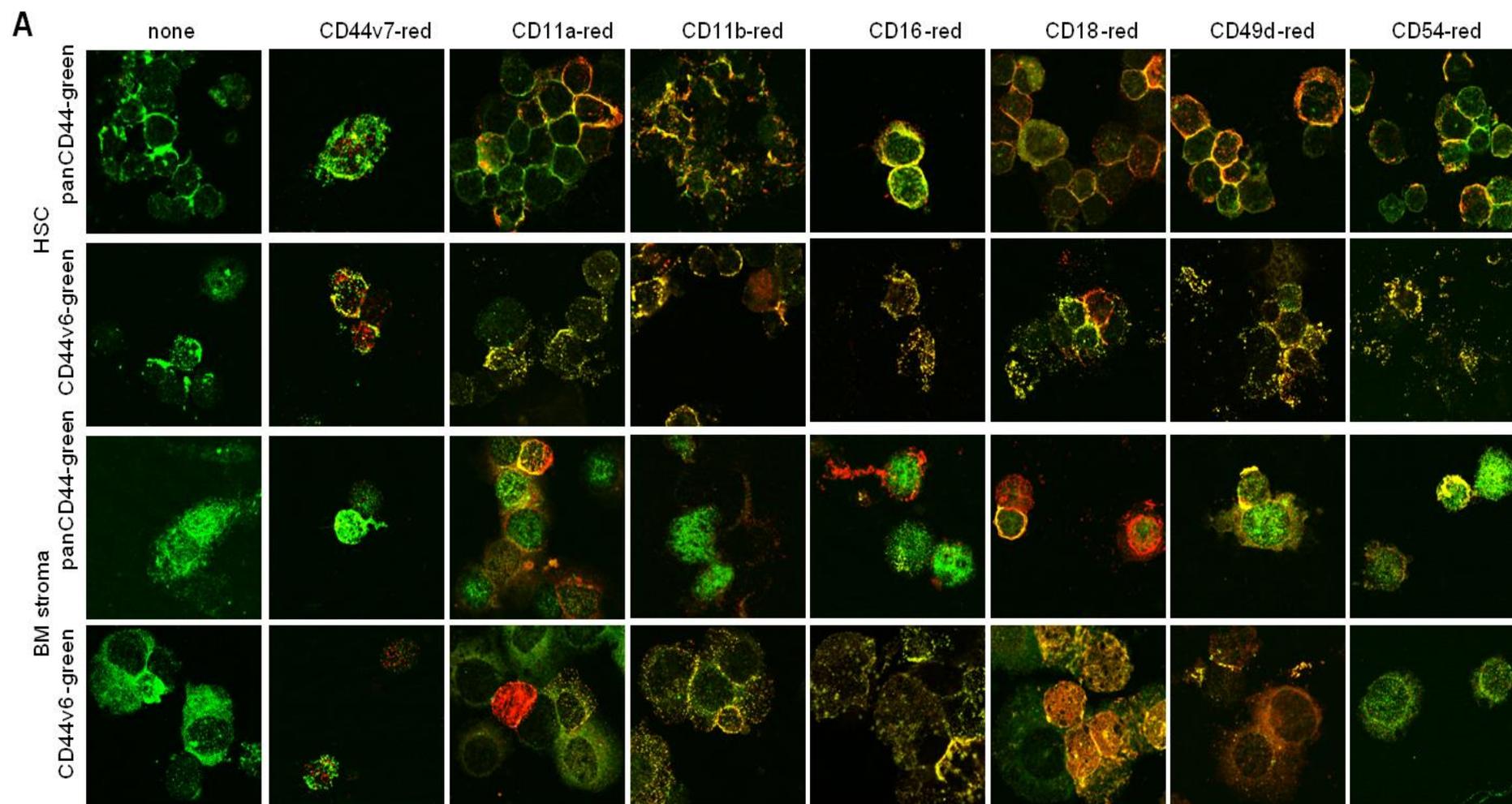
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Protein Description	BM-Str - coIP					
	panCD44		CD44v6		CD44v7	
	Match	Cov.[%]	Match	Cov.[%]	Match.	Cov.[%]
macrosialin			3	5.5		
major vault protein			3	4.8		
multidrug resistance-associated protein 5					2	3.7
nitric oxide-inducible gene protein			5	1.1		
peroxiredoxin-1	10	31.8	31	55.3	13	31.2
phosphatase 2A	2	2.9	3	6.4	2	2.5
phosphoglycerate mutase 1			2	4.3		
phospholipase D3	6	6.4	6	9.4	2	2.5
profilin-1	12	38.5	6	24.3		
protease, serine, 1	9	12.2	18	16.3	13	12.2
protein broad-minded					2	0.6
protein CREG1			2	5	2	5
protein disulfide-isomerase A3			6	8.9	2	2.5
rab-14			2	12.1		
rab-1B			6	25.9		
rab7	5	16.5	7	28		
radixin			30	17.5	6	4.1
ras-related protein Rab-5C			2	11.3		
rho GDP-dissociation inhibitor 2			5	22.1		
serotransferrin	2	6.1	9	13.8	12	13.5
serpin B6	3	6.9	6	13	4	7.1
spectrin	3	3.6			2	0.7
talin	3	0.8				
titin	5	0.1			4	0.1
tubulin	16	22.5	5	12.4		

Usual approach in mass spectrometry-based identification of proteins is to employ bottom-up strategy where the proteins are enzymatically digested and to make into smaller peptides which were then separated by liquid chromatography. But glycosylated peptides are difficult to cleave into peptides to give the sequence information [179]. Alternatively, I used confocal microscopy to obtain complementary information that were missed in the mass spectrometry

results. Some of the known proteins that were already known in the literature for interacting with CD44 have been probed on confocal microscopy.

Confocal microscopy shows the co-localization of CD44s and CD44v6 with the adhesion molecules CD11a, CD11b, CD16, CD18, CD49d and CD54 on HSC as well as on BM-Str. (Fig.6A, B). The association of CD44s and CD44v6 with actin, ezrin, moesin, tubulin, talin and HSP70 was found in both HSC and BM-Str (Fig.7B) with talin and HSP70 co-localized with BM-Str (Fig. 7A). In HSC, CD44v6 associated less with cathepsin G, PGK1 and SOD2 and more with PLC γ , and RhoGD1. In BM-Str, CD44v6 associates with cathepsin G, PGK1, PP1A, PolyADP and 14-3-3 (Fig.8).



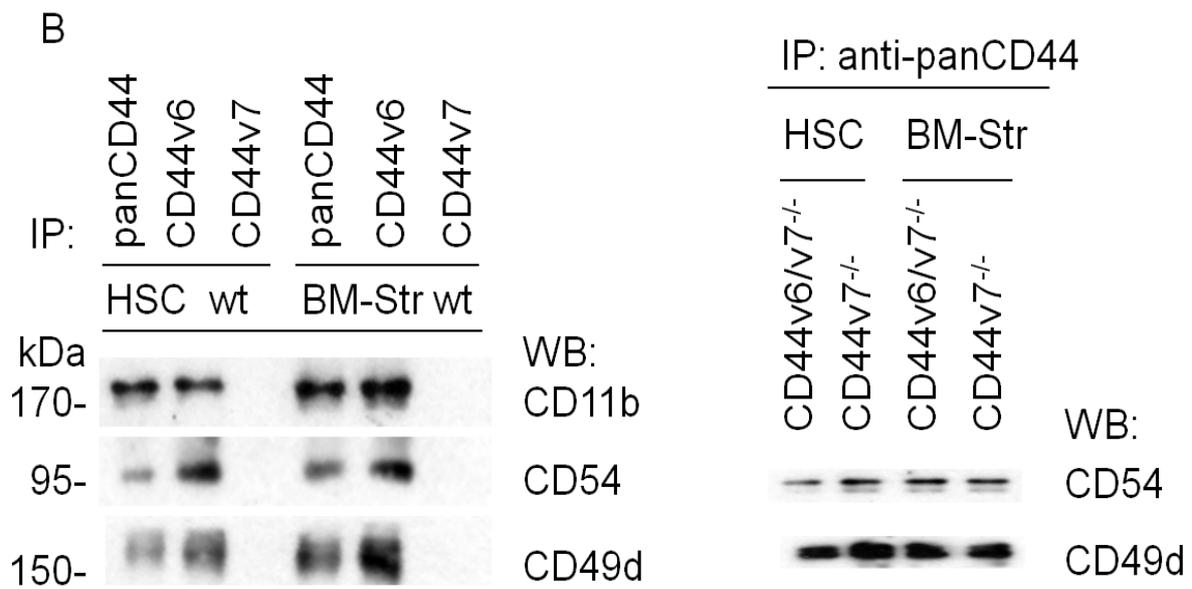
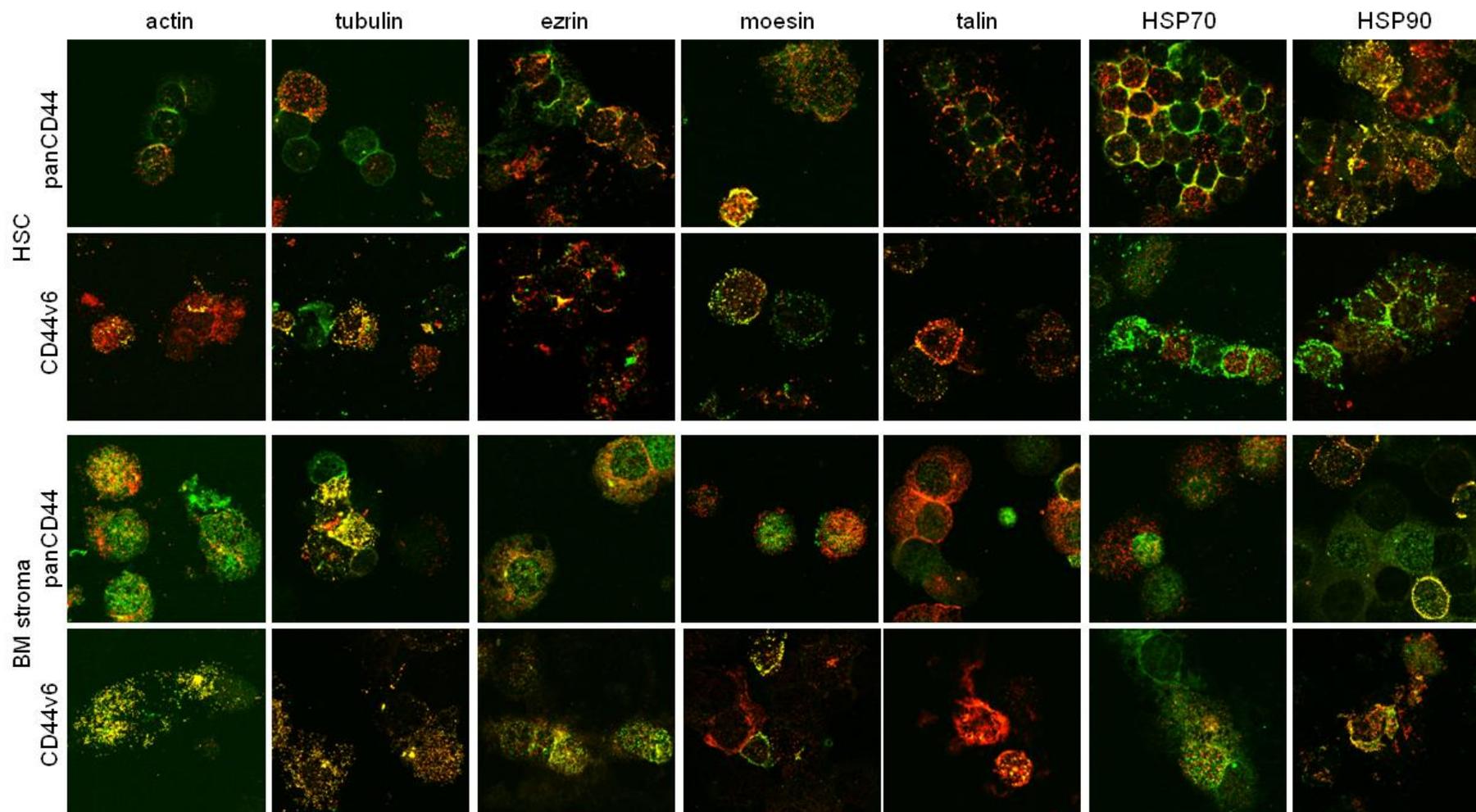


Figure 6: CD44 associating adhesion molecules on HSC and BM-Str: (A) The HSC and BM-Str of CD44wt mice are permeabilized and stained first with anti-panCD44 (green)/anti-CD44v6 (green), and then stained with anti-CD44v7 (red), anti-CD11a (red), anti-CD11b (red), anti-CD16 (red), anti-CD18 (red), anti-CD49d (red) and anti-CD54 (red) and then with secondary dye labeled antibody. Overlay of confocal microscopy are shown (scale bar: 10 μ m) with co-localized molecules shows are bright yellow color. (B) Lysates of HSC and BM-Str of CD44wt mice are immunoprecipitated with anti-panCD44, anti-CD44v6 and anti-CD44v7 and blotted with anti-CD11b, anti-CD49d and anti-CD54 and lysates of HSC and BM-Str of CD44v6/v7^{-/-} and CD44v7^{-/-} mice are immunoprecipitated with anti-panCD44 and blotted with anti-CD54 and anti-CD49d.

A



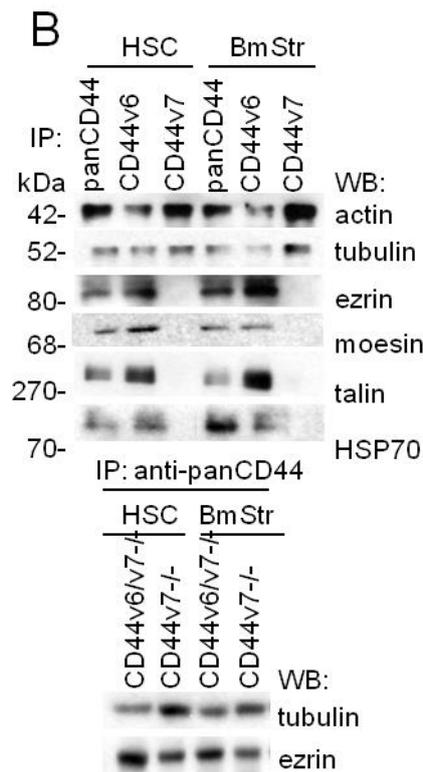


Figure 7: Co-localization of CD44 with cytoskeleton molecules on HSC and BM-Str: (A) The HSC and BM-Str of CD44wt mice are permeabilized and stained first with anti-panCD44 (green)/anti-CD44v6 (green), and then stained with anti-actin (red), anti-tubulin (red), anti-ezrin (red), anti-moesin (red), anti-talin (red), anti-HSP70 (red) and anti-HSP90 (red) and then with secondary dye labeled antibody. Overlay of confocal microscopy are shown (scale bar: 10 μ m) with co-localized molecules shows are bright yellow color. (B) Lysates of HSC and BM-Str of CD44wt mice are immunoprecipitated with anti-panCD44, anti-CD44v6 and anti-CD44v7 and lysates of HSC and BM-Str from CD44v7^{-/-} and CD44v6/v7^{-/-} are precipitated with anti-panCD44 and are resolved on SDS-PAGE gels, transferred to a nitrocellulose membrane, blotted with anti-actin, anti-tubulin, anti-ezrin, anti-moesin and anti-HSP70.

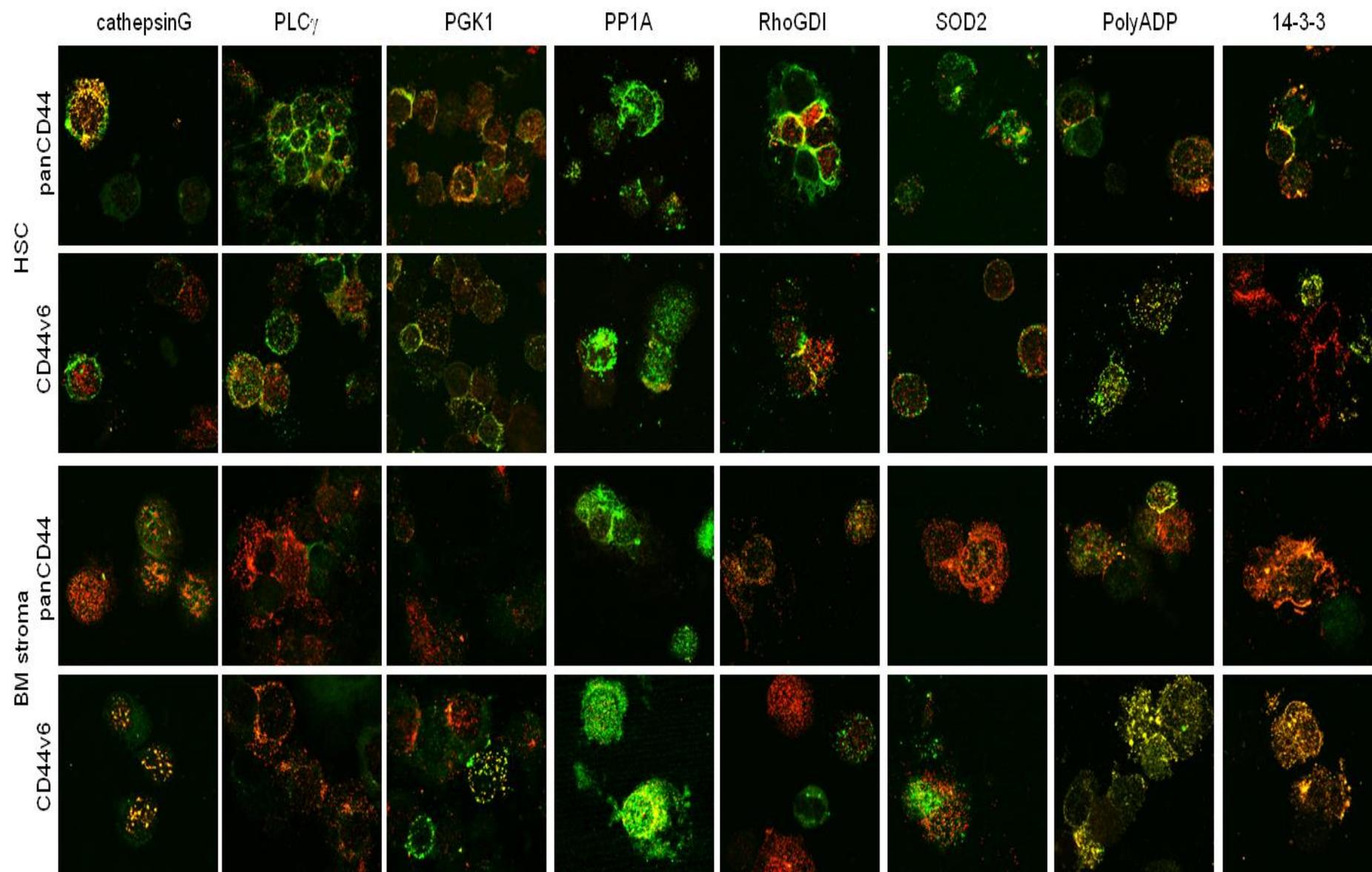


Figure 8: Signal transducing molecules in HSC and BM-Str. The HSC and BM-Str of CD44wt mice are permeabilized and stained first with anti-panCD44 (green)/anti-CD44v6 (green), and then stained with anti-cathepsinG (red), anti-PLCg (red), anti-PGK1 (red), anti-PP1A (red), anti-RhoGD1 (red), anti-SOD2 (red), anti-PolyADP (red), anti-14-3-3 (red) and then with secondary dye labeled antibody. Overlay of confocal microscopy are shown (scale bar: 10 μ m) with co-localized molecules shows are bright yellow color.

Cellular ligands

CD44 interacts with ECM proteins and growth factors to prepare a complex between CD44-ligand and receptor tyrosine kinase and initiate signal transduction [180]. The signaling pathways that are initiated decide the fate of the HSC in the osteogenic niche such as cell migration, homing and mobilization/extravasation. The hypothesis was that, the variable regions of CD44v6 and CD44v7 might have specific ligands. In order to find the CD44v6 and CD44v7 cellular ligands, HSC and BM-Str membrane proteins were allowed to bind to recombinant proteins of CD44s, CD44v6 and CD44v7 coupled to CNBr-sepharose resins. The bound resins were washed extensively to remove non-specific binding, eluted and analyzed the eluates by mass spectrometry. A detailed list of proteins that were obtained from the mass spectrometry analysis (that have at least 2 unique peptides) is given in the table below.

Table 3: Pulldown assay of HSC proteins on recombinant CD44s, CD44v6 and CD44v7. This table gives the detailed list of pulled down proteins that were obtained from passing the membrane proteins of HSC from CD44wt mice over CNBr-coupled CD44s, CD44v6 and CD44v7 columns. The columns were washed with phosphate buffer, pH7.2, eluted with glycine buffer, pH2.7, neutralized to pH8.0 and resolved on 10% SDS-PAGE gels, stained with coomassie blue and then destained. The individual proteins bands thus obtained were analyzed using mass spectrometry. Only protein hits identified with at least 2 unique peptides are listed in the table.

CD44 binding proteins

Protein Description	wt HSC pulldown					
	rCD44s		rCD44v6		rCD44v7	
	Match.	Cov.[%]	Match.	Cov.[%]	Match.	Cov.[%]
78 kDa glucose-regulated protein			9	12.4	7	8.1
actin, gamma	36	35.9	18	26.1	18	31

Results

AFP	101	8.1	19	6.5	27	9.6
ALDH	2	4.6	3	12.4	4	6.4
alpha-2-macroglobulin			2	1.1		
alpha-N-acetyl galactosaminidase			7	6.7		
ATP synthase alpha			2	1.8		
beta-galactosidase	2	1.4	4	5.1		
beta-globin	8	29.9	8	30.1	37	72.2
bone marrow proteoglycan	4	8.5	5	61.5	2	5.4
calcium binding and spermatid-specific protein 1	7	24.6	4	18.7	3	14.2
calreticulin precursor					3	4.6
carbonic anhydrase 2	23	46.5	5	7.3	23	48.1
carbonic anhydrase I	21	26.8	6	10.7	21	35.6
cathepsin B			11	20.4	6	12.1
cathepsin G	40	37.9	33	37.9	9	22.6
CD11b	3	1.9	9	5.3		
CD44	9	5.2	2	2.6	2	2.6
charged multivesicular body protein 4b	6	15.6	5	15.6	4	15.6
cofilin-2			4	19.7		
complement C1Q	4	11.8	37	41.4	7	17.2
complement C3			5	13.6		
cyclophilin			6	19.5	5	19.5
eosinophil cationic protein 2	4	13.5	6	25.6	3	13.5
ezrin	3	3.2	2	3.1		
Ferritin L subunit 1	15	39.9	14	31.1	2	11.5
flavin reductase			2	8.3	4	8.3
fructose-bisphosphate aldolase A2	4	7.4	3	5.8	6	14.8
Galectin-3	4	13.3	5	11.4	2	9.1
GAPDH	18	32.1	12	30.9	15	30.9
gelsolin			2	3.3	3	4.4
glutathione peroxidase 1			4	14.6	4	14.6
haemoglobin	54	79.5	19	71.2	46	83.6
haemoglobin beta-2 chain	32	53.6	16	59.6	32	76.8
HSP2			15	13.3	12	10.5
HSP70	17	16.4	12	12.7	38	27.9
HSP90	29	19.1	10	9.4	9	7.6
LAMP-1	2	2.7	7	8.4	5	8.4
L-lactate dehydrogenase A chain isoform 1	9	19.3	5	9.9	5	11.7
lysosomal membrane glycoprotein B			2	7.8		
lysozyme C-1					2	12.2
M2-type pyruvate kinase	13	19.4	10	13.9	14	21.1
myeloperoxidase	42	23.3	33	19.4	41	26.5
myosin II-A	25	10.2	3	1.6	9	3.2
neutrophil elastase	5	11.7	4	11.7	9	16.6
nitric oxide-inducible gene protein					3	1.1
nucleoside diphosphate kinase A			2	2.5		
peroxiredoxin-1			7	20.1	7	20.1
polyubiquitin C			2	12.7	2	19.8

protease, serine, 1	14	8.1	5	8.1	9	12.2
protein SET					3	11.4
radixin			3	2.9	5	4.6
serpin B6			2	2.6	4	4.2
superoxide dismutase			2	7.8	2	7.8
testin			3	6.5		
tubulin, alpha	14	25.3	2	5.3	6	12

Table 4: Pulldown assay of BM-Str proteins on recombinant CD44s, CD44v6 and CD44v7: This table gives the detailed list of proteins that were obtained from passing the membrane proteins of BM-Str from CD44wt mice over CNBr-coupled CD44s, CD44v6 and CD44v7 columns. The columns were washed with phosphate buffer, pH7.2, eluted with glycine buffer, pH2.7, neutralized to pH8.0 and resolved on SDS-PAGE gels, stained with coomassie blue and then destained. The individual proteins bands thus obtained were analyzed using mass spectrometry. Only protein hits identified with at least 2 unique peptides are listed in the table.

Protein Description	wt BMStr pulldown					
	rCD44s		rCD44v6		rCD44v7	
	Match	Cov.[%]	Match	Cov.[%]	Match	Cov.[%]
78 kDa glucose-regulated protein			3	4	3	4
actin gamma	36	35.9	21	25.5	29	40.8
adenine nucleotide translocase-1			6	7.1	187	22.9
AFP	2	3.1	8	3.1	7	6.5
ALDH	14	19.1	4	7.3	6	10.6
alpha-2-macroglobulin			6	1.6	4	1.1
annexin A1			3	4	8	9
annexin A2	18	23.3	26	38.6	27	35.4
beta2-microglobulin			3	14.3	2	9.8
beta-globin			8	42.9		
calcium-binding and spermatid-specific protein 1	3	14.2	2	2.4	14	25.4
cathepsin G preproprotein	5	11.1	9	16.5	7	11.1
CD44	9	5.2	4	4.5	3	4.5
chaperonin			18	25.2	6	8.5
cofilin-1			3	15.1	2	6.6
cold shock domain protein A-b			24	49.7		
collagen I					5	5.8
complement C3			5	2.2		
cyclophilin			9	33.2	11	28.8
dual specificity protein kinase TTK			3	3.2	5	8
E3 ubiquitin-protein ligase NEDD4			5	27.2	2	9.7
eukaryotic translation initiation factor 3 subunit H	3	7.1	11	25.8	9	20
ezrin	2	2.9	6	4.6	3	3.2
Ferritin light chain 1	3	11.5	9	32.8	4	11.5
fibronectin	45	13.2	14	4.6	30	10.3
filamin-A1			25	12.3		
flotillin-2	10	19	5	9	4	5.1

Results

fructose-bisphosphate aldolase A2			2	3.8		
galectin-3	18	29.2	15	29.2	8	15.9
GAPDH	18	32.1	25	30.9	21	33
haemoglobin alpha	32	53.6	11	23.2	9	23.2
HSP70	17	16.4	21	25.5	7	10.4
HSP90	6	3.6	25	30.9	13	13.3
inosine-5'-monophosphate dehydrogenase 2			11	16.3	11	14.8
lactadherin			17	19.2	11	14.3
LAMP-1			10	9.6	3	5.4
L-lactate dehydrogenase A1	9	19.3	8	14.5	8	14.2
lysozyme C-1			8	14.5	3	12.2
M2 pyruvate kinase	13	19.4	19	25.4	11	20.2
major vault protein			3	3	7	11.4
MHC I Heavy Chain			4	4.5	4	7.6
microtubule-associated protein 4	15	12	32	23.7	22	18
moesin	33	6.2	22	21.3	30	24.6
myeloperoxidase					11	9.6
myosin	25	10.2	37	12.7	2	9.5
myristoylated alanine-rich C-kinase substrate	7	11.3	5	12.5	8	13.3
nascent polypeptide-associated complex ab			6	19.5	3	13.5
nucleoside diphosphate kinase A			2	12.7	1	5.4
peroxiredoxin-1			37	12.7	10	35.7
phosphoglycerate kinase 1	20	8	11	15.3	11	16.5
plectin 1			4	11.6	10	1.6
profilin-1			4	11		
prohibitin			10	27		
proliferation-associated protein 2G4			6	11.8	10	17.6
protease, serine, 1		5	8.1	6	24.3	7
protein kinase C delta-binding protein			5	13.8	2	3.8
protein TFG			6	12.9	4	6
proteinase-activated receptor 2			6	7.6	9	16.1
rab 4			18	24.9		
rab-10			5	16.5		
rab-1B			13	43.8		
S100-A11			5	6.6	4	13.2
serum deprivation-response protein			7	10.3	3	8.1
t complex polypeptide 1			13	25.5	6	7.9
T-complex protein 1 subunit gamma			13	18.8	20	14
T-complex protein 1 theta			18	22.3	11	13.7
triosephosphate isomerase			5	16.5		
tropomodulin-3			2	3.1	14	5
tropomyosin					17	4.6
tubulin alpha-1C	16	16.7	5	10	7	12
vimentin					24	34.8
zinc finger C2HC domain-containing protein 1A	5	9.9	38	59	6	9.9

Cathepsin B, cyclophilin A, peroxiredoxin-1, polyubiquitin C, radixin, superoxide dismutase and complement C3 are found pulled down with HSC CD44v6 and CD44v7. ATP synthase alpha, nucleoside diphosphate kinase A and alpha-2-macroglobulin are found only with HSC CD44v6 (Table 3). 78 kDa glucose-regulated protein, alpha-2-macroglobulin, annexin A1, beta2-microglobulin, cheperonin, cofilin-1, cyclophilin, MHC I, nucleoside diphosphate kinase A and peroxiredoxin-1 are found pulled down with BM-Str CD44v6 and CD44v7. Beta globulin, complement C3, filamin-A1, fructose bisphosphate aldolase A2, profilin-1, prohibitin, rab4 and rab10 are found uniquely pulled down with CD44v6 (Table 4). The repeated observation of cytosolic proteins is due to their abundance in the isolation of membrane proteins that are difficult to remove which is a tradeoff between membrane protein recovery and abundance in undesired cytosolic proteins.

3.2.3 Cytokines that may influence motility, dormancy and differentiation

Cytokines within the bone marrow are available to HSC for different actions of motility to and from the osteogenic niche, maintenance of dormancy within the niche and differentiation into progenitor and specialized cells. Among them, SDF1 [181], G-CSF [182] and GM-CSF [183] are observed to induce motility; SCF [184, 185] is responsible for maintenance of dormancy in the cells and OPN is a negative regulator of HSC proliferation [82] and IL-3 [186], IL-6 [187-189], IL-7 [190] for the stem cells to differentiate into progenitor cells.

The signals from growth factors and cytokines are sensed and transduced by CD44 to the interior of the cells and stimulate the cells to undergo cell division, motility and differentiation. To understand the relevance of each of CD44s and variant forms of CD44v6 and CD44v7 on HSC in the crosstalk with cytokines, I performed functional assays that are relevant for HSC motility, dormancy and differentiation. Adhesion of HSC CD44wt, CD44v7^{-/-} and CD44v6/v7^{-/-} on cytokines showed that they had different abilities in binding to the various molecules of cytokines and chemokines with HSC from CD44v7^{-/-} and

CD44v6/v7^{-/-} bound less efficiently to SDF1, OPN and SCF as well (Fig. 9A). They bound equally to the inflammatory cytokine IL6 and to bFGF and GMCSF.

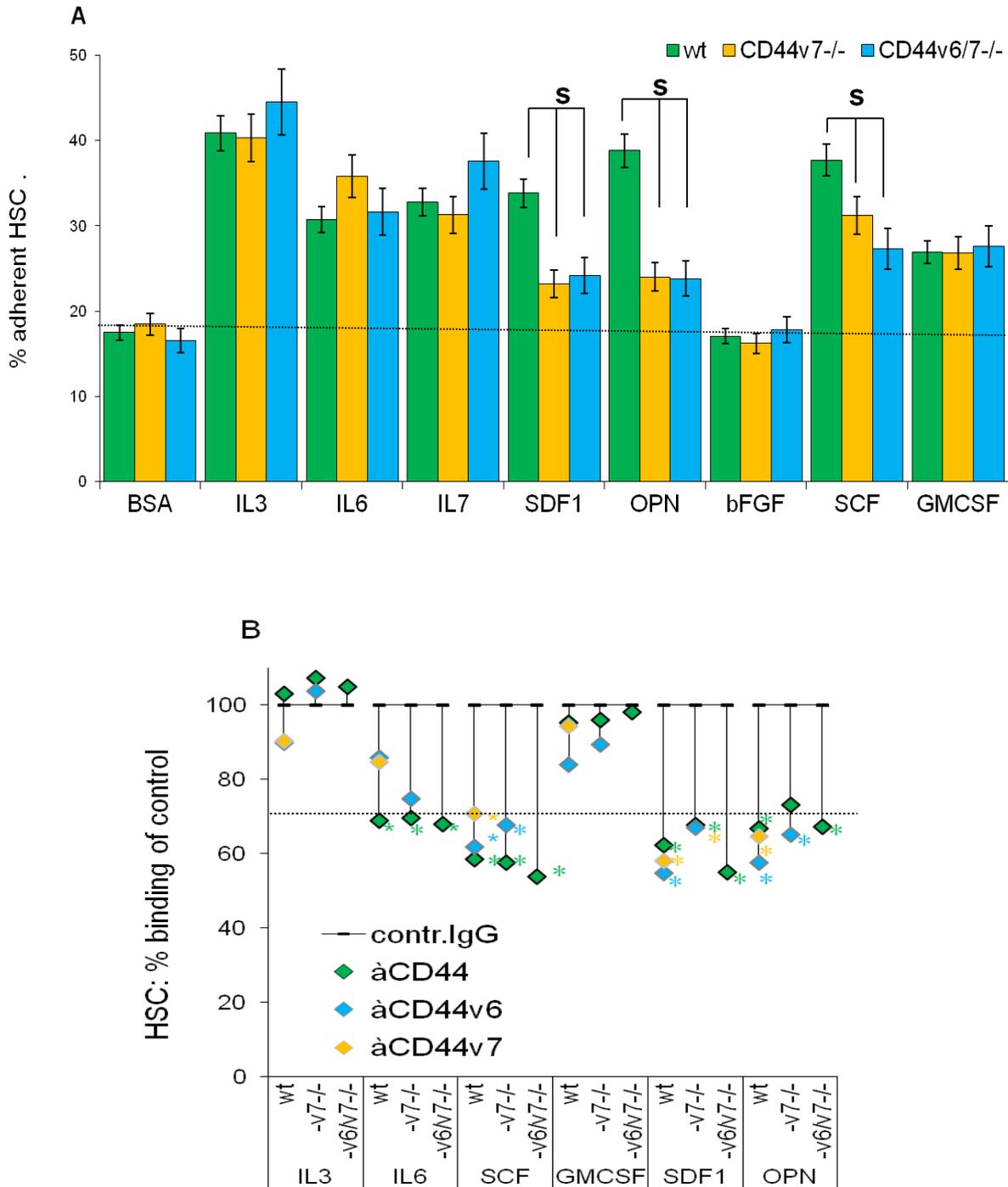


Figure 9: Adhesion of HSC to various cytokines and chemokines: (A) The evaluation of HSC from CD44wt, v7^{-/-} and v6/v7^{-/-} mice adhering to cytokines and chemokines are shown with mean percentage of adherent HSC +/- SD (triplicates). The significant drop in adhesion among the knockout cells are indicated by 'S'. (B) Antibody blocking experiments to evaluate

the contribution of variants towards HSC binding to cytokines/chemokines in the presence of control IgG (=100%), anti-panCD44, anti-CD44v6 and anti-CD44v7. Significant differences in binding between CD44s, CD44v6 and CD44v7 are indicated by '*'. CD44v6 and CD44v7 contribute to SCF, OPN and SDF1 binding.

The blocking of CD44v6 and CD44v7 by anti-CD44v6 and anti-CD44v7 affected the binding of HSC to SDF1 and OPN (Fig.9B) with strong decrease in the number of adherent cells whereas the adherence of HSC to SCF decreased on blocking with anti-CD44v7.

CD44 binds equally, without any additional contribution of CD44v6 and CD44v7, to IL3, IL6, IL7 and GMCSF. CD44v6 and CD44v7 are important for the binding to SDF1, OPN and SCF.

Migration assay of HSC with IL6, GMCSF, SCF, bFGF, SDF1, OPN and BSA (as negative control) as stimulus indicated that migration of HSC to GMCSF, bFGF and SCF is unaffected and is independent of the CD44 variant forms (Fig.10A). Migration of HSC from CD44v7^{-/-} to IL6 was unaffected but the migration was significantly reduced when HSC from CD44v6/7^{-/-} was seeded over IL6. This indicated a contribution of CD44v6 to migration of HSC towards IL6. CD44v6 and CD44v7 contributes to migration of HSC to OPN and SDF1 which was evident from the reduction in the number of HSC from CD44v7^{-/-} and CD44v6/7^{-/-} migrating towards them (Fig.10A).

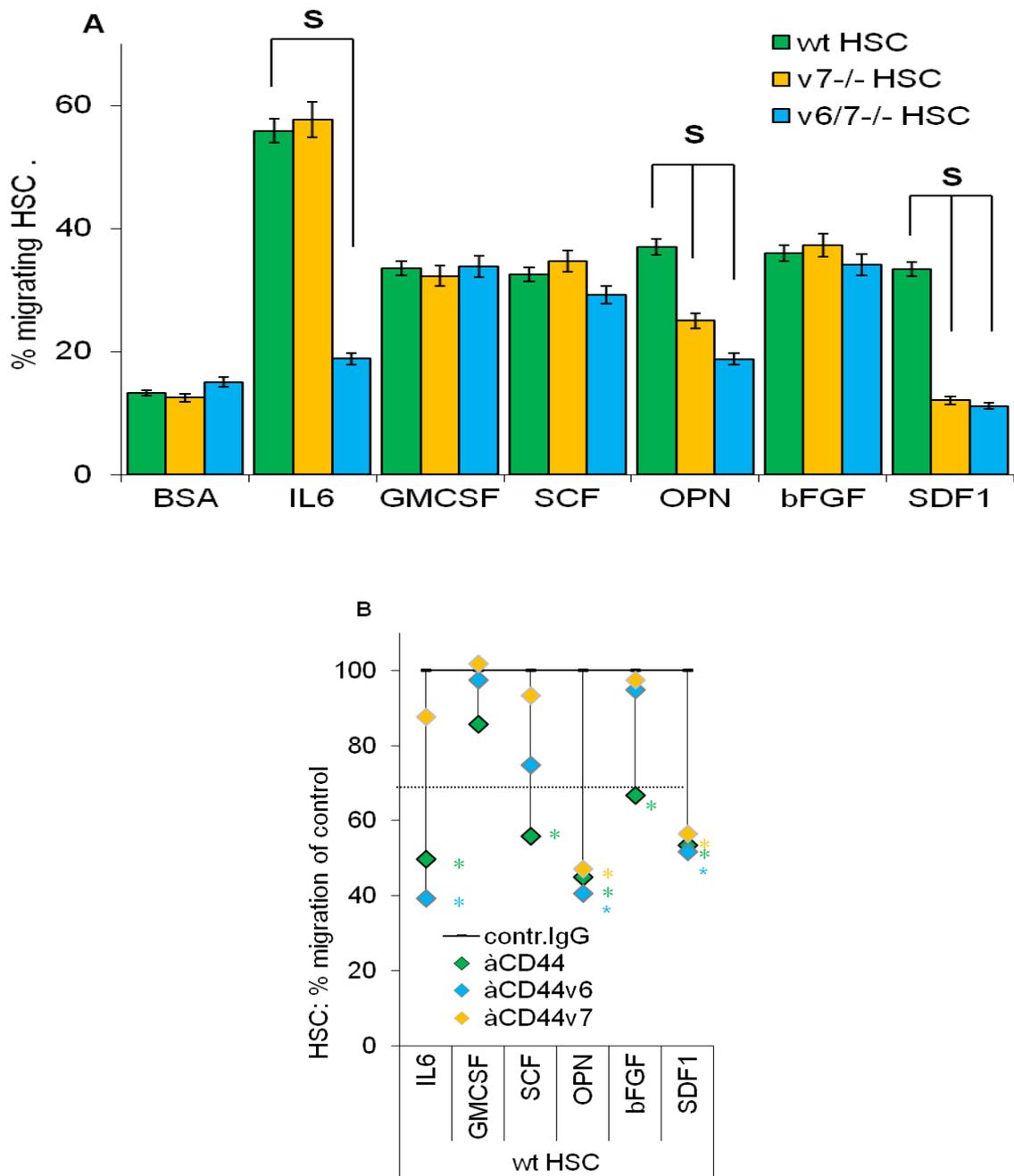


Figure 10: Migration of HSC towards cytokines and growth factors: CD44wt, CD44v7^{-/-} and CD44v6/7^{-/-} HSC were seeded onto the top of the Boyden chamber which contains (A), (B) IL6, GMCSF, SCF, OPN, bFGF, SDF1 as stimulus are at the bottom of the assembly. After 6h of migration, the cells at the bottom chamber are counted. The mean percentage +/- SD (triplicates) of migrating HSC with significant differences in the migration of cells between CD44wt, CD44v7^{-/-} and CD44v6/7^{-/-} HSC are indicated by ‘S’. (B) Antibody inhibition of migrating HSC against CD44s, v6 and v7 and with IgG (=100%) as control indicates the importance of the variant isoforms of CD44v6 and v7 for migration. Significant antibody inhibition on cells is denoted by ‘*’.

Antibody inhibition of CD44 variants (Fig.10B) also indicated that there was no participation of CD44v6 and CD44v7 in the migration of HSC towards GM-CSF and SCF. Migration was significantly reduced towards IL6, SDF1 and OPN when CD44v6 was blocked.

3.3 CD44 and maintenance of HSC quiescence

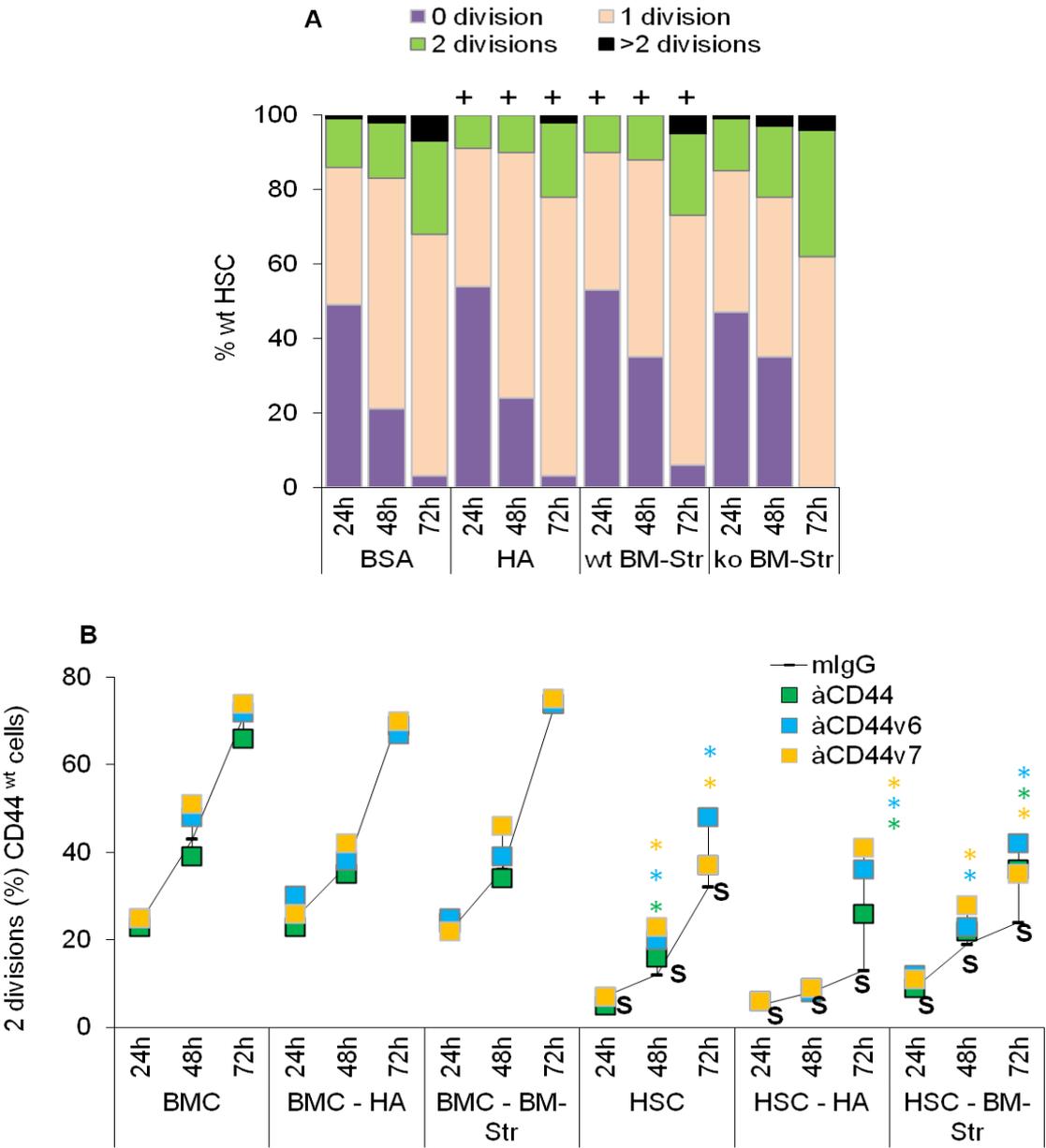
HSC mostly remain quiescent to maintain the supply of mature blood cells and to prevent HSC exhaustion during the lifetime of an individual. They maintain themselves mostly in the G0 phase of the cell cycle [191] with occasional cell division to replace aging cells. Also, critical are the mutations associated with replication, stem cells need to be quiescent [192]. HSC quiescence supported by the interactions between BM-Str and CD44 and/or its variants CD44v6 and CD44v7 will help in understanding and treatment of hematological disorders.

The interactions of HSC with osteogenic niche across cell-cell, cell-ECM and receptor-ligand maintain the quiescence [193]. HSC quiescence is positively coordinated by extrinsic regulators such as BM-Str of osteogenic niche [194], secreted transforming growth factor-beta (TGF- β) [195, 196] and Wnt signaling pathway [197]. These factors in the regulation of HSC quiescence in the context of CD44 and CD44v6/v7 are presented in the following.

BMC and HSC were seeded on BSA, HA or BM-Str and the contribution of CD44 towards proliferation of BMC and HSC in the presence or absence of anti-panCD44, anti-CD44v6 and anti-CD44v7 were evaluated for a period of 72h. Cell division was evaluated by CFSE dilution.

HSC divided less frequently (Fig.11A) when compared to bulk BMC, where even after 48h only 10% of HSC, but more than 40% of BMC have divided. The HSC cells were more quiescent when they were seeded on HA and on CD44wt BM-Str whereas there was no role for CD44v6/v7^{-/-} BM-Str towards HSC proliferation. BMC division remained unaffected even after incubating with blocking antibodies against CD44, CD44v6 and CD44v7. HSC which

remained quiescent by rarely dividing entered into cell division with blocking CD44v6 and CD44v7 by neutralizing antibodies. This was significant with a predominant effect seen after 72h. This indicated a contribution of CD44v6/CD44v7 to HSC quiescence (Fig.11A, 11B).



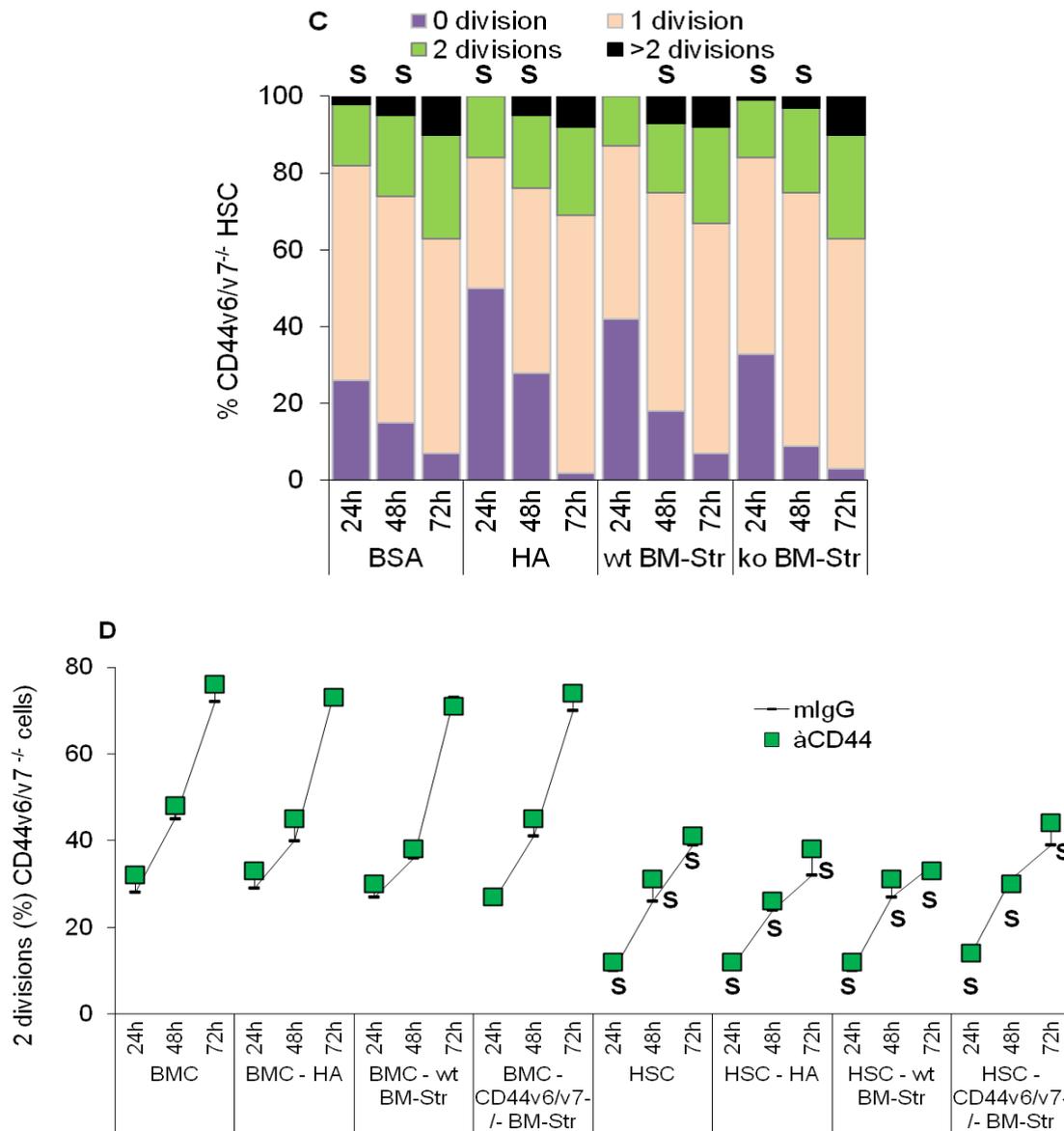


Figure 11: HSC-BM-Str crosstalk in maintaining HSC quiescence: (A,B) CFSE-labeled BMC and HSC were seeded on BSA, HA, CD44wt BM-Str and CD44v6/v7^{-/-} BM-Str, evaluating the number of cell division at 24h, 48h, 72h. Where indicated the cells were preincubated with anti-panCD44, anti-CD44v6 and anti-CD44v7. (A) Mean percentage of cells (triplicates) that did not divide or divided 1-time, 2-times or >2-times after 48h of culture. (B) Mean percent of cells (triplicates) that has divided 2 times. (C, D) The experimental setup described in (A, B) was repeated with CD44v6/v7^{-/-} BMC and HSC. (A) Significant differences in HSC proliferation depending on culture conditions (BSA, HA, BM-Str) are indicated by '+'. (A, C) Significant differences in proliferation of CD44v6/v7^{-/-} and CD44wt HSC are indicated by 'S'. (B, D) Significant differences between HSC and BMC are indicated by 'S' and significant differences by anti-CD44 by '*'. CD44wt HSC rarely proliferate on CD44wt BM-Str and CD44v6/v7^{-/-} HSC proliferation is not affected by BM-Str.

The influence of CD44v6/CD44v7 and BM-Str to HSC quiescence was confirmed by repeating the proliferation experiment with CD44v6/v7^{-/-} HSC. Although BMC from CD44wt and BMC from CD44v6/v7^{-/-} did not differ much in their proliferation profile, HSC from CD44v6/v7^{-/-} mice showed a higher proliferation compared to HSC from CD44wt (Fig.11C). The HSC proliferation did not differ between HSC cultured on CD44wt BM-Str and CD44v6/v7^{-/-} BM-Str. But HA and CD44wt BM-Str had some effect on proliferation (Fig.11D). Anti-panCD44 did not promote proliferation.

Cell cycle progression of HSC

Apart from proliferation, cell cycle progression is also influenced by HSC CD44v6/v7. In general, HSC remained mostly in the G0 phase of the cell cycle. When grown on HA or CD44wt BM-Str, the number of CD44wt HSC in G0 phase was increased with a complementary reduction in percentage of cells in G1/S and G2/M phase. Few cells of CD44wt HSC were in G2/M phase when cultured on HA or CD44wt BM-Str. The percentage of CD44v6/v7^{-/-} HSC in G2/M phase was not affected by HA or CD44wt BM-Str. Moreover, a minor increase in the percentage of CD44v6/v7^{-/-} HSC was observed in G2/M phase when compared with CD44wt HSC in G2/M phase (Fig.12A).

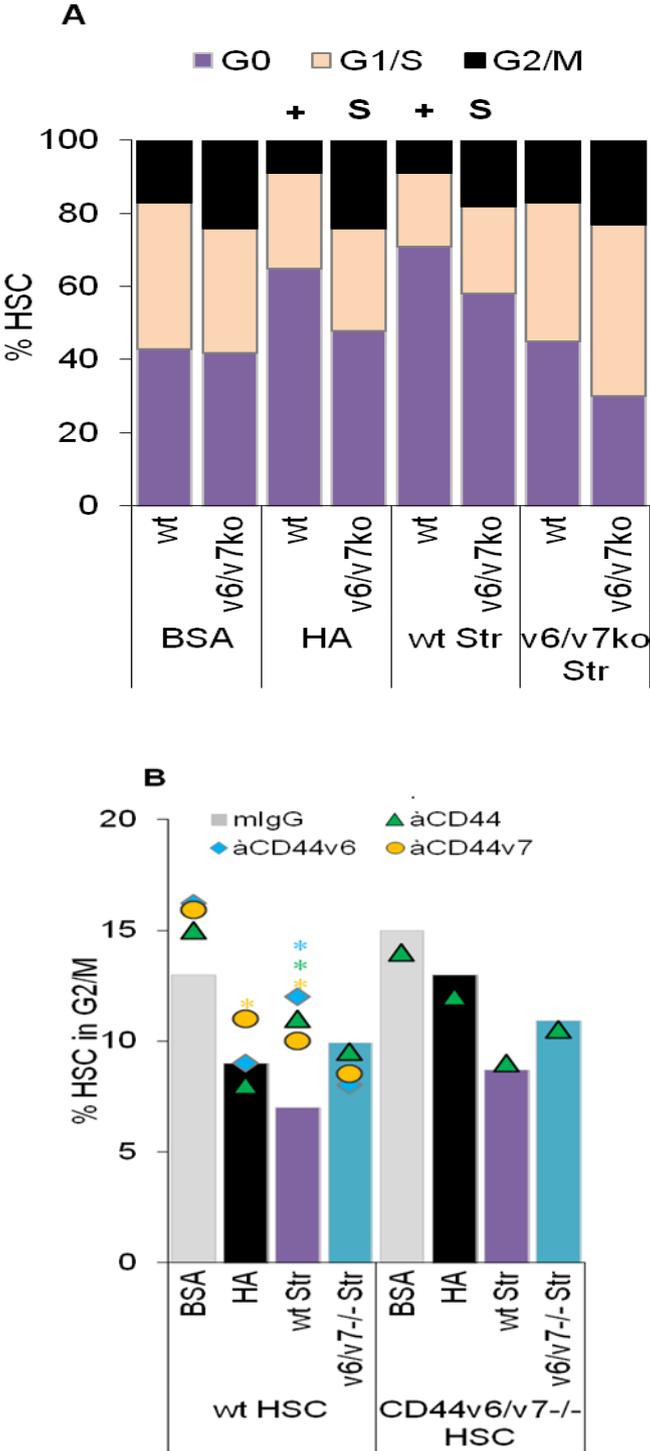


Figure 12: Cell cycle progression of HSC grown on HA, CD44wt BM-Str and CD44v6/v7^{-/-} BM-Str: Cell cycle progression by PI staining was evaluated in CD44wt and CD44v6/v7^{-/-} HSC grown on BSA, HA, CD44wt and CD44v6/v7^{-/-} BM-Str. (A) The percentage of cells in G0, G1/S and G2/M phase is shown, significant differences between CD44wt and CD44v6/v7^{-/-} HSC are denoted by ‘S’ and significant differences by culture condition by ‘+’. (B) Significant differences by antibody blocking is denoted by ‘*’.

Anti-panCD44 had no effect on percentage of CD44v6/v7^{-/-} HSC in G2/M phase. Whereas, CD44wt HSC when grown on HA were driven into mitosis by anti-CD44v6 and when grown on BM-Str by anti-panCD44, anti-CD44v6 and anti-CD44v7 (Fig.12B).

These observations show that CD44v6/v7 contribute to maintaining HSC in G0 phase when supported by CD44v6/v7-competent BM-Str.

HSC proliferation is retarded by CD44wt and CD44v6/v7^{-/-} BM-Str. CD44v6/v7^{-/-} BM-Str did not affect the cell cycle progression and thus having opposing influences on HSC proliferation and cell cycle progression. Hence I looked for extrinsic regulators on BM-Str that might influence HSC quiescence. They included SDF1, OPN, bone morphogenetic protein 4 (BMP4), inflammatory cytokine IL-6, TGF- β and its family including CD105 and inhibin, Wnt and leukemia inhibitory factor (LIF).

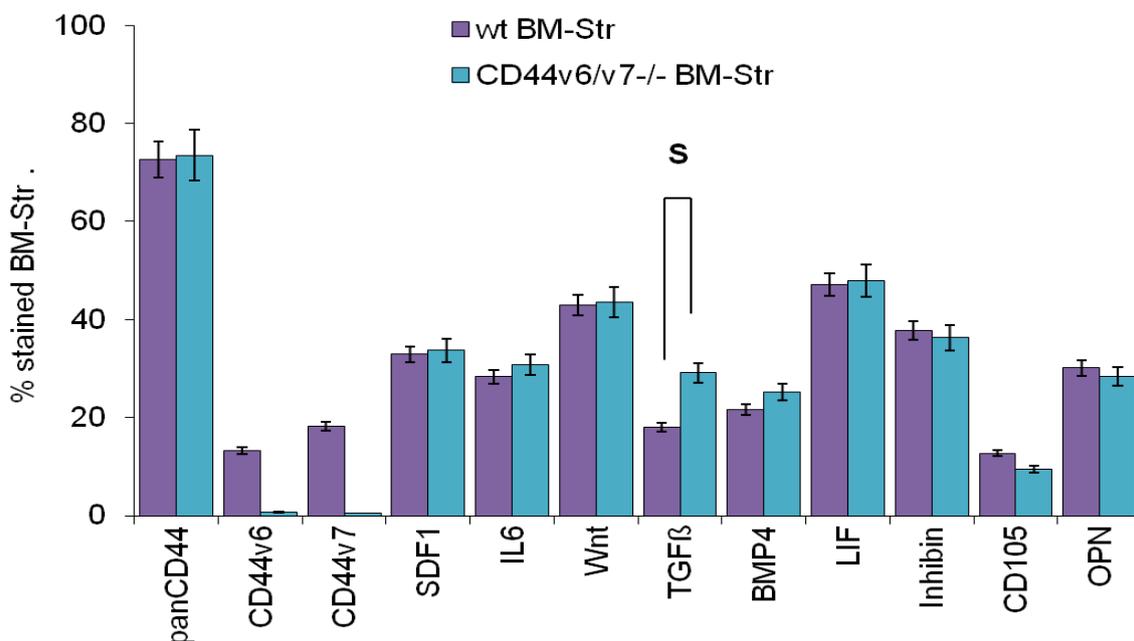


Figure 13: Expression of genes in CD44wt BM-Str and CD44v6/v7^{-/-} BM-Str that participate in the quiescence: Flow-cytometry analysis of the indicated markers in CD44wt and CD44v6/v7^{-/-} BM-Str; percent stained cells (mean \pm SD, triplicates); significant difference between CD44wt and CD44v6/v7^{-/-} BM-Str: S.

Among them, CD44v6/v7^{-/-} BM-Str displayed a slightly higher level of TGF- β (Fig.13).

When HSC were cultured on CD44wt and CD44v6/v7^{-/-} BM-Str, I noted a decrease in the CD117 expression in CD44v6/v7^{-/-} HSC when cultured on CD44v6/v7^{-/-} BM-Str. The expression of Fms-like tyrosine kinase 3 (Flt3), known for HSC maintenance, was not affected. The expression of CD126 on CD44v6/v7^{-/-} HSC was not upregulated when compared to CD44wt HSC when cultured on BM-Str (Fig.14).

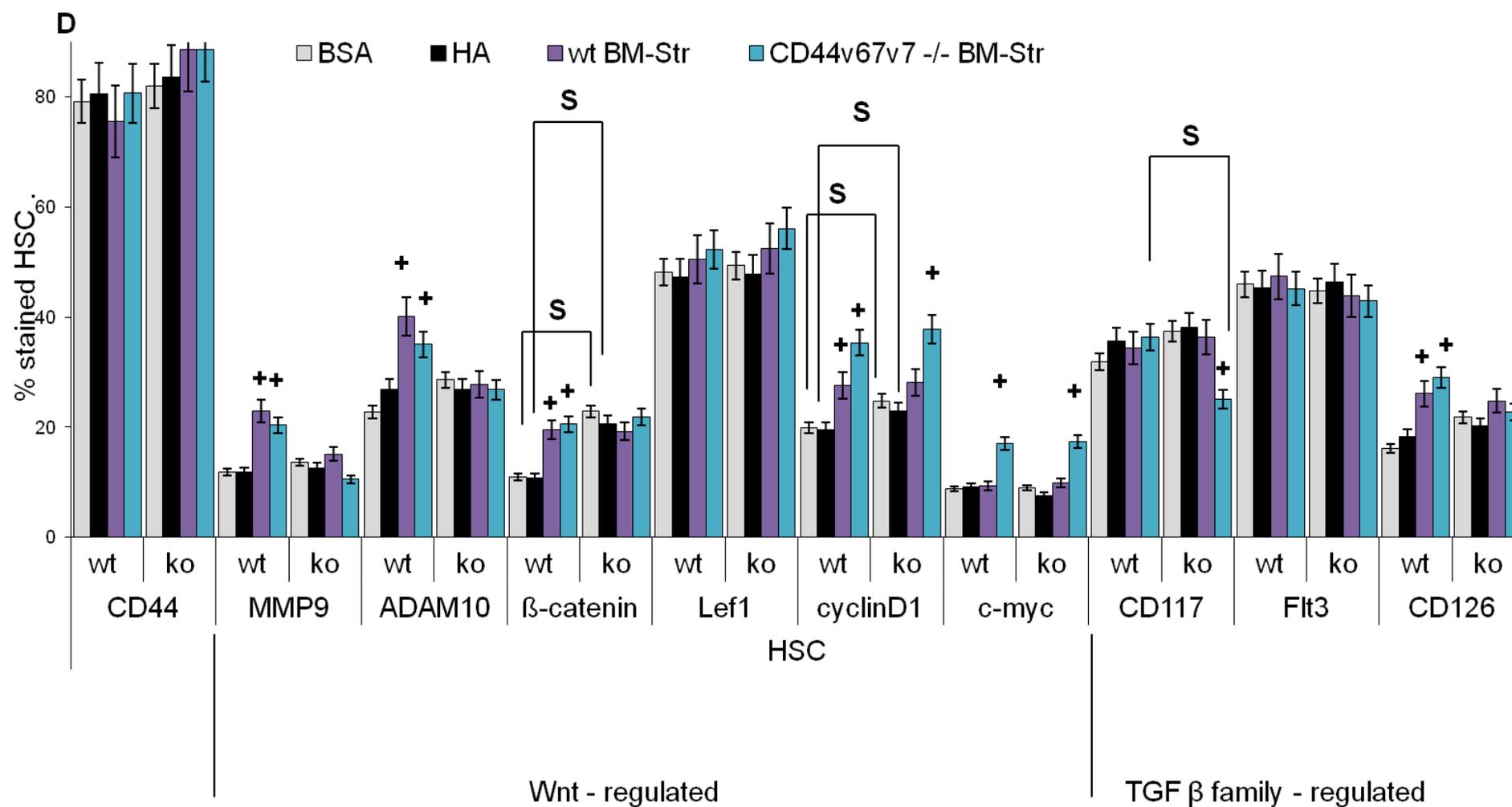


Figure 14: Wnt and TGF- β regulated genes on HSC quiescence: Flow cytometry of markers expressed on CD44wt and CD44v6/v7^{-/-} HSC cultured on BSA, HA, CD44wt and CD44v6/v7^{-/-} BM-Str. Percent stained HSC (mean +/- SD, triplicates) and significant differences in culture conditions are denoted by '+' and significant differences between CD44wt and CD44v6/v7^{-/-} HSC are denoted by 'S'.

Wnt-regulated MMP9 and A Disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) expressions became stimulated only in CD44wt HSC cocultured with BM-Str. Major Wnt targets like β -catenin and cyclin D1 are upregulated on CD44wt HSC cocultured with BM-Str but not in CD44v6/v7^{-/-} HSC that displayed higher expression levels than CD44wt HSC. The expression of Lef1 remains unaffected by CD44v6/v7^{-/-} in HSC or BM-Str. The expression of cyclin D1 and c-Myc expression became upregulated in cocultures with CD44v6/v7^{-/-} BM-Str (Fig.14). The base line cyclin D1 and β -catenin expression was higher in CD44v6/v7^{-/-} HSC.

The expression of CD44v6/v7 in HSC contributes to maintaining HSC quiescence as more CD44v6/v7^{-/-} HSC than CD44wt HSC were cycling. This fits to the poorer recovery of HSC from CD44v6/v7^{-/-} mice (data not shown) and a reduced number of CD117⁺ cells when cultured on CD44v6/v7^{-/-} BM-Str. Also observed was an increase in expression of β -catenin and cyclin D1 in CD44v6/v7^{-/-} HSC. The impact of CD44v6/v7^{-/-} BM-Str on cyclin D1 and c-Myc was less pronounced. The expression of markers belonging to Wnt pathway indicated that CD44wt BM-Str had a greater impact on quiescence of CD44v6/v7-competent HSC.

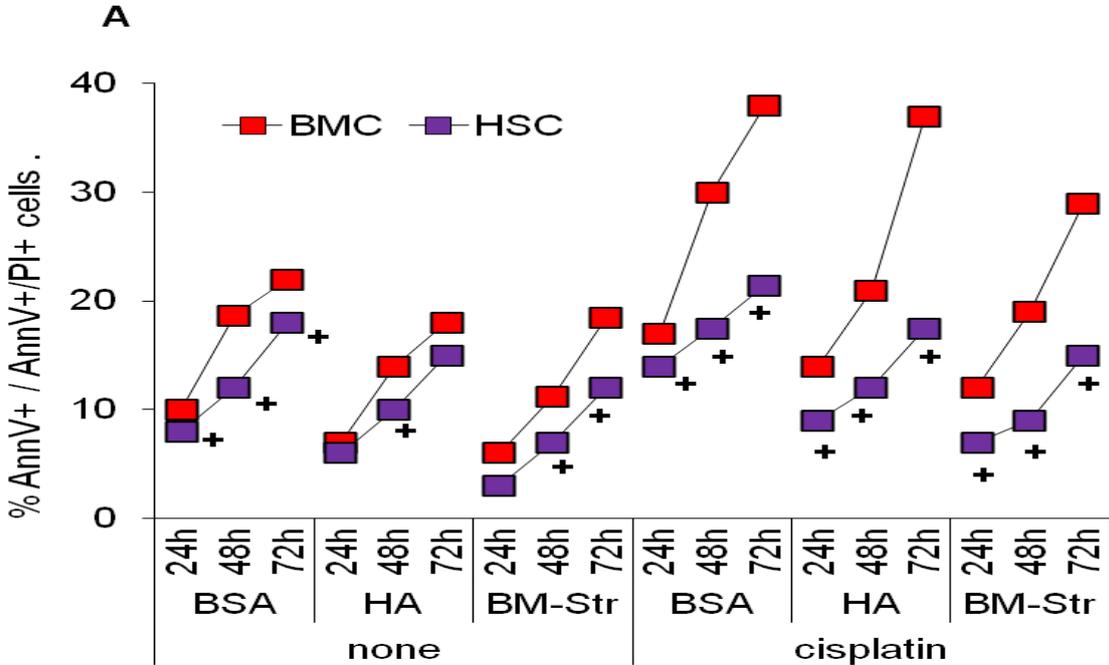
3.4 CD44 and apoptosis resistance of HSC

Apoptosis resistance of HSC grown on BM-Str

HSC are characterized by their relatively high apoptosis resistance [198, 199]. The resistance to apoptosis is contributed by BM-Str [200]. HSC and BMC were grown on BSA, HA or BM-Str for a time period of 24-72h either in the presence or absence of cisplatin and analyzed for apoptosis resistance by flow cytometry using Annexin/PI staining.

HSC in general displayed a lower apoptotic rate than BMC. This effect was more pronounced when HSC were grown on BM-Str. The presence of cisplatin (5µg/ml) increased the apoptotic rate of BMC significantly but only a small increase in the apoptotic rate was seen in HSC (Fig.15A) when cultured on HA and BM-Str.

The resistance to apoptosis by CD44wt HSC was CD44v6 and CD44v7 dependent (Fig.15B). Apoptosis resistance was strongly reduced in CD44v7^{-/-} HSC and, more pronounced in CD44v6/v7^{-/-} HSC. HSC apoptosis resistance was supported by HA and CD44wt BM-Str, but less efficiently by CD44v6/v7^{-/-} BM-Str.



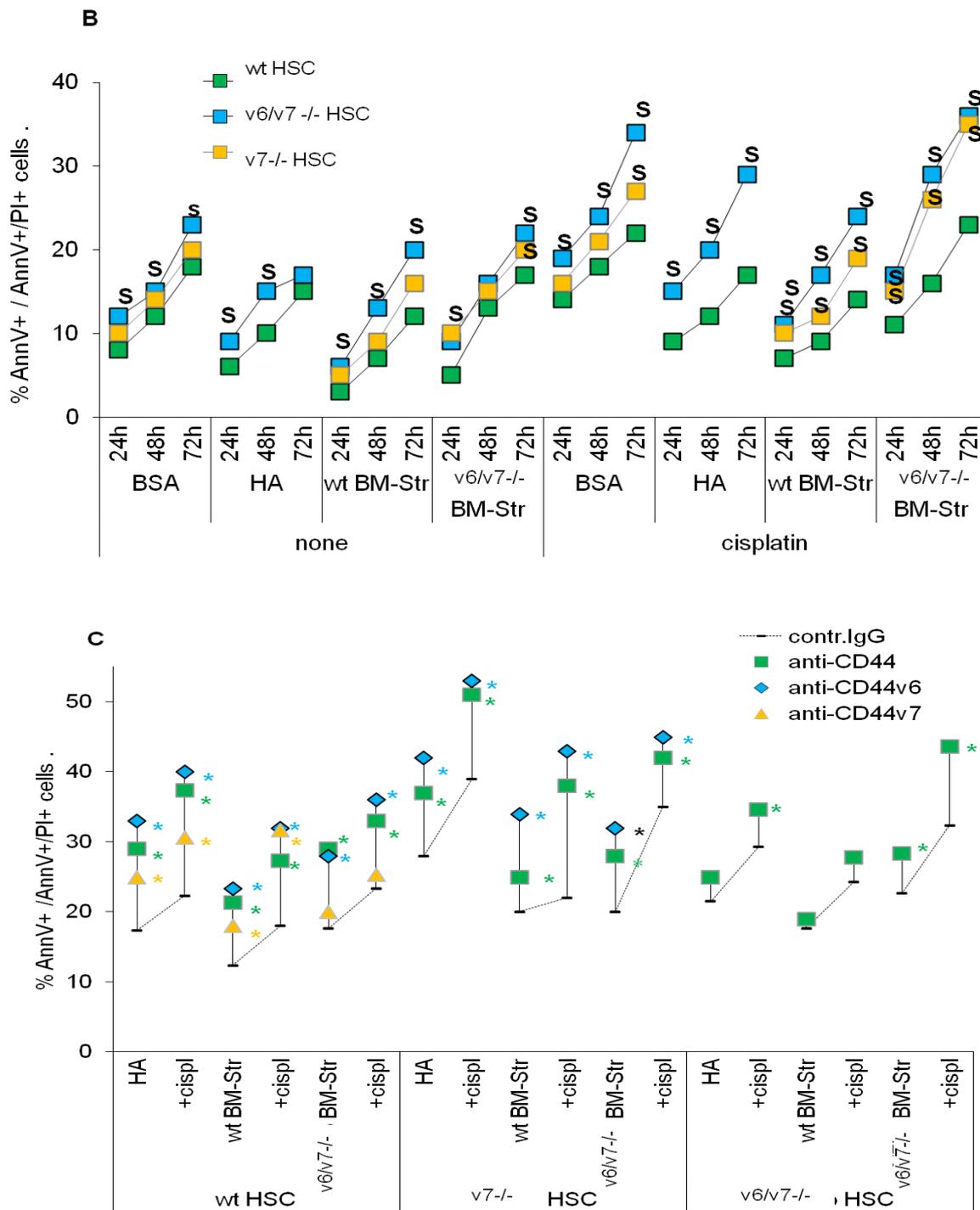


Figure 15: Apoptosis resistance of HSC supported by BM-Str: (A) CD44^{wt} BMC and HSC and (B, C) HSC from CD44^{wt}, CD44^{v7^{-/-}} and CD44^{v6/v7^{-/-}} were cultured for 48h in the absence or presence of 5µg/ml of cisplatin on BSA, HA and BM-Str and (C) cells were preincubated with anti-panCD44, anti-CD44v6 and anti-CD44v7. Mean percentage of AnnV/PI stained cells (+/-SD) of triplicates are shown. (A) Significant differences in the percentage of apoptotic cells between BMC and HSC are indicated by '+'. (B) Significant difference between CD44^{wt}, CD44^{v7^{-/-}} and CD44^{v6/v7^{-/-}} HSC are indicated by 'S'. (C) Significant differences due to antibody inhibition by '*'.

Antibody blocking studies show the role played by CD44 variants in the gain of apoptosis resistance. CD44wt, CD44v7^{-/-} and CD44v6/v7^{-/-} HSC were grown on HA, CD44wt BM-Str and CD44v6/v7^{-/-} BM-Str in the absence or presence of cisplatin and using antibodies against anti-panCD44, anti-CD44v6 and anti-CD44v7.

HSC from CD44wt were rather resistant to apoptosis even in the presence of cisplatin when grown on HA and/CD44wt BM-Str. Apoptosis resistance was weakened in the presence of anti-panCD44 and anti-CD44v6. There was slight or no effect when anti-CD44v7 was used. The impact of anti-CD44 was weaker on CD44v6/v7^{-/-} HSC. To differentiate between the impact of CD44v6 and CD44v7 on HSC or on BM-Str, the antibody blocking experiment was repeated with CD44wt HSC cocultured with CD44v6/v7^{-/-} BM-Str. Anti-CD44v6 still promoted apoptosis. Use of anti-CD44v7 did not increase HSC apoptosis indicating that CD44v7 on HSC does not contribute to HSC apoptosis resistance in the osteogenic niche (Fig. 15C).

Apoptosis can be initiated by pro-apoptotic death receptors. Cells under stress produce pro-apoptotic ligands such as CD95L and TNF-related apoptosis-inducing ligand (TRAIL). The pro-apoptotic ligands and their receptors belong to tumor necrosis factor (TNF) family which includes death receptors such as CD95, TNFR1 and TNFR2. The ligands bind to the death receptors, recruit adapter proteins and form death-inducing signaling complex that catalyze the caspase cascade, caspase 8, caspase 3 and caspase 9. These trigger apoptosis causing DNA and proteolytic damage. So, the expression of death receptors and their corresponding ligands on CD44wt and CD44v6/v7^{-/-} HSC were analyzed by flow cytometry.

Analysis of death receptor expression in CD44wt and CD44v6/v7^{-/-} HSC

CD44wt and CD44v6/v7^{-/-} HSC were grown on BSA, HA, CD44wt and CD44v6/v7^{-/-} BM-Str with cisplatin. From the flow cytometry results, it was observed that only CD95L was constantly upregulated in cisplatin-treated CD44v6/v7^{-/-} HSC. Coculturing CD44wt and CD44v6/v7^{-/-} HSC with BM-Str was accompanied by a slight downregulation in CD95, and a minor upregulation of TRAIL and TNFR1 which was independent of CD44v6/v7 expression (Fig.16).

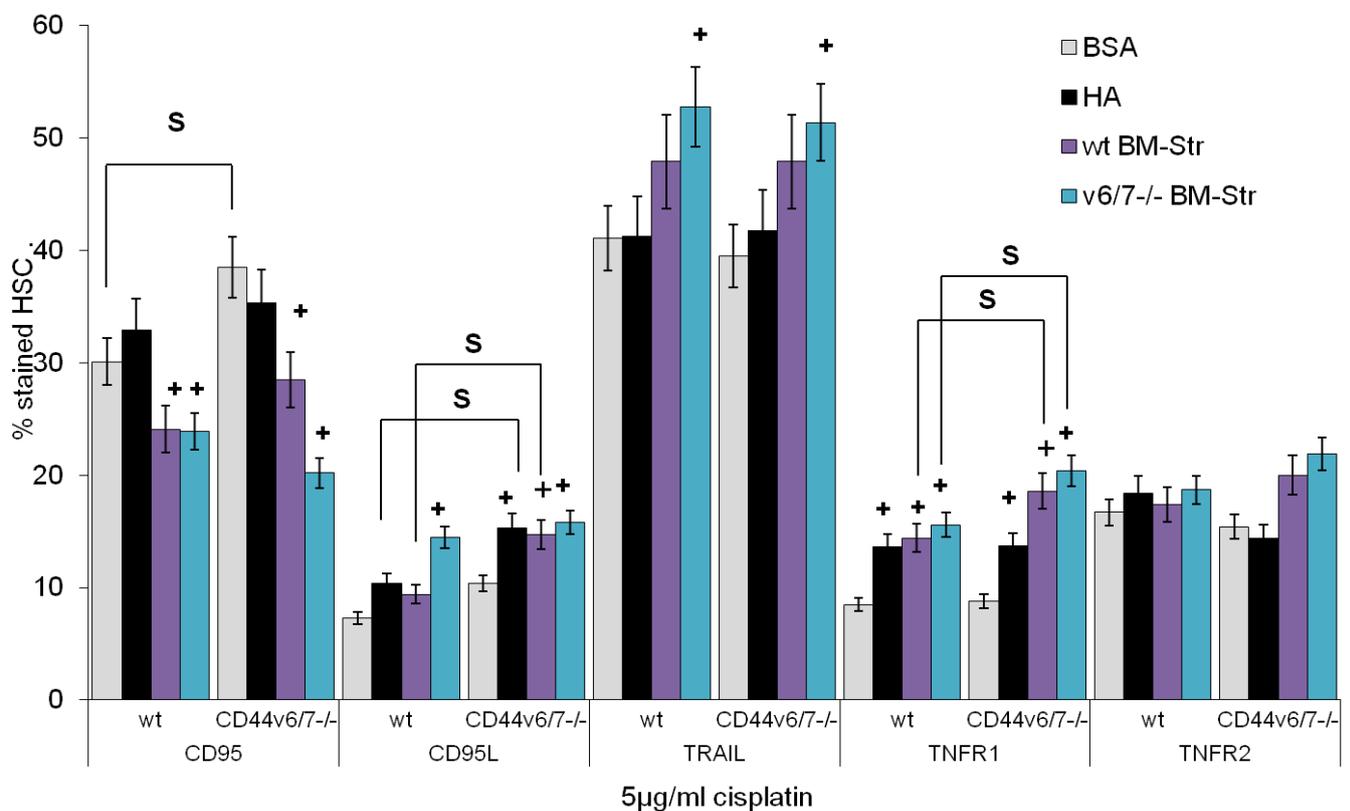


Figure 16: Analysis of pro-apoptotic molecule expression in HSC: Flow-cytometry analysis of pro-apoptotic receptor molecules in CD44wt and CD44v6/v7^{-/-} HSC grown on BSA, HA, CD44wt and CD44v6/v7^{-/-} BM-Str cultured for 48h with 5μg/ml of cisplatin. Expressed as mean percentage HSC +/- SD (in triplicates). Significant differences in culture condition is denoted as '+' and significant differences between CD44wt and CD44v6/v7^{-/-} HSC is denoted as 'S'.

Caspases are proteolytic enzymes whose expressions get upregulated with the activation of death receptor-mediated apoptosis.

Analysis of caspase expression in CD44wt and CD44v6/v7^{-/-} HSC

To differentiate between receptor or mitochondrial damage induced apoptosis, CD44wt and CD44v6/v7^{-/-} HSC grown on BSA, HA, CD44wt BM-Str and CD44v6/v7^{-/-} BM-Str with cisplatin were analyzed by flow cytometry for the expression of caspase8, activated caspase3 and cleaved caspase9. The analysis of caspase activity confirmed the independence of death receptors as caspase activity was similar on CD44wt and CD44v6/v7^{-/-} HSC with lower expression in HSC cultured with HA or CD44wt BM-Str. Activated caspase3 and cleaved caspase9 did not increase in cisplatin-treated CD44wt HSC protected by HA or CD44wt BM-Str. CD44v6/v7^{-/-} BM-Str exerted a weaker protective effect than CD44wt BM-Str (Fig.17).

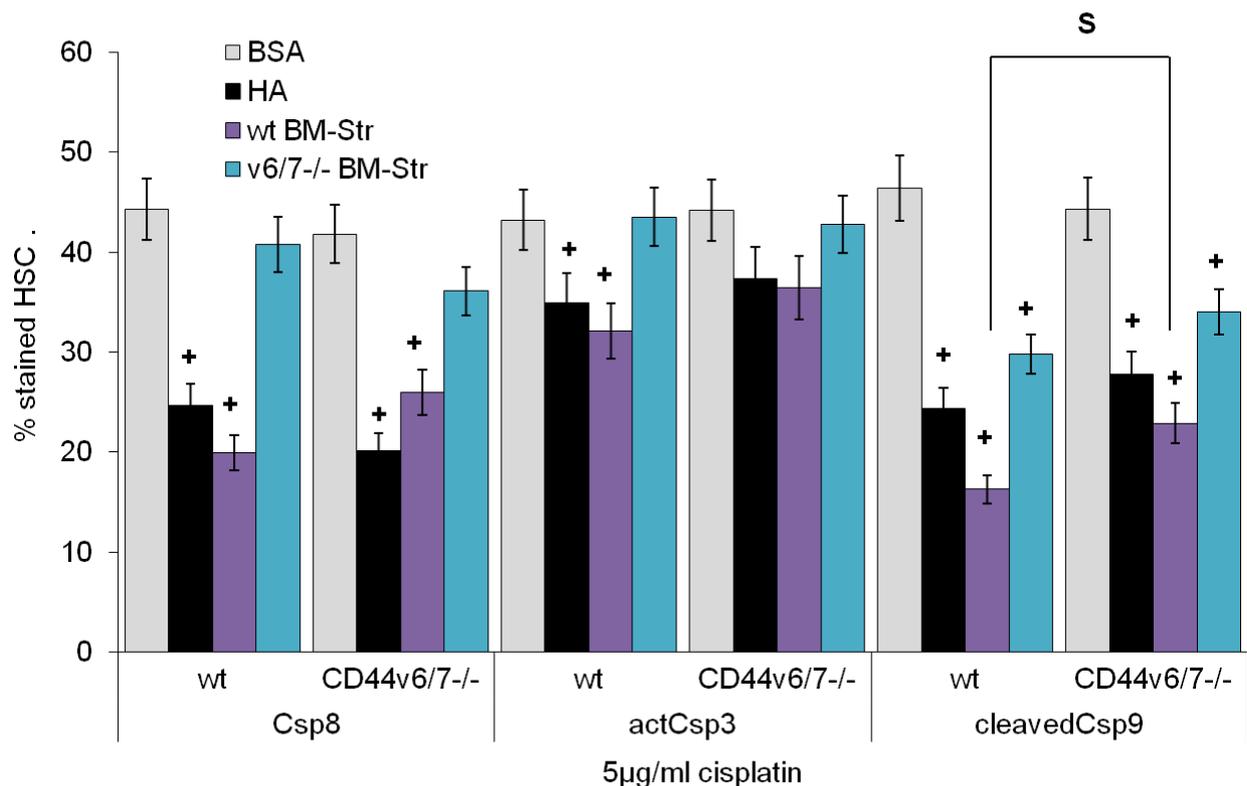


Figure 17: Analysis of caspase expression in HSC: Flow cytometry analysis of caspase expression on CD44wt and CD44v6/v7^{-/-} HSC grown on BSA, HA, CD44wt and CD44v6/v7^{-/-} BM-Str cultured for 48h with 5µg/ml of cisplatin. Mean percentage of stained HSC +/- SD (in triplicates). Significant differences by culture condition is denoted as '+' and significant differences between CD44wt and CD44v6/v7^{-/-} HSC is denoted as 'S'.

Caspase3 activation and caspase9 cleavage did not strongly differ between CD44wt and CD44v6/v7^{-/-} HSC. HA and more pronounced CD44wt BM-Str protected HSC from caspase activation.

These findings point towards the activation of PI3K/Akt pathway in HSC by HA or BM-Str.

Activation of PI3K/Akt pathway in HSC by HA and BM-Str

To study the anti-apoptotic pathway that was activated in HSC, HSC from CD44wt, CD44v7^{-/-} and CD44v6/v7^{-/-} grown on BSA, HA, CD44wt and CD44v6/v7^{-/-} BM-Str in the presence of cisplatin for a period of 48h were evaluated for the expression of anti-apoptotic molecules by flow cytometry. Phosphorylation of PI3K was strengthened, when HSC were cultured on HA or BM-Str (Fig.18A). An increase in pBAD and Bcl-xl was also seen when the cells were activated upon HA and BM-Str. Cells grown on HA and BM-Str showed upregulated expression of pAkt. The expression of Bcl2 was BM-Str dependent (Fig.18B). Lower expression of apoptosis regulator - BAX was observed on CD44wt HSC grown on HA and CD44wt BM-Str but not on CD44wt HSC grown on CD44v6/v7^{-/-} BM-Str. These observations were different from those in cisplatin-treated CD44v6/v7^{-/-} HSC, where pPI3K, pAkt and pBAD expression was less significantly promoted by HA or BM-Str and BAX expression increased more strongly. The CD44v6/v7^{-/-} BM-Str did not suffice to strengthen anti-apoptotic protein expression in CD44v7^{-/-} HSC (Fig.18).

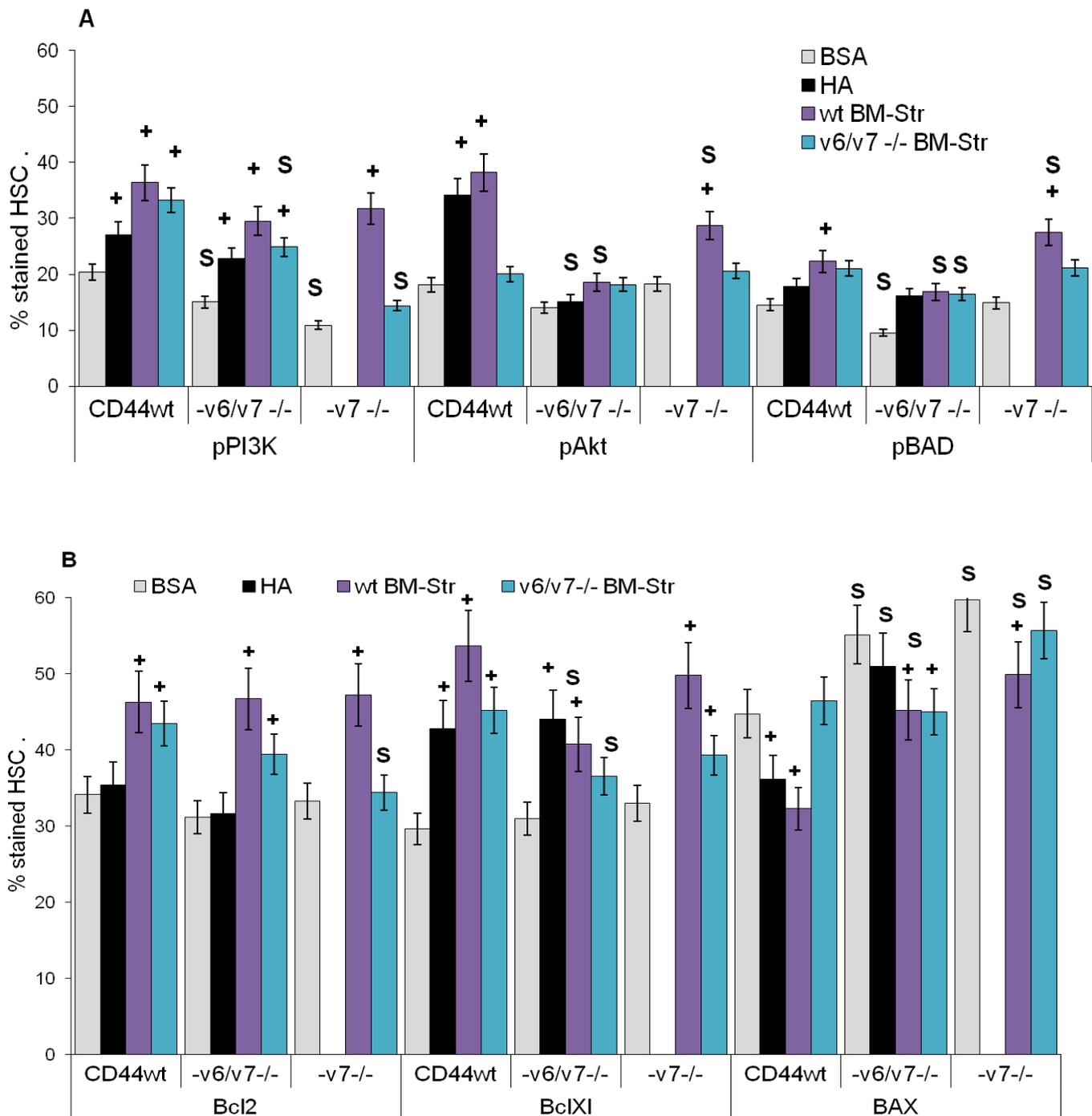


Figure 18: Activation of PI3K/Akt pathway in the interaction between HA-BM-Str: Flow cytometry analysis of anti-apoptotic molecules in CD44wt, CD44v7^{-/-} and CD44v6/v7^{-/-} HSC cultured for 48h on BSA, HA, CD44wt, CD44v7^{-/-} and CD44v6/v7^{-/-} BM-Str in the presence of cisplatin; (A,B) mean percent +/- SD (triplicates) of HSC stained for anti-apoptotic proteins. Significant differences by culture conditions is indicated by '+' and significant differences between CD44wt, CD44v7^{-/-} and CD44v6/v7^{-/-} HSC is indicated by 'S'.

CD44v6 is important in the apoptosis resistance of HSC. CD44v6 expressing HSC gain apoptosis resistance by the coculturing of HSC in HA and CD44/CD44v6-competent BM-Str.

4. Discussion

It can be summarized that mostly CD44s accounts for matrix binding. CD44v6 is engaged in the migration of HSC on HA and CD44v7 promotes homing. CD44v6 played a major role in the crosstalk of HSC with HA and BM-Str in supporting quiescence and apoptosis resistance.

I want to discuss particularly the following points.

4.1 HSC adhesion to matrix and engraftment

HSC interact with cells and ECM components in the bone marrow. These selective interactions take place at specific sites or 'niches'. Adhesion provides 'contact-mediated cues' [67] by presenting GM-CSF, bFGF, associated with the ECM components, to HSC and regulates hematopoiesis. CD11a/CD54 as ligand-receptor help hematopoietic progenitors to attach to cytokine activated BM-Str. CD49d expressed on HSC mediate adhesion to ECM, CD44 attaches HSC to HA and FN. Attachment to FN is essential for differentiation of erythroid progenitors [201]. Interaction of B-cell progenitors with FN and IL6 through CD49d helps in differentiation into mature B-cells [202]. Antibodies against CD49d release progenitors from the bone marrow into the blood. CD44 is important for binding of HSC to coll I, coll IV and HA [203]. The binding affinity of CD44 to HA varies by the insertion of the variant exon products and glycosylations. Absence of CD44v6/v7 in HSC and in BM-Str is reflected in slightly reduced binding to HA. So, the observed differences among CD44 and its variants in binding to HA could alter the adhesion of HSC to BM-Str. Co-stimulatory signals arising from CD44 binding to SCF activate CD49d to bind to FN with strong affinity [204]. The coordinated interaction among adhesion receptors is helpful in maintaining the hematopoiesis.

4.2 HSC mobilization

Role of GTPases in HSC mobilization

CD44 expressed on both HSC and BM-Str is involved in HSC cell migration on BM-Str. This is a multistep process that is activated by cell-ECM interactions that activate signaling events towards changes in the cell cytoskeleton and migration. CD44 by interacting with ERM proteins mediates cell migration [63]. Intracellular signaling enzymes such as Rac and Rho are Rho guanosine triphosphatases (GTPases) that regulate actin cytoskeleton, adhesion, HSC migration and associated gene expression. SDF1 gradients controls the mobilization of HSC by effector protein Wiskott-Aldrich syndrome protein family verprolin-homologous protein (Wave) that acts as downstream effector of Rac and is required for the formation of lamellipodia [205, 206]. HA binding to CD44 activates Rac1 and the interaction with ezrin contributes to loosening of intercellular contacts and promotes cytoskeletal rearrangements [207]. HA-mediated Rac activation is possible only through the interaction between CD44 and HA. Hence CD44v7^{-/-} HSC showed decreased chemotaxis towards SDF-1 in the migration experiment similar to migration of Rac-deficient HSC towards SDF-1 [208]. CD44 interacts with IQGAP1 [136] and we found that HSC CD44v7 in particular associates with IQGAP1. F-actin interacts with N-terminus of IQGAP1 and Rac interacts with C-terminus of IQGAP1. IQGAP1 acts as effector downstream of RhoGTPase and alters the E-cadherin- β -catenin equilibrium at the cell junctions [209]. Increase in F-actin filaments at the lamellopodia is possible by the HA-mediated IQGAP1 [135] providing the conducive environment for cell migration.

OPN-mediated HSC egress

The idea that OPN binding affect negatively the HSC retention in the niche can be correlated with the HSC migration towards OPN. CD44v6/v7 is important for HSC adhesion to OPN

and CD44v7 contributes to HSC migration towards OPN as CD44v7-deficient HSC cannot respond to OPN expressed by bone-lining osteoblasts and do not migrate towards it. The CD44v7-OPN interaction supports towards HSC mobilization. HSC from OPN^{-/-} mice get accumulated in BM and spleen [210].

Innate immunity in HSC mobilization

Components of the innate immunity, in particular C1q of the complement system participate in cell mobilization [211]. The molecular association between HSC CD44v7 and C1q receptor is shown in coimmunoprecipitation experiment. C1q receptor forms a complex with CD44, β 1-integrin, complement C1q [212], HA, FN and VN [213]. C1q receptor helps in lamellopodia formation which contains CD44 along with ERM proteins, actin and Rho GTPases [214, 215]. The concept that C1q receptor regulates lamellopodia formation and helps cell migration by coupling with receptor tyrosine kinase (RTK) signaling is shown by C1q-knockdown in lung adenocarcinoma cells. Also, in breast cancer cell line, immunoprecipitation experiment showed the molecular association between C1q receptor and CD44. Activation of RTK by ligand requires C1q receptor and CD44 association. C1q receptor mediates the crosstalk between migration and epidermal growth factor receptor (EGFR) signaling through its interaction partner CD44 which is known to regulate the EGFR, FGFR and c-Met in ligand-induced activation and subsequent migration [211].

4.3 HSC quiescence regulated by extrinsic factors

HSC maintain a balance between the mature blood cell supply and HSC population number by remaining quiescent mostly with only a small number participating in mature cell production. By remaining relatively quiescent HSC avoid replication-associated mutations. HSC respond to stress by exiting G0 phase and entering cell proliferation and differentiation.

HSC achieve this balance between stress response and population number by cell-cell, cell-ECM and receptor-ligand interactions in the osteogenic niche [216].

SCF-CD117 interaction

Adherence of HSC CD44v6/v7 to SCF is important for the SCF-CD117 signaling to maintain HSC dormancy and function [185] where a decrease in HSC correlates with mutations in CD117 and SCF [217]. Also, in coculture experiments of CD44v6/v7^{-/-} HSC with CD44wt BM-Str and CD44v6/v7^{-/-} BM-Str, there was a reduction in CD117⁺ cells in the CD44v6/v7^{-/-} HSC cocultured with CD44v6/v7^{-/-} BM-Str. The decrease in the expression of CD117 corresponds to an increase in the number of CD44v6/v7^{-/-} HSC in the G2/M phase when cultured on CD44v6/v7^{-/-} BM-Str.

Hypoxic environment

HSC reside inside the hypoxia environment of the endosteum of the bone. Inside the hypoxia environment HSC undergo non-oxidative glycolysis to derive their energy. Hypoxia prevents cells from undergoing oxidative metabolism of mitochondria and increases 'stemness' in HSC [218]. Cells in undifferentiated state produce lactate for their energy needs by opting anaerobic glucose metabolism. This is accompanied by a corresponding increase in the expression of glycolytic enzymes, the prominent among them is lactate dehydrogenase (LDH) [219] which is necessary for conversion of pyruvate to lactate. The measurement of lactate production has been a benchmark for the measurement of long-term repopulating ability in bone marrow transplantation or its success in engraftment in hypoxic niche [220]. The association of LDH found with CD44v6/v7 on HSC helps long-term survival by maintaining a dormant state and resisting differentiation.

TGF- β -mediated quiescence of HSC

Stromal microenvironment produces TGF- β and inhibits proliferation of HSC [221]. Blocking TGF- β releases the progenitor cell from quiescence in the presence of cytokines [222]. Antibodies against TGF- β in human hematopoietic progenitors blocked the expression of the HSC marker CD117 [223]. Flt3-ligand [224] which push forward cell cycle to differentiate into lymphoid and myeloid progenitors and IL-6 [225] are some of the cytokines that release the cells from quiescence. In a study, it was demonstrated that the expression of Flt3, CD126 (IL-6R) and CD117 get regulated by anti-TGF- β serum [226]. Autocrine secretion of TGF- β reduced in CD44wt BM-Str mice, and increased in CD44v6/v7^{-/-} BM-Str. HSC quiescence by BM-Str was studied by growing HSC on CD44wt BM-Str and CD44v6/v7^{-/-} BM-Str. The results showed the regulation of CD117, Flt3 and CD126 by BM-Str probably by autocrine secretion of TGF- β . The expression of IL6-R and Flt3 remain unchanged irrespective of the expression of CD44v6/v7 on BM-Str, whereas expression of CD117 was reduced in cocultures with CD44v6/v7^{-/-} BM-Str. Physiologically, the number of quiescent cells gets reduced with CD117 reduction and the recovery of HSC from BMC population in CD44v7^{-/-} and CD44v6/v7^{-/-} mice was less.

β -Catenin is required for HSC quiescence

Wnt signals are important in mediating HSC quiescence [197]. Bone vasculature and bones of the limbs which are derived from mesoderm germ layer are important for hematopoiesis. β -Catenin is necessary for the development of mesoderm germ layer [227]. β -Catenin expressed on BM-Str support HSC and promote hematopoiesis [228]. β -Catenin is necessary for the osteoblasts generation and helps osteoblasts to secrete factors such as bFGF, SCF for hematopoiesis [228]. Also, β -Catenin interacts with E- or N- cadherins in stroma and form adherens junction complex [229] that help in maintaining Wnt signaling. Reduced β -Catenin

affects quiescent HSC and induces cell cycling. β -Catenin is the prime regulator of canonical Wnt signaling. Its expression gets upregulated when HSC are grown on CD44wt BM-Str. Our data are in line with CD44v6/v7 competent HSC to contribute to maintaining HSC quiescence in the BM microenvironment.

4.4 Resistance to TRAIL-mediated apoptosis and induction of apoptosis resistance through PI3K/Akt signaling

HSC and BM-Str reside in the hypoxic environment of the niche. CD44 associates with glycolytic enzymes, chaperones and proteins against oxidative damage to maintain them. Thus we see a great number of molecules belonging to glycolytic pathway, heat shock proteins and superoxide dismutase (SOD).

HSC resistance to cytotoxic drugs and to apoptosis can be explained by Galectin-3 induction in the signal transduction. Galectin-3 is induced when HSC are cocultured with BM-Str [230]. In a similar setup of coculture between CML-cell line and HS-5, a human bone marrow stromal-derived cell line, induction of Galectin3 expression has been observed [230]. CD44v6 is expressed in CML [231, 232]. CD44v6 and Galectin-3 are lectin-related molecules and their coexpression in malignant transformation and cell growth deregulation has been described [233].

Galectin3 is normally expressed in BM microenvironment and overexpression of Galectin3 promotes multidrug resistance [234]. Expression of CathepsinB and Annexin A2 along with CD44v6 is identified as another characteristic towards apoptosis resistance [235].

CD44wt HSC grown on CD44wt BM-Str acquires apoptosis resistance and reduced caspase expression [236] and an increase in the expression of anti-apoptotic protein, Bcl2 [237]. Galectin3 is an anti-apoptotic molecule inhibiting the Fas-induced and cisplatin-induced apoptosis in cells [238]. It regulates CD95-activated [239] and inhibits TRAIL-induced

apoptotic pathways [240]. TRAIL is proapoptotic and transmits signals through death-domain containing receptors. TRAIL induces the formation of a death-inducing signaling complex (DISC). DISC and caspase8 activate the executioner caspases and Bcl2 family proapoptotic member Bid, links this extrinsic apoptotic pathway with the intrinsic pathway by ensuring mitochondrial membrane permeabilization, activation of caspase9 and caspase3 and advancing cell death. Similarly other TNF family of death ligands such as TNF and CD95L induce apoptosis by the internalization of their death receptors. Galectin3 blocks the death receptor internalization and prevents apoptosis.

Resistance to TRAIL is mediated internally by the serine/threonine protein kinase, Akt which inhibits apoptosis and stimulates cell survival. Activation of PI3K/Akt pathway results in phosphatidylinositol-3,4,5-triphosphate binding to Akt, recruiting it to the cell membrane, phosphorylating Akt and activating it. Galectin-3 mediated Akt-activity increase [240] confers TRAIL resistance in cells. Activated Akt enters into the nucleus and inactivates FOXO transcription factors and upregulates ATP synthase for energy production, HSP70 chaperones and 14-3-3 proteins to integrate prosurvival signals mediated by Akt in HSC [241] by sequestering FOXO3a and BAD.

Taken together I propose that Galectin-3 promotes PI3K/Akt pathways by conferring TRAIL resistance to CD44v6-competent CD44wt HSC cells grown on CD44wt BM-Str.

5. Summary and outlook

Summary

The importance of CD44v6 and CD44v7 in adhesion, migration, quiescence/proliferation and apoptosis resistance of HSC in the bone marrow microenvironment is elaborated. HSC crosstalk with BM-Str in these physiological processes mediated by CD44v6 and CD44v7 and their associated molecules is explained. CD44v6 is needed for the strong binding to HA, FN and Coll I, which helps in the interaction with the cytokines localized in the BM niches. Adhesion to cytokine, SDF1 needs coordinated effort from CD44v6 and CD44v7 on HSC that promote homing and CD44v6 for adhesion to SCF for hematopoiesis. CD44v6 contributes to migration of HSC on HA and expression of CD44v7 on both HSC and BM-Str supports migration on BM-Str. HSC CD44v7 associates with IQGAP1 and complement C1q assisting in migration. When HSC were cocultured with BM-Str, the interaction between CD44v6 on HSC and BM-Str contributes to the cell cycle of HSC supporting the HSC in the quiescent state. This is achieved by TGF- β -regulated expression of CD117, marker for multipotential stem cells, and Wnt-regulated expression of β -Catenin that promotes the expression of bFGF and SCF for hematopoiesis and formation of adherens junction complex in sustaining Wnt signaling. Also, CD44v6/v7 help HSC in switching the energy requirements of the cell from normal glycolytic pathway towards lactate production under hypoxic conditions by the expression of lactate dehydrogenase for pyruvate conversion. Lactate helps HSC survive long-term by maintaining themselves in undifferentiated state. HSC inside the niche interact with BM-Str and express molecules like HSP, SOD that protect from stress-related damage. CD44wt HSC cocultured with CD44wt BM-Str acquire resistance to TRAIL- and caspase-mediated apoptosis possibly by recruiting Galectin3. The resulting antiapoptotic pathway continues via Akt activation and expression of HSP and inactivation of proapoptotic BAD.

Outlook

The contribution of CD44v6 to homing, quiescence maintenance and apoptosis resistance could be used in therapies against leukemia stem cells/leukemia initiating cells (LSC/LIC). Since HSC and LIC frequently express CD44s, which is important for HSC homing and adhesion, blocking CD44s could affect HSC reconstitution. This problem can be circumvented by targeting variants of CD44 that are expressed higher on LIC than on HSC. CD44 variants along with their associated molecules could interrupt the LIC quiescence and to disrupt LIC association with the osteogenic niche inside the bone marrow and to drive them to the peripheral blood towards differentiation.

Cells survive with the energy produced by glycolysis. Pyruvate produced in the final step of glycolysis is converted either into lactate or to acetyl coenzyme A. Cells decide about pyruvate conversion by the enzymatic activity of pyruvate kinase M2 (PKM2). Low-activity PKM2 converts pyruvate to lactate.

Galectin3 [242], EGFR and CD44v6 [243] expression are elevated in leukemia cells. Galectin3 binds to leukemia cell surface and binds to N-glycans on EGF, TGF- β [244]. These bound Galectin monomers come together and form multimerization and create crosslinking along with mucin1 [245]. Mucin1, another glycoprotein highly expressed in leukemia [246] when glycosylated, binds to Galectin3 and is crosslinked to EGFR [245]. TGF- β -activated EGFR phosphorylates mucin1. Phosphorylated mucin1 binds to PKM2 and suppresses its activity [247]. Thus Galectin3 plays a crucial role in bringing together the regulatory molecules of cell activation/proliferation to make the leukemic cell tolerant to apoptosis-inducing drugs. Also mucin1-Galectin3 colocalizes into the nucleus and activates along with β -catenin/Tcf in gene transcription [248].

CD44v6 and Galectin3 are expressed less on HSC and high on leukemia cells, a bi-specific antibody that recognizes the epitopes of CD44v6 and Galectin3 would help in formulating a formidable therapy against resistant LIC to force them into differentiation without affecting the HSC.

6. References

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