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Reciprocal upregulation of Notch signaling molecules in hematopoietic progenitor and mesenchymal stromal cells

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Although mesenchymal stem cells (MSCs) play pivotal supportive roles in hematopoiesis, how they interact with hematopoietic stem cells (HSCs) is not well understood. We investigated the interaction between HSCs and surrogate MSCs (C3H10T1/2 stromal cells), focusing on the molecular events induced by cell contact of these bipartite populations. C3H10T1/2 is a mesenchymal stromal cell line that can be induced to differentiate into preadipocytes (A54) and myoblasts (M1601). The stromal cell derivatives were cocultured with murine HSCs ($Lineage^{-}Sca1^{+}$), and gene expression profiles in stromal cells and HSCs were compared before and after the coculture. HSCs gave rise to cobblestone areas only on A54 cells, with ninefold more progenitors than on M1601 or undifferentiated C3H10T1/2 cells. Microarray-based screening and a quantitative reverse transcriptase directed-polymerase chain reaction showed that the levels of Notch ligands (Jagged1 and Delta-like 3) were increased in A54 cells upon interaction with HSCs. On the other hand, the expression of Notch1 and Hes1 was upregulated in the HSCs cocultured with A54 cells. A transwell assay revealed that the reciprocal upregulation was dependent on cell-to-cell contact. The result suggested that in the hematopoietic niche, HSCs help MSCs to produce Notch ligands, and in turn, MSCs help HSCs to express Notch receptor. Such a reciprocal upregulation would reinforce the downstream signaling to determine the fate of hematopoietic cell lineage. Clarification of the initiating events on cell contact should lead to the identification of specific molecular targets to facilitate HSC engraftment in transplantation therapy.

Keywords: mesenchymal stromal (stem) cell, hematopoietic stem cell, cell contact, Notch signaling

Introduction

Hematopoietic stem cells (HSCs) are capable of self-renewing and differentiating into all the blood cell lineages, and the property allows them to reconstitute adult hematopoiesis following transplantation. The growth and differentiation of HSCs is regulated by orchestrated signals from various soluble factors and the hematopoietic microenvironment, or 'niche'. With the aid of many other cell types, osteoblasts and vascular endotherial cells maintain the balance of dormant and active HSCs in the osteoblasic and vascular niches [1-4].

The interaction between HSCs and the niche cells comprises cytokines and cell-to-cell contact. The involved cytokines include stem cell factor (SCF), stromal-derived factor 1 (SDF1), angiopoietin1 (Ang1) and osteopontin, and the functions of these factors have been studied extensively [5-9]. On the other hand, molecular events of the direct cell contact are mostly unclear. Wagner et al. investigated the behavioral and molecular changes in hematopoietic progenitors upon interaction with a stromal cell line AFT024 [10]. They found that the genes involved in the cytoskeleton reorganization, DNA stabilization and methylation were upregulated. However, molecular events in the niche cells have not vigorously explored.

Mesenchymal stem cells (MSCs) in the bone marrow play a vital role in supporting hematopoiesis, therefore they are considered as niche cells, too [11,12]. To further explore the hematopoiesis-supporting ability of MSCs, we have used a surrogate MSC line C3H10T1/2 (10T1/2) and its derivative preadipocytes (A54) and myoblasts (M1601). Among these cells, only A54 preadipocytes helped the expansion of hematopoietic progenitors with an augmented production of SCF, SDF1 and Ang1 [13,14]. In the present study, we investigated the cellular and molecular events in the interactive communication between HSCs and stromal cells using this differentiation-inducible system, particularly focusing on the changes in the stromal cells.

Materials and methods

Cells

10T1/2 cell line (from Riken Biological Resource Center, Tsukuba, Japan) was used as an inducible MSC model. A54 preadipocytes and M1601 myoblasts were established as described previously [13]. All the cell lines were cultured in Iscove's Modified Dulbecco's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA). Bone marrow mononuclear cells were separated from C57BL/6 mouse femurs using

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Lympholyte-M (Cedarlane, Hornby, ON, Canada), and serially fractionated with immunomicrobeads and AutoMACS (Miltenyi Biotech, Bergisch Gradbach, Germany). Lineage-negative (Lin^-) and –positive (Lin^+) cells were separated with a Lineage Cell Depletion Kit (Miltenyi Biotech). Sca1-, CD45R (B220)- and CD90 (Thy1.2)-positive cells ($Sca1^+$, $B220^+$ and $Thy1.2^+$ cells, respectively) were separated with appropriate microbeads (Miltenyi Biotech). Lin^-Sca1^+ cells were used as HSCs. Lin^+B220^+ cells and $Lin^+Thy1.2^+$ cells were used as B-lymphocytes and T-lymphocytes, respectively.

Coculture of HSCs and stromal cells

10T1/2, A54 and M1601 stromal cells were inoculated (5 x 10^3 cells/well) on a 12-well culture plate (Falcon 3043, BD, Franklin Lakes, NJ, USA). After 2 days, the near-confluent cells (ca. 2 x 10^4 cells/well) were gamma-irradiated (30 Gy) using a Gammacell 40 Exactor (Nordion International, Ottawa, ON, Canada) to prevent hyperproliferation. Then 1 x 10^4 HSCs (Lin^-Sca1^+ cells) were added to the irradiated stromal cells and cocultured for 5 days in α-Minimum Essential Medium (Invitrogen) supplemented with 10% FBS. A coculture was also carried out using a Cell Culture Insert (Falcon 353090, BD) on a 6-well culture plate (Falcon 3046, BD) for 5 days, with HSCs seeded in the upper wells and stromal cells in the lower wells. After coculture, cells were examined for cobblestone formation (30 or more clustered HSCs) on an inverted fluorescent microscope (Olympus IX-70, Tokyo, Japan), and the stromal cells and HSCs were separated with MACSelect K^k microbeads (Miltenyi Biotech) and AutoMACS for further analyses. The stromal cells derived from C3H mouse ($H\text{-}2^k$) were separated as a positive fraction, and HSCs derived from C57BL/6 mouse ($H\text{-}2^b$) were separated as a negative fraction. The separated cells were stained with a phycoerythrin-conjugated anti-mouse $H\text{-}2K^k$ antibody and a fluorescein isothiocyanate-conjugated anti-mouse $H\text{-}2K^b$ antibody (BD Biosciences, San Jose, CA, USA), and the purity was evaluated with BD LSR and CellQuest software (BD Immunosystems, Mountain View, CA, USA). For colony asaay after coculture, separated HSCs were cultivated (1 x 10^3 cells/35 mm dish) in growth factor-containing methylcellulose medium (MethoCult GF M3434, StemCell Technologies, Vancouver, BC, Canada) and colonies were counted on day 7.

Gene expression analysis

To quantify several signaling molecules, the reverse transcriptase-directed polymerase chain reaction (RT-PCR) was performed. Total RNA was extracted from the cells using an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and cDNA was synthesized with a SuperScript First-Strand Synthesis Kit (Invitrogen). PCR was carried out with a QuantiTect SYBR Green PCR Kit (QIAGEN), and the incorporation of the fluorescent dye into the PCR products was monitored with ABI Prism 7700 (Applied Biosystems, Foster City, CA, USA). As internal controls, beta-actin was used for the stromal cells and glyceraldehyde-3-phosphate dehydrogenase was used for HSCs. The primers for PCR are listed in Table 1. Gene expression profiles of A54 before and after coculture with HSCs were compared using a FilgenArray Mouse 32K (Filgen, Nagoya, Japan).

Table 1. RT-PCR primer design.

Target	Forward primer	Reverse primer
Ang1	CCAATCTAAATGGAATGTTCT	CAGAGCACCTTCAAAAGTCCA
beta-actin	CCATCATGAAGTGTGACGTTG	GTCCGCCTAGAAGCACTTGCG
BMP6	TTCTCCCCACATCAACGACACC	AAACTCCCCACCACACAGTCC
Dll3	CCAGTAGCTGCCTGAACTCC	ATTGAAGCAGGGTCCATCTG
GAPDH	CCTGGAGAAACCTGCCAAGTATG	AGAGTGGGAGTTGCTGTTGAAGTC
Hes1	AAAGCCTATCATGGAGAAGAGGCG	GGAATGCCGGGAGCTATCTTTCTT
Jag1	CCGTAATCGCATCGTACTGC	GGCCTCCACCAGCAAAGTGT
Notch1	TGTTAATGAGTGCATCTCCAACCCA	CATTCGTAGCCATCAATCTTGTCC
SCF	ATGGACAGCCATGGCATTGC	CACCTCTTGAAATTCTCTCT
SDF1	GCCCTTCAGATTGTTGCA	CGTCTGACTCACACCTCACA

Immunostaining

A54 cells and HSCs were cocultured on Culture Slides (Falcon 354111, BD), and immunostained on day 5. The cells were fixed with 4% paraformaldehyde, and incubated with a rabbit anti-mouse Jag1 antibody (ab7771, Abcam, Cambridge, UK) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (A11008, Invitrogen) for 30 minutes on ice, respectively. Slides were inspected and photographed under an IX-70 microscope.

Statistical analysis

The statistical analysis was performed using a Statcel2 (OMS Publishing, Saitama, Japan) add-on package for Microsoft Excel. Student's t-test was used for comparison between two groups, and a multiple comparison test (the Turkey-Kramer method) was used for comparison among groups of three or more. A P-value < 0.05 was considered to be significant for all analyses.

Results

Requirement of cell contact in supporting hematopoeisis

In coculure experiments with 10T1/2 or its derivatives (A54 and M1601), HSCs formed cobblestone areas only on A54 at day 5 (12.5 ± 0.6 cobblestone areas/10^4 cells), as we reported previously [14]. We also confirmed that the murine hematopoietic progenitors were expanded by ninefold (36.0 ± 4.0 colonies/10^3 cells post-coculture with A54 vs. 4.0 ± 2.0 colonies/10^3 cells pre-coculture, Figure 1) whereas the number of hematopoietic progenitors was not significantly increased after the coculture with parental 10T1/2 cells or M1601 myoblasts (3.3 ± 1.5 colonies/10^3 cells with 10T1/2, and 6.3 ± 1.5 colonies/10^3 cells with M1601; Figure 1). Without stromal cell support, HSCs completely lost their progenitor activity in the same medium used for coculture ('No stroma' in Figure 1). Indeed, only few monocyte/macrophage-like cells were found after 5-day-incubation in this population; we speculated that all the clonogenic cells fell into apoptosis because no additional cytokine was provided. As we previously reported, expression of SCF, SDF1 and Ang1 in A54 was greater than that in 10T1/2 and M1601cells [14]. Such an overexpression of SCF, SDF1 and Ang1 in A54 cells was not further augmented or cancelled by addition of interleukin-1 or coculture with HSCs (data not shown).

Next, we addressed whether soluble factors from A54 preadipocytes were sufficient for supporting hematopoiesis. To discriminate the contribution of cell communication via direct adhesion, mouse bone marrow-derived HSCs were cultivated on a microporous membrane (1 micron Cell Culture Insert) placed in a well containing A54 cells underneath. We found that physical separation from A54 cells totally abrogated the progenitor activity of HSCs, in spite of the availability of soluble molecules from the stromal cells (Figure 1). This observation indicated that A54 cells supported hematopoiesis by cell contact in addition to secreting cytokines.

Gene expression changes in preadipocytes cocultured with HSCs

Upon learning that cell adhesion is required for A54 cells to support HSCs, we speculated that some signaling from HSCs to stromal cells might occur during coculture. This idea prompted us to search for molecules whose expression changed in A54 cells cocultured with HSCs. Total RNA was extracted from A54 cells before and after the coculture and gene expression profiles were compared by microarray. Out of 33696 genes screened, 353 genes were upregulated by twofold or more after the coculture with HSCs, and 13 genes were downregulated to the levels less than a half of starting value (data not shown). Among them, 29 genes (21 upregulated and 8 downregulated; Table 2) were implicated to be involved in hematopoiesis. Therefore we focused on these molecules for further investigation. A quantitative RT-PCR analysis confirmed the upregulation of Jagged 1 (Jag1), Delta-like 3 (Dll3) and bone morphogenetic protein 6 (BMP6) in A54 cells after coculture with HSCs (Figure 2). On the other hand, upregulation of Jag1, Dll3 and BMP6 was not observed in the undifferentiated 10T1/2 cells or M1601 myoblasts after coculture with HSCs (data not shown). Expression of Jag1 was also increased in A54 cells cultured with differentiated cells such as B- and T-lymphocytes, but to a lesser extent (Figure 2A).

Figure 1

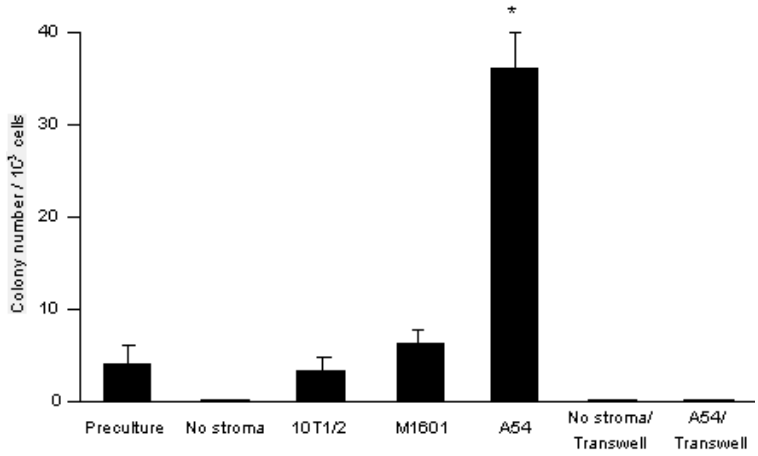

Figure 1. Requirement of direct contact for preadipocytes to support hematopoiesis. 1 x 10^4 mouse bone marrow Lin^-Sca1^+ cells (HSCs) were cocultured with 2 x 10^4 C3H10T1/2 cells (10T1/2), 10T1/2-derived myoblasts (M1602) or preadipocytes (A54) for 5 days. After coculture, HSCs were separated with immunomagnetic beads and subjected to colony assay. HSCs were seeded on MethoCult GF M3434 (1 x 10^3 cells/dish) and colonies were counted on day 7. Preculture: colonies from HSCs prior to coculture. No stroma: colonies from HSCs incubated 5 days without stromal support. No stroma/Transwell: colonies from HSCs seeded on the top wells, with no stromal cells in the bottom wells. A54/Transwell: HSCs were coincubated with A54 but separated by Cell Culture Insert. *: P < 0.01 (n = 3).

Figure 2

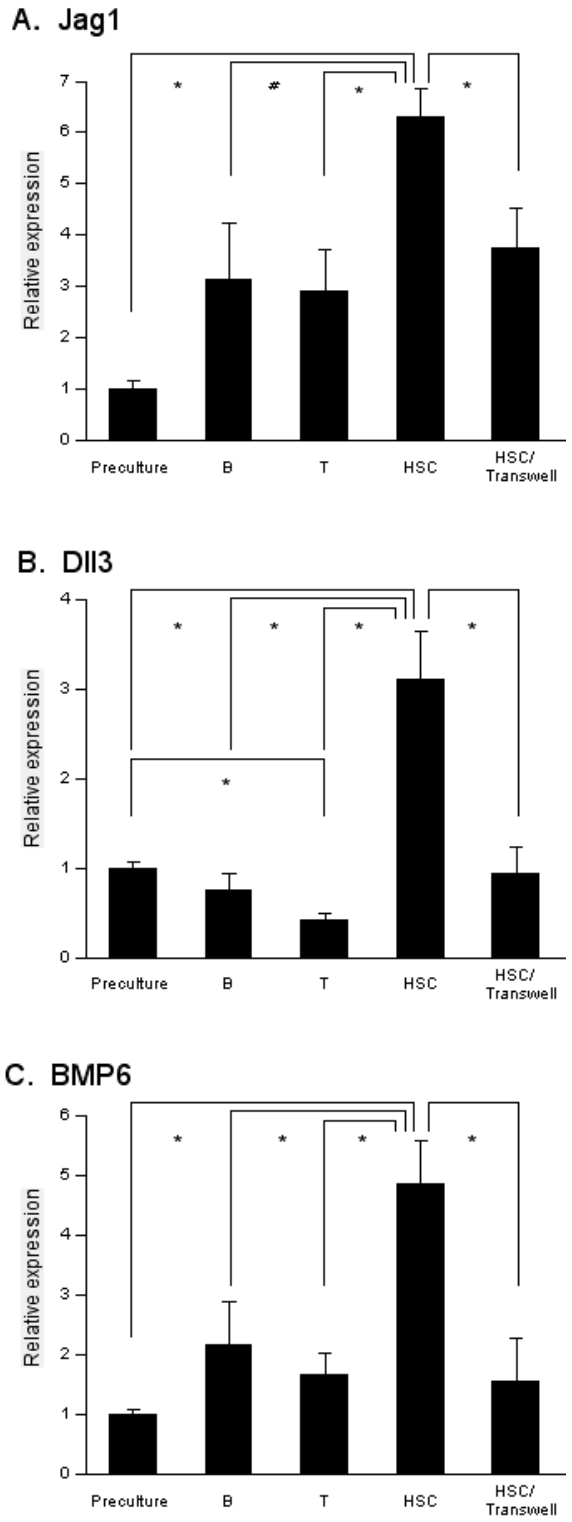

Figure 2. Elevated levels of Jag1, Dll3 and BMP6 in A54 cells cocultured with HSCs. Total RNA was isolated from 2 x 10^4 A54 cells prior to coculture (Preculture) and after 5-day-coculture with 1 x 10^4 B-lymphocytes (B), 1 x 10^4 T-lymphocytes (T) or 1 x 10^4 bone marrow Lin^-Sca1^+ cells (HSC). Expression of Jag1 (A), Dll3 (B) and BMP6 (C) was measured by quantitative RT-PCR with the primers listed in Table 1. HSC/Transwell: A54 cells were coincubated with HSCs but separated by Cell Culture Insert. *: $P < 0.01$ ($n = 3$).

Dll3 expression in A54 was decreased after coculture with T-cells, while it was not significantly changed after coculture with B-cells (Figure 2B). BMP6 expression was mildly upregulated in A54 cells cocultured with mature lymphocytes, but the difference was not statistically significant (Figure 2C). These results suggested that the upregulation of Jag1, Dll3 and BMP6 was induced by interaction with immature hematopoietic cells. Furthermore, such elevation was cancelled out when A54 cells were separated from HSCs with a microporous membrane (Figure 2). Immunostaining with an anti-Jag1 antibody confirmed that hematopoietic progenitors stimulated Jag1 expression in the neighboring A54 preadipocytes. Jag1 signal was apparently greater in A54 cells in cobblestoned areas than those in the surrounding regions (Figure 3). Taken together, mesenchymal stromal cell-derived preadipocytes received signals from HSCs through cell contact, resulting in multiple events including upregulation of Jag1, Dll3 and BMP6.

Activation of Notch signaling in HSCs

The coculture experiment showed that the expression of multiple Notch ligands (Jag1 and Dll3) was upregulated in A54 preadipocytes during their interaction with HSCs. We next addressed whether the expression of Notch receptors and downstream factors was altered in the HSCs.

Figure 3

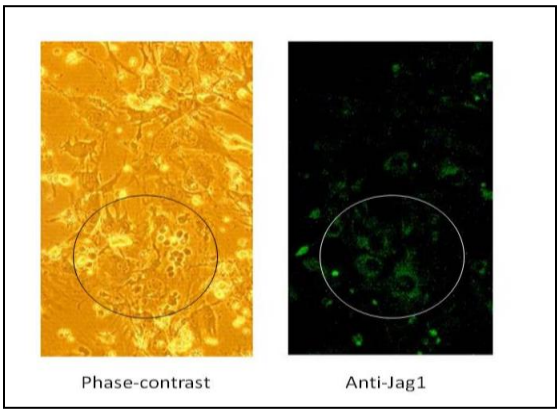

Figure 3. Jag1 expression in a cobblestone area. 1 x 10^4 murine bone marrow HSCs were incubated on 2 x 10^4 A54 cells for 5 days. Cells were immunostained with a rabbit anti-mouse Jag1 antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG. In the left panel (phase-contrast bright field, magnification × 400), a cobblestone area with small, rounded hematopoietic cells is indicated with a circle. In the right panel (immunofluorescent view, magnification × 400) showing the same location, several large, stretched stromal cells are positively immunostained (green), wheras stromal cells outside the cobblestone area are negative or weakly positive.

Table 2. Hematopoiesis-related genes upregulated or downregulated in cocultured A54 cells.

Upregulated or downregulated molecule	Before [a]	After [b]	Ratio [c]
Upregulated			
Mcl1 (Myeloid cell leukemia sequence 1)	116	514	4.42
Stom (Stomatin)	126	481	3.83
Foxk1 (Forkhead box K1)	129	464	3.58
Foxf2 (Forkhead box F2)	133	455	3.43
Bmp6 (Bone morphogenetic protein-6)	133	451	3.38
Itgbl1 (Integrin , beta-like 1)	135	447	3.30
Dll3 (Delta-like 3)	136	446	3.29
Arts1 (Adipocyte-derived leucine aminopeptidase)	136	446	3.28
Sox13 (SRY-box containing gene 13)	136	444	3.27
Cdc40 (Cell division cycle 40 homolog)	136	443	3.26
Jag1 (Jagged 1)	137	443	3.24
Shh (Sonic hedgehog)	137	440	3.20
Cdc16 (Cell division cycle 16 homolog)	143	423	2.95
Sox17 (SRY-box containing gene 17)	146	416	2.85
Cdc2l6 (Cell division cycle 2-like 6)	144	408	2.83
Col15a1 (procollagen , type XV)	147	408	2.77
Rras (Ras-related protein)	149	405	2.72
Pik3cb (phosphatidylinositol3-kinase, catalytic, b polypeptide)	147	401	2.72
Hoxa3 (Homeobox A3)	149	378	2.54
Adfp (Adipophilin, Adipose differentiation-related protein)	150	361	2.41
Cdc91l1 (cell division cycle 91-like 1)	146	324	2.22
Downregulated			
Nudt2 (nucleoside diphosphate linked moiety X - type motif 2)	3155	1337	0.42
Lrp2 (LDL receptor-related protein 2)	2210	963	0.44
TPO (thrombopoietin)	866	419	0.48
Taar1 (trace amine-associated receptor 1)	892	432	0.48
Centg3 (centaurin , gamma 3)	1932	942	0.49
Ccdc66	777	382	0.49
Olig1 (oligodendrocyte transcription factor 1)	1293	639	0.49
AA474455	1809	897	0.49

[a] Before: Expression (net intensity) in A54 cells before coculture.
[b] After: Expression in A54 cells after 3 days of coculture with HSCs.
[c] Ratio = After/Before.

A quantitative RT-PCR analysis revealed that the message level of Notch1 was elevated in HSCs cocultured with A54 cells by 3.6-fold (Figure 4A). Simultaneously, the expression of Hairy enhancer of split-1 (Hes1), a target of Notch1, was upregulated by 27-fold, indicating that the authentic signaling pathway was actually viable (Figure 4B). The results clearly showed that Notch ligands and receptors were upregulated in the stromal cells and HSCs in a reciprocal fashion through cell contact.

Discussion

In the present study, we investigated the molecular events in hematopoiesis supported by mesenchymal stromal cell-derived preadipocytes. We and other investigators reported that several preadipocyte-like stromal cells were capable of supporting hematopoiesis [13–16]. While osteoblasts enhance HSC self-renewal in the hematopoietic niche [2,3], preadipocytes may have additional or different function, such as promoting the asymmetric division of HSCs and proliferation of early progenitors. Our finding that A54 preadipocytes promoted the formation of cobblestone areas and colonies supports this idea, but such function may not be completely cell-autonomous. Assuming that some instructive signals from HSCs, we investigated the gene expression profile of A54 cells in a coculture with HSCs. Among the molecules upregulated or downregulated by more than twofold in a microarray screening, an increase of Notch ligands (Jag1 and Dll3) and BMP6 was confirmed in A54 cells. This finding is intriguing, because signals from Notch and BMP receptors are integrated in osteoblasts and other cell types [17-20]. Notch is a single-pass transmembrane receptor interacting with cell-bound ligands. So far, four Notch receptors (Notch1-4) and five ligands (Jag1-2 and Dll1, 3 and 4) have been identified in mammals. As in many cell systems, Notch signaling is essential for regulating HSCs and blood cell differentiation [21,22].

Previous studies showed that Jag1 in marrow stromal cells and osteoblasts promotes HSC proliferation [3,23], Dll1 produces cobblestone areas [24]. In the present study, we demonstrated that coculturing resulted in concomitant increases in Notch ligands (Jag1 and Dll3) in A54 preadipocytes and Notch signaling in HSCs. Notably, in accordance with an elevation in levels of the Notch1 receptor, expression of the transcription factor Hes1 was also upregulated. Hes1, one of the targets of Notch signaling, regulates specific groups of genes to maintain HSCs and early progenitors [25,26]. Thus, upregulation of the Notch1-Hes1 axis in HSCs strongly suggests that this signaling pathway is actually viable with the increase in Notch ligands in neighboring preadipocytes. Since Notch activation plus cytokine receptor signaling has a combined effect on hematopoietic cells [27], production of Notch ligands and cytokines (SCF, SDF1 and Ang1) by A54 cells is likely to represent a physiological role of marrow niche cells.

Figure 4

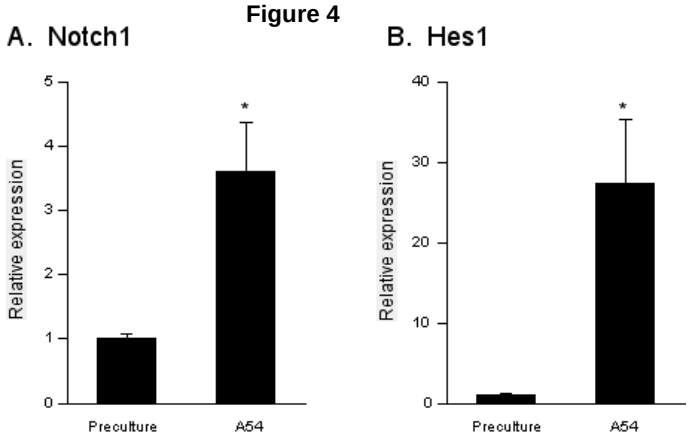

Figure 4. Expression of Notch1 and Hes1 in HSCs. Total RNA was isolated from 1 x 10^4 bone marrow Lin^-Sca1^+ cells prior to coculture and after 5-day-coculture with 2 x 10^4 A54 cells. Notch1 and Hes1 message levels were estimated by quantitative RT-PCR before (Preculture) and after coculture with A54 preadipocyres (A54). The primers for PCR are listed in Table 1. Both Notch1 (A) and Hes1 (B) were significantly upregulated in HSCs after coculture with A54 cells. * : $P < 0.01$ ($n = 4$).

Figure 5

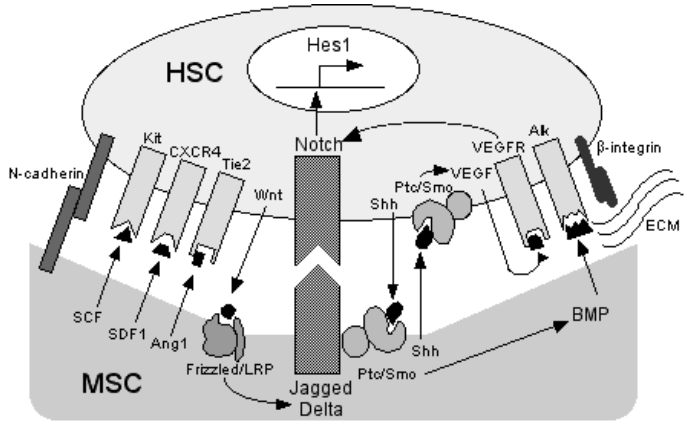

Figure 5. Interaction of MSCs and HSCs. Various factors are involved in the cell-to-cell interaction between MSCs and HSCs with spatial and temporal complexity. HSCs attach to MSCs via adhesion molecules such as N-cadherin and b-integrins (ECM: extracellular matrix secreted from MSC-derived cells). Soluble cytokines from MSCs such as SCF, SDF1 and Ang1 support the growth and differentiation of HSCs through cognate receptors (Kit, CXCR4 and Tie2). When HSCs and MSCs are in close proximity, expression of Notch ligands (Jagged and Delta-like) is upregulated in MSCs by Wnt from HSCs, while that of Notch receptors is upregulated in HSCs by Sonic hedgehog (Shh) from MSCs and HSCs. Vascular endothelial growth factor (VEGF) can further upregulate Notch receptor expression in an autocrine or paracrine fashion. The upregulated Notch signaling pathway induces the expression of downstream targets such as Hes1. The expression of bone morphogenic protein (BMP) is upregulated in MSCs by Shh signaling. Frizzled, Ptc/Smo, VEGFR, Alk: receptors for Wnt, Shh, VEGF and BMP, respectively.

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-b superfamily and have pleiotropic effects on tissue development. They have been implicated as key regulators in hematopoiesis: for example, BMP4 promotes the self-renewal of HSCs [28] and BMP6 enhances the formation of colonies [17]. Because of the redundancy of ligands/receptors and the dependence on the context and stage of differentiation of target cells, a detailed understanding of BMP signaling awaits further investigation.

The mechanisms responsible for the reciprocal upregulation of the expression of Notch ligands and receptors have yet to be clarified. We found that mature lymphocytes poorly induced Jag1, Dll3 and BMP6 expression in A54 cells, thus the stromal cells appear to receive the induction signal mainly from immature cells such as HSCs. Direct cell-to-cell contact with, or close proximity to HSCs was required for the upregulation of Jag1, Dll3 and BMP6 expression in A54 cells. Thus far several classes of molecules have been implicated to act upstream of Notch signaling. For example, the Wnt canonical pathway regulates the expression of Notch ligands in various cell types [29-31]. Sonic hedgehog (Shh) and receptors for Shh (Ptc/Smo) are expressed on primitive hematopoietic cells and marrow stromal cells and induce BMP expression [32,33]. In addition, Shh can increase levels of Notch receptors via vascular endothelial growth factor (VEGF) [34]. Of note, Wnt and Shh are lipid-modified proteins, and therefore appropriate for exerting effects on short-ranged target cells in a paracrine and/or autocrine fashion [35,36]. In the hematopoietic niche, these signaling networks must be precisely orchestrated to regulate HSC behavior, presumably through cross-talk with signals from adhesion molecules such as integrins (Figure 5). Further investigation of such communicative events will identify target molecules to improve engraftment in transplantation therapy.

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