NOTCH-Mediated Maintenance and Expansion of Human Bone Marrow Stromal/Stem Cells: A Technology Designed for Orthopedic Regenerative Medicine

YUFENG DONG, TENG LONG, CUICUI WANG, ANTHONY J. MIRANDO, JIANQUAN CHEN, REGIS J. O’KEEFE, MATTHEW J. HILTON

ABSTRACT

Human bone marrow-derived stromal/stem cells (BMSCs) have great therapeutic potential for treating skeletal disease and facilitating skeletal repair, although maintaining their multipotency and expanding these cells ex vivo have proven difficult. Because most stem cell-based applications to skeletal regeneration and repair in the clinic would require large numbers of functional BMSCs, recent research has focused on methods for the appropriate selection, expansion, and maintenance of BMSC populations during long-term culture. We describe here a novel biological method that entails selection of human BMSCs based on NOTCH2 expression and activation of the NOTCH signaling pathway in cultured BMSCs via a tissue culture plate coated with recombinant human JAGGED1 (JAG1) ligand. We demonstrate that transient JAG1-mediated NOTCH signaling promotes human BMSC maintenance and expansion while increasing their skeletogenic differentiation capacity, both ex vivo and in vivo. This study is the first of its kind to describe a NOTCH-mediated methodology for the maintenance and expansion of human BMSCs and will serve as a platform for future clinical or translational studies aimed at skeletal regeneration and repair.

INTRODUCTION

Bone marrow stromal/stem cells (BMSCs) are an adherent, nonhematopoietic fraction of the bone marrow stroma that contains a subset of clonogenic and multipotent cells, referred to as skeletal stem cells (SSCs), that are capable of regenerating a complete bone organ in vivo [1]. Human BMSCs have been studied with great interest because of their therapeutic potential for treating skeletal disease and facilitating skeletal repair, although maintaining their multipotency and expanding this heterogeneous group of cells ex vivo has proven to be very difficult, thereby limiting their use in clinical orthopedics [2–4]. Although BMSCs and other so-called “mesenchymal stem cells” have been used for a variety of preclinical and clinical therapeutic purposes by taking advantage of their suggested paracrine-based immune modulatory and anti-inflammatory properties [2, 5], relatively few preclinical or clinical trials are under way that focus on the capacity of BMSCs to act as true stem cells for the direct regeneration of skeletal tissues [3, 4]. The primary reason for this is that we are still learning the appropriate methods for BMSC enrichment of SSCs, the direct isolation of SSCs, and the appropriate BMSC/SSC ex vivo maintenance, expansion, and transplantation procedures. Though plastic adherence and clonal density culturing of BMSCs can serve to enrich this population with SSCs, there remain major hurdles to expanding these cells ex vivo while limiting their tendency to inappropriately differentiate and/or undergo senescence during cell passaging [2, 6–8]. Because large segmental bone grafting and repair of nonunions are some of the most critical and challenging uses for BMSCs/SSCs in orthopedic regenerative medicine, the ability to generate large numbers of the appropriate BMSCs/SSCs via the use of novel ex vivo isolation and culturing methods stands out as an issue of paramount importance [3, 4].

Various signaling factors have been implicated in the regulation of BMSC maintenance and expansion, including the NOTCH signaling pathway. In humans and mice, NOTCH signaling is initiated when the JAGGED1 (JAG1) or JAGGED2 (JAG2) or DELTA-LIKE 1, 3, or 4 (DLL1, DLL3, or DLL4) NOTCH ligands bind to cell surface NOTCH receptors (NOTCH1, NOTCH2, NOTCH3, or NOTCH4) on neighboring cells [9]. This interaction induces cleavage and release of the NOTCH intracellular domain (NICD), which translocates from the cell surface into the nucleus to activate gene expression via a NICD-RBPjκ–MAML transcriptional complex [9]. Some of the most well-defined canonical,
or RBPjκ-dependent, NOTCH target genes include specific members of the HES/HEY family of transcription factors: HES1, HES5, HES7, HEY1, HEY2, and HEY3, which are thought to mediate much of NOTCH function [10, 11]. Canonical NOTCH signaling is well-recognized as a regulator of stem cell differentiation, proliferation, and self-renewal in the hematopoietic, neural, pancreatic, intestinal, and skeletal muscle systems [12–17]. Recently, our group and others have identified the RBPjκ-dependent NOTCH pathway as an important inducer of mesenchymal progenitor cell (MPC) and BMSC proliferation and an inhibitor of MPC/BMSC differentiation during mouse limb-bud and postnatal bone development [18–20]. These mouse genetic studies demonstrated that conditional ablation of the NOTCH pathway components (NOTCH1, NOTCH2, RBPjκ, or HES/HEY factors) in early MPCs and BMSCs of the limbs resulted in enhanced chondrogenic and osteogenic differentiation followed by a depletion of the BMSC pool [18–20]. We also showed that sustained NOTCH activation in MPCs arrested chondrogenic and osteogenic differentiation, while expanding the targeted population of MPCs [18]. Several recent lines of data have suggested that this MPC/BMSC inhibition of differentiation may be mediated by one or more of the HES/HEY NOTCH-related factors [18, 20]. Consistent with our data, other studies have shown that sustained activation of the NOTCH pathway in cultured human BMSCs via viral infection with JAG1 or NICD1 suppressed chondrogenic differentiation [21]. Furthermore, treatment of cultured human BMSCs with the NOTCH inhibitor N-[25-3,5-difluorophenyl] acetyl-l-alanyl-2-phenyl-1,1-dimethylglycine (DAPT) reduced their proliferative capacity ex vivo [22]. Collectively, these data implicate the NOTCH pathway as an important regulator of BMSC proliferation and differentiation.

In this study, we set out to determine whether transient activation of the NOTCH pathway could promote the expansion and maintenance of human BMSCs/SSCs in culture for multiple passages, while preserving their skeletogenic differentiation capacity. Here we have investigated the following: (a) NOTCH component expression in human BMSCs, (b) human BMSC responsiveness to transient NOTCH activation via recombinant JAG1-coated plates, (c) the ability of JAG1-mediated NOTCH activation to induce human BMSC expansion and maintenance over multiple cell passages, and (d) the skeletogenic differentiation capacity of human BMSCs both in vitro and in vivo following their removal from JAG1-mediated and transient NOTCH activation.

**Materials and Methods**

**BMSC Culture and Selection**

Human BMSCs were purchased from Lonza (Walkersville, MD, http://www.lonza.com) and designated for our purposes as passage 0 (P0) cells. Cells were seeded at 5,000 cells per cm² in T75 flasks in 25 ml of BMSC growth medium (MSCGM; Lonza) until ~80% confluence (day 3 or 4) for expansion. A full medium exchange was performed on day 3 only if additional time in culture was needed to meet the desired confluence. Expanded BMSCs were harvested as passage 1 (P1), and a portion of these cells were further sorted using an immunomagnetic bead separation (Dynabeads; Invitrogen, Carlsbad, CA, http://www.invitrogen.com) approach, in which the NOTCH2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) was conjugated to magnetic beads. Both the P1 total BMSCs and NOTCH2-selected BMSCs were collected for further culture.

**Recombinant JAGGED1-Coated Culture Plates**

The human JAG1 recombinant protein (Enzo Life Sciences, Farmingdale, NY, http://www.enzolifesciences.com) contains the signal peptide and extracellular domain of JAG1 fused at the C terminus to the Fc portion of human IgG. JAG1-coated plates were generated using the protocol as previously described [23, 24]. Briefly, culture plates were coated with anti-human IgG (10 μg/ml) (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) in PBS at 4°C overnight and then incubated in a solution containing three different doses of recombinant JAG1 protein (5, 10, and 15 μg/ml) at 4°C overnight. The same concentrations of human IgG (Sigma-Aldrich) were used to coat plates as controls. JAG1 binding to culture plates was measured by chromogenic detection using JAG1 antibody (C-20; Santa Cruz) and an alkaline phosphatase-conjugated secondary antibody. Color reactions were visualized by incubation of one-step nitro blue tetrazolium /5-bromo-4-chloro-3-indolyl phosphate (Pierce, Rockford, IL, http://www.piercenet.com) for 60 minutes. All P1 BMSCs were cultured on JAG1- and IgG-coated plates for an additional 1, 4, and 9 passages prior to harvest at passages 2, 5, and 10 (P2, P5, and P10), respectively, for the following assays.

**Bromodeoxyuridine Labeling**

Cell proliferation assays were performed using a bromodeoxyuridine (BrdU) enzyme-linked immunosorbent assay (ELISA) kit (Roche, Indianapolis, IN, http://www.roche.com). Briefly, expanded P1 BMSCs were cultured on IgG- and JAG1-coated plates at 4,000 cells per cm² for 24 hours and then exposed to BrdU labeling reagent for 6 hours followed by incubation with FixDenat buffer for 30 minutes and detection with anti-BrdU-peroxidase working solution. Absorbance values were measured by a multi-mode microplate reader (BioTek Instruments, Winooski, VT, http://www.biotek.com) at 450 nm.

** Colony-Forming Unit-Fibroblast Assays**

Clonogenicity of BMSCs were analyzed using colony-forming unit-fibroblast (CFU-F) assays following one and nine passages on JAG1- and IgG-coated plates using P1 cells from each experimental and control group. CFU-F assays were performed as previously described [19]. Briefly, BMSCs were reseeded as single-cell suspensions of 100 BMSCs per well on 6-well plates for 14 days without a change of media. Cells were washed and fixed in 100% methanol followed by 5 minutes of Giemsa stain (Sigma-Aldrich). CFU-F colonies were identified as those containing more than 50 round or spindle-shaped cells in direct contact with one another. The frequency of CFU-Fs at each passage is correlated to the number of multipotent, clonogenic SSCs remaining during continuous passages of BMSCs.

**Flow Cytometry**

Flow cytometry was used for the detection of positive BMSC surface markers, CD105, CD166, and CD29. P2, P5, and P10 BMSCs cultured on standard 6-well plates or IgG/JAG1-coated plates were detached, washed, and stained with antibodies conjugated to either FITC, PE, or APC for 30 minutes. Flow cytometry was performed on a LSR-II flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). The data were analyzed using FlowJo software (Tree Star, Ashland, OR, http://www.treestar.com).
BMSC Differentiation Assay

Differentiation assays of BMSCs were performed using the following differentiation kits. For osteoblastic differentiation, BMSCs were removed from JAG1- or IgG-coated plates following passage 5 and cultured at 200,000 cells per well to confluence on standard culture dishes. Stem cell growth media were then replaced with osteogenic media supplied with the Differentiation Media BulletKit-Osteogenic (Lonza). RNA isolation and alkaline phosphatase staining were performed at 14 days of culture as previously described [18]. Quantitative alkaline phosphatase activity was also measured by StemTAG alkaline phosphatase activity assay kit (Cell Biolabs, Inc., San Diego, CA, http://www.cellbiolabs.com).

For chondrogenic differentiation, cell pellet cultures were performed using a BulletKit-Chondrogenic (Lonza) as per the manufacturer’s instructions and harvested for RNA isolation and histology at 21 days. Histological sections were stained with Alcian blue and toluidine blue according to our online protocols [25]. Quantitative analyses of alkaline phosphatase staining and Alcian blue- and toluidine blue-positive areas were performed using Visiomorph image analysis software (Visiopharm, Hørsholm, Denmark, http://www.visiopharm.com).

In Vivo Ectopic Bone-Formation Assay in Immune-Deficient Mice

1.0 × 10^6 total or NOTCH2-selected P5 BMSCs from JAG1-coated plates were resuspended in 80 μl of BMSC growth media and gently mixed with 50 mg of hydroxyapatite (HA) powder (Himed, Old Bethpage, NY, http://www.himed.com). The composites were then centrifuged for 2 minutes at 200 rpm to form a BMSC/HA pellet. For ectopic bone formation surgery, ten 8-week-old NOD.CB17-Prkdcscid/J mice (Charles River, Kingston, NY, http://www.criver.com) (up to two implants for each animal) were anesthetized, and the dorsal skin was cleaned with 70% ethanol.

Statistical Analysis

The results are expressed as means ± SD. Differences between groups were examined for statistical significance using Student’s t test or analysis of variance.

Expression of NOTCH and Stem Cell-Related Molecules in Cultured Human BMSCs

To determine whether the NOTCH pathway could be used to promote the maintenance and expansion of BMSCs derived from human bone marrow, we first analyzed the expression of all NOTCH receptors and each of the HES/HEY genes using human BMSCs cultured over multiple passages. Our results demonstrate that all NOTCH genes (NOTCH1, NOTCH2, NOTCH3, and NOTCH4) and most of the HES/HEY genes were expressed in BMSCs. Interestingly, NOTCH2 (Fig. 1A) and HES1 (Fig. 1B) were identified as the most highly expressed NOTCH components in early passaged BMSCs. This is consistent with our previous data analyzing NOTCH component expression and function in MPCs isolated from the embryonic mouse limb bud [18]. Because NOTCH signaling is thought to be an early regulator of MPC “stemness,” it would be likely that the expression of NOTCH molecules identified in early passaged BMSCs (NOTCH2 and HES1) would decrease as cells were passaged for several generations, slowly losing their stem-like properties. A similar rational would also apply to important transcriptional regulators of stemness including OCT4, SOX2, and NANOG, all of which are expressed in BMSCs. Although we did detect protein expression of these transcription factors in BMSCs (data not shown), here only real-time qPCR data are analyzed. Therefore, we performed real-time qPCR experiments analyzing the gene expression of NOTCH2, HES1, OCT4, SOX2, and NANOG from P2 and P10 BMSCs. Cells were passaged on standard culture plates in MSCGM, so as not to induce their differentiation. Our data indicate that the NOTCH molecules (NOTCH2 and HES1) and the multipotent stem cell regulators (OCT4, SOX2, and NANOG) displayed reduced expression in P10 as compared with P2 BMSCs (Fig. 1C). To further confirm the age-related loss of NOTCH2 expression in passaged BMSCs, we also performed flow cytometry using antibodies against the extracellular domain of NOTCH2. These data demonstrated a greater than 50% reduction in NOTCH2-positive BMSCs at P10 as compared with P2 (Fig. 1D). We also analyzed the presence of a typical BMSC surface marker, CD105, on P2 and P10 BMSCs to confirm the altered stem cell-like phenotype generated during passaging. These data show...
a significant decrease in CD105-positive cells when comparing the P2 and P10 BMSCs (Fig. 1D). Collectively, these results indicate a potential role for NOTCH2 and NOTCH signaling in maintaining BMSC stemness during ex vivo passaging.

JAG1-Mediated NOTCH Activation in Cultured BMSCs

Previously, we determined that NOTCH signaling maintains and expands mouse MPCs via a JAG1-NOTCH2-HES1 signaling axis during mouse limb-bud development and that this sustained NOTCH signal blocks chondrogenic and osteogenic differentiation from MPCs [18]. From these data, we hypothesized that transient activation of the NOTCH pathway in cultured human BMSCs would promote cell proliferation and maintain their stem-like phenotype in culture while also allowing the cells to undergo chondrogenic or osteogenic differentiation when removed from the temporary NOTCH signal. To test this hypothesis, we established a protocol for coating culture dishes with recombinant JAG1 protein using 5, 10, and 15 μg/ml concentrations of JAG1 and 10 μg/ml of IgG as controls. Ligand-mediated NOTCH activation requires that the ligand be tethered to a substrate (i.e., cell, culture dish, or artificial clustering of the ligand). To test for even coating of the JAG1 recombinant protein, we performed a chromogenic staining of the coated plates using an anti-JAG1 antibody followed by color reaction. These data demonstrate that a maximal and even coating of the plates is achieved at a concentration of 10 μg/ml recombinant JAG1 (Fig. 2A). Higher concentrations did not appear to increase the yield of JAG1 bound to the dish. Alternatively, the 5 μg/ml JAG1 concentration exhibited a reduced staining intensity and an uneven distribution of protein throughout the dish. IgG control plates showed no color reaction as expected for a plate lacking JAG1 protein (Fig. 2A). To further confirm JAG1-mediated induction of HES1, we performed Western blots to detect HES1 protein expression. Our results demonstrate that the JAG1-coated plates significantly induce HES1 in cultured BMSCs at 24 hours (Fig. 2D). It is also of note that the BMSCs grown on both IgG- and JAG1-coated plates display no obvious changes in cell size, shape, or cell survival.
induction of this network of stem cell factors. To determine whether NOTCH activation regulates the proliferation of BMSCs. We performed BrdU ELISAs using P2 BMSCs cultured on JAG1- and IgG-coated plates for 24 hours. Our data demonstrate that JAG1-mediated NOTCH signaling increases BrdU incorporation by more than 50% as compared with BMSCs grown on IgG plates (Fig. 2F). Taken together, these data support the concept that JAG1-coated plates can induce NOTCH signaling in cultured BMSCs and that JAG1-mediated NOTCH activation can promote the expression of stem cell regulatory molecules and BMSC proliferation.

**Transient NOTCH Activity Maintains Human BMSCs While Preserving Their Differentiation Potential**

A common assay performed to measure the clonogenic potential (or stem cell nature) of BMSCs is the CFU-F assay, which measures the ability of bone marrow-derived cells to grow in a density-insensitive fashion. To determine whether JAG1-mediated NOTCH activation of BMSCs can maintain clonogenic populations over multiple cell passages, we cultured human BMSCs on JAG1 and IgG-coated plates for one and nine passages followed by plating at clonal density. Data from these experiments indicated that JAG1-mediated NOTCH activation maintains almost 1.5-fold more clonogenic cells as compared with control (IgG) cultured BMSCs after 9 passages (Fig. 3A). We did not see a significant difference in CFU-Fs from P2 BMSC cultures, which were only grown on JAG1 or IgG plates for a single passage. Furthermore, our data show that CFU-Fs are reduced from P10 BMSCs as compared with those from P2 cultures, suggesting a loss of clonogenicity with time and BMSC passage (Fig. 3A). Collectively, these data demonstrate that JAG1-mediated NOTCH signaling suppresses time- and passage-dependent loss of BMSC clonogenicity, which is consistent with our prior data demonstrating significant induction of stem cell regulatory factors.

To determine whether prolonged and exogenous activation of NOTCH signaling in cultured BMSCs impairs their differentiation potential, we performed osteogenic and chondrogenic differentiation assays of human BMSCs after four passages on JAG1 or IgG plates. Following the fifth passage, BMSCs from each group were cultured in osteogenic conditions or in chondrogenic pellet cultures and assayed for osteogenic activity (alkaline phosphatase [ALP] staining), ALP activity (Fig. 3B), and chondrogenic differentiation (toluidine blue staining and COL2A1 IHC).
ALP staining demonstrated that BMSCs receiving JAG1-mediated NOTCH activation for four passages were not only able to undergo osteoblastic differentiation, but they also showed a mild enhancement of ALP activity as compared with control BMSCs (Fig. 3B). Similarly, toluidine blue and COL2A1 staining and quantification of sections from chondrogenic pellet cultures indicated that JAG1-mediated NOTCH activation over four passages had no detrimental effect on BMSCs undergoing chondrogenesis (Fig. 3C). Because these cells were grown at a higher than standard density, we cannot exclude the possibility that the lack of a major difference between the groups during in vitro differentiation and the CFU-F assay is due to the more frequent cell-cell contacts. These data provide further proof that exogenous and transient NOTCH signals can promote the maintenance and expansion of BMSCs ex vivo without interfering with normal human BMSC differentiation.

**NOTCH2-Positive BMSCs May Be an Important Subpopulation of Total BMSCs**

Because JAG1-mediated NOTCH activation appears to promote the ex vivo maintenance and expansion of traditionally isolated BMSCs (total BMSCs), we set out to determine whether a NOTCH2-selected or enriched human BMSC population would represent a more readily expandable and clonogenic group of BMSCs when combined with our JAG1 activation approach. To determine the purity of NOTCH2-selected BMSCs, flow cytometry was performed using a NOTCH2 antibody. Our data clearly showed that more than 90% of NOTCH2-selected BMSCs were positive for NOTCH2 receptors (supplemental online Fig. 1). We first compared gene expression profiles for NOTCH signaling components and important BMSC regulatory genes from P5 NOTCH2-selected and total BMSCs that were cultured on IgG or JAG1 plates for four passages. The data from qPCR studies show that JAG1-mediated NOTCH activation in NOTCH2-selected BMSCs show elevated expression for \( \text{NOTCH2} \) and \( \text{HES1} \) as compared with either BMSC group cultured on IgG plates or total BMSCs cultured on JAG1 plates (Fig. 4A). Similar results were also observed when analyzing the expression of the stem cell and proliferation marker genes (\( \text{SOX2}, \text{OCT4}, \text{NANOG}, \) and \( \text{CCND1} \)). As previously demonstrated in Figure 2E, JAG1-mediated NOTCH activation of total human BMSCs enhances the expression of stem cell regulatory genes. Interestingly, this JAG1-induced expression of \( \text{SOX2}, \text{OCT4}, \) and \( \text{NANOG} \) is further potentiated in NOTCH2-selected BMSCs when compared with total BMSCs cultured on JAG1-coated plates (Fig. 4A). Based on these data, we next used flow

**Figure 3.** JAG1-mediated maintenance of total human bone marrow-derived stromal/stem cell (BMSC) clonogenicity and differentiation capacity. (A): CFU-F assays performed using total P2 BMSCs (cultured on IgG- or JAG1-coated plates for 1 passage) and P10 BMSCs (cultured on IgG- or JAG1-coated plates for nine passages). The results are representative of three independent experiments and are expressed as means ± SD. #, \( p < .05 \) compared with P2 IgG/total BMSCs; *, \( p < .05 \) compared with P10 IgG/total BMSCs. (B): ALP staining and quantitative ALP activity was performed using passage 5 (P5) BMSCs previously cultured on IgG- or JAG1-coated plates for 4 passages before being reseeded and cultured in osteogenic media for 14 days (magnification, \( \times 5 \)). (C): Toluidine blue and COL2A1 staining were used to evaluate chondrogenesis of P5 BMSCs previously cultured on IgG- or JAG1-coated plates for 4 passages before being reseeded for 21-day pellet culture. Quantifications of Tol Blue staining were performed using Visiomorph software. Abbreviations: ALP, alkaline phosphatase; CFU-F, colony-forming unit-fibroblast; JAG1, JAGGED1; P2, passage 2; P10, passage 10; Tol Blue, toluidine blue.
cytometry analysis for the BMSC surface marker, CD105, to determine whether NOTCH2-selected BMSCs represent a population of cells with a higher capacity to maintain their stem cell-like phenotype than total BMSCs. To test this, we cultured NOTCH2-selected and total BMSCs on IgG or JAG1 plates for 5 or 10 passages and performed flow cytometry for CD105. These results are consistent with previous data (Fig. 1D), such that both total BMSCs and NOTCH2-selected BMSCs cultured on IgG plates show a reduced percentage of CD105 expressing BMSCs at P10 as compared with P5, although NOTCH2-selected BMSCs retain approximately 10% more CD105-positive cells than total BMSCs at P10 (Fig. 4B). Furthermore, JAG1-mediated NOTCH activation of total BMSCs maintains a higher percentage of CD105 expressing cells at P10 than those cultured on IgG plates (76.9% vs. 59.8%; Fig. 4B). Consistent with our gene expression data, JAG1 induction of NOTCH2-selected BMSCs demonstrated the greatest maintenance of CD105 expressing BMSCs at P10 as compared with all other BMSC groups (87.5% vs. 76.9%, 69.9%, and 59.8%; Fig. 4B). Similar results were also obtained for the human BMSC markers, CD29 and CD166 (data not shown). Finally, to determine whether the NOTCH2-selected BMSCs are a more clonogenic population than total BMSCs, we performed CFU-F assays with these two BMSC populations after four passages on JAG1-coated plates. These data demonstrate that NOTCH2-selected BMSCs have an approximate 75% increase in CFU-F colonies when compared with total BMSCs cultured on JAG1-coated plates for four passages (Fig. 4C). Combined, our data suggest that NOTCH2-selected BMSCs may represent a more sustainable and clonogenic subset of human BMSCs, which can be further maintained via JAG1-mediated NOTCH activation.

The proliferative capacity of BMSCs plays a significant role in their utility as a potential therapeutic tool in orthopedic regenerative medicine. During ex vivo culture of human BMSCs, it has been noted that their proliferative capacity diminishes over time, limiting the number of BMSCs available for therapeutic approaches. This is further demonstrated in Figure 1A, where P10 BMSCs show a near 50% decrease in CCND1 expression. Here we also show that JAG1-mediated NOTCH activation can significantly induce CCND1 expression in both total and NOTCH2-selected BMSCs (Fig. 4A). Finally, we performed proliferation studies with a BrdU ELISA using total and NOTCH2-selected BMSC populations cultured on JAG1 plates for 24 hours. Our data show that NOTCH2-selected BMSCs have a 2.5-fold increase in cell proliferation as compared with total human BMSCs.
following NOTCH activation (Fig. 4D). These data further indicate that NOTCH2-selected BMSCs represent a cell population with an enhanced potential for BMSC maintenance and expansion.

**NOTCH2-Selected, Maintained, and Expanded BMSCs Show a Robust Differentiation Potential Following JAG1-Mediated NOTCH Activation**

To compare the differentiation potential of total and NOTCH2-selected human BMSCs, we performed both in vitro and in vivo differentiation assays. First, we repeated the in vitro chondrogenic differentiation cultures following JAG1-mediated maintenance and expansion of total and NOTCH2-selected BMSCs. BMSCs were cultured for up to five passages on JAG1 plates and then cultured as chondrogenic pellets for 21 days in chondrogenic media. Following chondrogenic differentiation, some pellets were sectioned for histology, whereas others were used to extract RNA for qPCR analyses. Alcian blue staining of chondrogenic cell pellet sections and quantification of positive area show that the pellets from NOTCH2-selected BMSCs had a more intense cartilage matrix staining as compared with total BMSC chondrogenic pellets (Fig. 5A). Real-time qPCR results also indicate an increased expression of chondrogenic markers, SOX9, COL2A1, and ACAN1, in NOTCH2-selected chondrogenic pellets (Fig. 5A). This further confirmed an enhanced chondrogenic differentiation capacity of NOTCH2-selected BMSCs.

We also performed the in vitro osteogenic differentiation cultures following JAG1-mediated maintenance and expansion of total and NOTCH2-selected BMSCs. BMSCs were cultured for up to five passages on JAG1 plates and then cultured in monolayer for 14 days in osteogenic media on standard dishes. Following osteogenic differentiation, some cultures were assayed for ALP staining and quantification of positive area, whereas others were used to extract RNA for qPCR. Similar to the chondrogenic differentiation assays, NOTCH2-selected BMSCs demonstrated an enhanced capacity for osteogenic differentiation when compared with total BMSCs as assessed by ALP staining (Fig. 5B). Additionally, osteogenic cultures from NOTCH2-selected BMSCs exhibited enhanced expression of osteogenic markers such as COL1A1, ALP, and OCN as compared with cultures from total BMSCs (Fig. 5B).

To further confirm the capacity of these human BMSCs to develop cartilage and bone tissue in vivo, we performed ectopic ossicle assays using 8-week-old nude mice. Total and NOTCH2-selected BMSCs were first cultured on JAG1 plates for five passages, mixed with HA ceramic powder, and centrifuged into BMSC/HA pellets prior to subcutaneous transplantation into nude mice. After 8 weeks of growth and differentiation, we harvested the pellets for histological and immunohistochemical analyses. These analyses showed that only pellets from NOTCH2-selected cells cultured on JAG1 plates exhibited significant bone-like matrix as revealed by trichrome, picrosirius red, and H&E staining (Fig. 5C, 5D). Approximately 22% of the pellet area from NOTCH2-selected BMSCs cultured on JAG1 plates stained positive for a type I collagen bone matrix as compared with approximately 4% of the pellet area from total BMSCs cultured on JAG1 plates (Fig. 5C). Furthermore, H&E staining of BMSC/HA pellet sections demonstrated that the NOTCH2-selected BMSC pellets displayed both a well-developed solid bone-like matrix (right side of image) with transitions to a loose bone-like matrix (left side of image), whereas total BMSC pellets displayed only a loose bone-like matrix or cell dense and fibrotic regions with little to no bone matrix. Immunohistochemical analyses showed an abundance of OSX, a marker of immature osteoblasts, expressing flattened and cuboidal osteoblast-like cells adjacent to either the bone-like matrix and/or HA scaffold in the NOTCH2-selected BMSC/HA pellet sections (Fig. 5D). Comparatively little OSX expression was observed in the sections of pellets from total BMSCs cultured on JAG1 plates (Fig. 5D). It is also of note that only the NOTCH2-selected BMSC pellets developed sporadic clusters of chondrocytes near the periphery of the BMSC/HA pellets, whereas total BMSC/HA pellets did not develop any chondrocyte clusters (data not shown). These data further suggest an enhanced capacity for both osteogenic and chondrogenic differentiation using NOTCH2-selected BMSCs cultured on JAG1 plates. Interestingly, cell pellets produced from human BMSCs without culturing on JAG1 plates demonstrated hardly any signs of bone or cartilage formation in vivo (data not shown). Based on all of our findings, we propose that the NOTCH2-selected population of BMSCs represents a uniquely expandable and maintainable subset of BMSCs with an enhanced capacity for osteogenic and chondrogenic differentiation that could be exploited for orthopedic regenerative medicine and therapeutic applications.

**Discussion**

The ability of BMSCs to induce skeletal tissue regeneration or repair can be largely predicted based on CFU-F concentrations, or in other terms, the concentration of SSCs in heterogeneous BMSC populations is critical for successful bone regeneration and translation to the clinic. This has been highlighted by several basic and clinical studies using BMSC populations and/or bone marrow aspirates (BMAs) for transplantation and bone regeneration [4]. In one study of more than 60 patients undergoing bone reconstruction and being treated with BMSCs/BMA, 7 patients failed to heal properly, and it was only these patients that showed dramatically reduced numbers and concentrations of CFU-Fs from the transplanted BMSCs/BMA [27]. Studies like this one suggest that a major shortcoming of orthopedic regenerative medicine lies in our inability to appropriately select, enrich, maintain, and expand useful BMSC/SSC populations. To address these issues, research efforts have recently focused on a variety of approaches to select, expand, and maintain BMSCs/SSCs ex vivo.

To discriminate BMSCs/SSCs from other bone marrow-derived stem cells including hematopoietic stem cells (HSCs) or endothelial progenitor cells, a number of BMSC surface markers including CD29, CD90, CD105, CD106, CD146, and CD166 [28] have been used for positive selection or identification, whereas the hematopoietic and endothelial lineage markers including CD11b, CD34, CD31, and CD45 [29, 30] are used for negative selection/identification. Here we demonstrated that following adherence to plastic, BMSCs could be further enriched for NOTCH2-positive cells and that these cells may represent a unique subpopulation of BMSCs that are expandable and can be maintained over multiple passages using our transient NOTCH activation strategy.

However isolated or selected, BMSCs tend to lose their proliferative capacity, suggesting senescence [6], while also demonstrating an impaired differentiation capacity when extensively passaged ex vivo. Growth factors such as FGF2, FGF4, and several WNT agonists can promote the expansion of BMSC populations and prevent senescence ex vivo, although their effects on BMSC differentiation are variable and potentially deleterious for bone regeneration applications [31–34]. Recently, the NOTCH ligand,
DLL1, via signaling through the NOTCH1 receptor was shown to be important in regulating the expansion and repopulating ability of human HSCs and umbilical cord blood progenitors in vivo and ex vivo [16, 35–39]. Here we analyzed the ability of JAG1-mediated, transient NOTCH2 signaling to maintain and expand human BMSCs ex vivo, because (a) JAG1 is significantly expressed in BMSC populations both in vivo and ex vivo [40] (Fig. 1); (b) mouse genetic studies demonstrated that NOTCH loss of function leads to enhanced chondrogenic and osteogenic differentiation followed by a depletion of BMSCs/MPCs [18–20], whereas sustained NOTCH activation induced MPC proliferation and arrested MPC differentiation [18]; (c) JAG1 and NOTCH2 human mutations are linked to BMSC-associated skeletal diseases such as low bone mineral density, osteoporotic fractures, and Hedjui-Cheney syndrome [41–43]; and (d) JAG1 signals preferentially via NOTCH2 in other cell signaling contexts [44]. Collectively, our data demonstrate that transient NOTCH activation, specifically via a JAG1-NOTCH2 signaling axis, can promote BMSC proliferation and maintenance ex vivo while preserving their chondrogenic and osteogenic differentiation potential.

To assess BMSC/SSC maintenance of clonogenic potential, researchers have used the CFU-F assay to estimate the number of SSCs (clonogenic and self-renewing fraction) within the heterogeneous BMSCs [1]. Forced expression of some of the stem cell

**Figure 5.** NOTCH2-selected human BMSCs show enhanced chondrogenic and osteogenic capacity following JAG1-mediated expansion. (A): Alcian blue staining of pellet culture sections, quantification of Alcian blue-positive area, and quantitative polymerase chain reaction (qPCR; SOX9, COL2A1, and ACAN1) analyses of chondrogenesis from 21-day chondrogenic pellet cultures. Total and N2+ BMSCs were previously cultured for 4 passages on JAG1-coated plates prior to chondrogenic differentiation (magnification, ×2.5). (B): ALP staining, quantification of ALP-positive area, and qPCR (COL1A1, ALP, and OC) analyses of osteogenesis from 14-day osteogenic differentiation cultures. Total and N2+ BMSCs were previously cultured for 4 passages on JAG1-coated plates prior to osteogenic differentiation. Gene expression data are represented as means ± SD of three independent experiments performed in triplicate. Total BMSC gene expression levels were set at 1 (magnification, ×1). (C): Trichrome and picrosirius red staining of tissue sections from the ectopic ossicle formation assays performed with total and N2+ BMSCs that were previously cultured for 4 passages on JAG1-coated plates and transplanted to nude mice. Red staining denotes a COL1A1 rich bone-like matrix in the N2+ BMSC ossicles with limited staining of the total BMSC sections. Areas of bone-like matrix staining on trichrome-stained sections were quantified using OsteoMeasure software for both groups (n = 3; magnification, ×20). (D): H&E staining and OSX IHC analyses of tissue sections from N2+ BMSC ossicles and total BMSC tissues. Note the OSX-positive osteoblastic cells near the surface of bone matrix and/or hydroxyapatite particles. *p*, significant differences between N2+ and total BMSCs (p < .05). Abbreviations: ALP, alkaline phosphatase; BMSCs, bone marrow-derived stromal/stem cells; H&E, hematoxylin and eosin; IHC, immunohistochemistry; N2+, NOTCH2-selected; OSX, Osterix.
transcriptional regulators (SOX2, OCT4, and NANOG) can promote the maintenance and clonogenicity of BMSCs in culture [45–48]. Our data clearly show a NOTCH-mediated enhancement in both the expression of these stem cell regulatory factors and in CFU-Fs of cultured BMSCs during passaging, suggesting a potential functional link between JAG1-induced NOTCH signaling, SOX2, OCT4, NANOG, and the ex vivo expansion and maintenance of human BMSCs.

NOTCH regulation of bone and bone marrow-related cells is complex. Mouse genetic studies have provided striking evidence that sustained activation of NOTCH signals in MPCs inhibits cartilage and bone formation while inducing MPC proliferation [18]. Alternatively, NOTCH sustained activation in committed osteoblasts promotes osteoblast proliferation and activity, leading to an osteosclerotic high bone mass phenotype [49]. NOTCH loss-of-function studies have also indicated that removal of NOTCH signaling components in MPCs enhances early cartilage and bone formation followed by a depletion of the BMSCs/MPCs, whereas loss of NOTCH in differentiated osteoblasts primarily results in low bone mass phenotypes caused by an osteoblast-mediated osteoclasic enhancement [19, 20, 50]. This complexity is further observed in the ex vivo culture of BMSCs exposed to JAG1 in either maintenance/growth conditions (as seen here) or in the context of differentiation cues. We have demonstrated that JAG1-mediated NOTCH activation of BMSCs in MSCGM (Lonza) induces BMSC expansion and maintenance over multiple cell passages, whereas a recent report indicates that NOTCH signaling activation of BMSCs via JAG1 can promote osteoblastic differentiation or expansion when cultured in osteogenic differentiation media [51].

CONCLUSION

The NOTCH pathway is an important regulator of human BMSC maintenance and expansion ex vivo. We have shown for the first time that (a) NOTCH2 may be used as an additional selection criteria for human BMSCs when maintaining and expanding cells ex vivo; (b) immobilized, recombinant JAG1-coated plates can be used as a tool for the NOTCH-mediated maintenance and expansion of BMSCs; (c) NOTCH2-selected and JAG1-activated BMSCs have enhanced chondrocyte and osteoblast differentiation capacity ex vivo and in vivo; and (d) JAG1-mediated and transient NOTCH activation of BMSCs may serve as an important step in generating clinically relevant numbers of BMSCs/SSCs for use in skeletal tissue regeneration and repair.

ACKNOWLEDGMENTS

This work was supported in part by the following U.S. National Institute of Health and foundation grants: NIH Grants R01-AR057022 and R01-AR063071 (to M.J.H.), NIH Grant R21-AR059733 (to M.J.H.) and R21-AR063803 (to Y.D.), Airlift Research Foundation Grant 108468 (to Y.D.), and NIH Core Center Grant P30-AR061307. We also acknowledge the technical expertise and assistance of Sarah Mack, Kathy Maltby, and Ashish Thomas at the University of Rochester Center for Musculoskeletal Research Histology, Biochemistry, and Molecular Imaging Core.

AUTHOR CONTRIBUTIONS

Y.D.: financial support, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; T.L., C.W., A.J.M., and J.C.: collection and/or assembly of data, final approval of manuscript; R.J.O.: administrative support, final approval of manuscript; M.J.H.: conception and design, financial support, administrative support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflict of interests.

REFERENCES

1. Bianco P, Robey PG, Saggio I et al. “Mesenchymal” stem cells in human bone marrow (skeletal stem cells): A critical discussion of their nature, identity, and significance in incurable skeletal disease. Hum Gene Ther 2010;21:1057–1066.
2. Bianco P, Cao X, Frenette PS et al. The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat Med 2013;19:35–42.
3. Chatterjee A, Meijer G, van Blitterswijk C et al. Clinical application of human mesenchymal stromal cells for bone tissue engineering. Stem Cells Int 2010;2010:215625.
4. Dawson JJ, Kanczler J, Tare R et al. Concise review: Bridging the gap: Bone regeneration using skeletal stem cell-based strategies: Where are we now? Stem Cells 2014;32:35–44.
5. Burdon TJ, Paul A, Noisoux N et al. Bone marrow stem cell derived paracrine factors for regenerative medicine: current perspectives and therapeutic potential. Bone Marrow Res 2011;2011:207326.
6. Degroof CM, Stokes D, Colter D et al. Propagation and senescence of human marrow stromal cells in culture: A simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Br J Haematol 1999;107:275–281.
7. Li Z, Liu C, Xie Z et al. Epigenetic dysregulation in mesenchymal stem cell aging and spontaneous differentiation. PLoS One 2011;6:e20526.
8. Stenderup K, Justesen J, Clausen C et al. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. Bone 2003;33:919–926.
9. Kopan R, Ilagan MX. The canonical Notch signaling pathway: Unfolding the activation mechanism. Cell 2009;137:216–233.
10. Iso T, Kedes L, Hamamori Y. HES and HERP families: Multiple effectors of the Notch signaling pathway. J Cell Physiol 2003;194:237–255.
11. Iso T, Sartorelli V, Poirzet C et al. HERP, a novel heterodimer partner of HES/ESPIN in Notch signaling. Mol Cell Biol 2001;21:6080–6088.
12. Apelqvist A, Li H, Sommer L et al. Notch signalling controls pancreatic cell differentiation. Nature 1999;400:877–881.
13. Imayoshi I, Sakamoto M, Yamaguchi M et al. Essential roles of Notch signaling in maintenance of neural stem cells in developing and adult brains. J Neurosci 2010;30:3489–3498.
14. Jensen J, Pedersen EE, Galante P et al. Control of endodermal endocrine development by Hes-1. Nat Genet 2000;24:36–44.
15. Mourikis P, Sambasivan R, Castel D et al. A critical requirement for notch signaling in maintenance of the quiescent skeletal muscle stem cell state. Stem Cells 2012;30:243–252.
16. Stier S, Cheng T, Dombkowski D et al. Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. Blood 2002;99:2369–2378.
17. Varnum-Finney B, Xu L, Brashem-Stein C et al. Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. Nat Med 2000;6:1278–1281.
18. Dong Y, Jesse AM, Kohn A et al. RBPJKappa-dependent Notch signaling regulates mesenchymal progenitor cell proliferation and differentiation during skeletal development. Development 2010;137:1461–1471.
19. Hilton MJ, Tu X, Wu Y et al. Notch signaling maintains bone marrow mesenchymal progenitors by suppressing osteoblast differentiation. Nat Med 2008;14:306–314.
20. Tu X, Chen J, Lim J et al. Physiological notch signaling maintains bone homeostasis via RBPjk and Hey upstream of Nfatc1. PLoS Genet 2012;8:e1002577.
21. Oldershaw RA, Tew SR, Russell AM et al. Notch signaling through Jagged-1 is necessary...
to initiate chondrogenesis in human bone marrow stromal cells but must be switched off to complete chondrogenesis. Stem Cells 2008;26:666–674.

22 Vujovic S, Henderson SR, Flanagan AM et al. Inhibition of gamma-secretases alters both proliferation and differentiation of mesenchymal stem cells. Cell Proli 2007;40:185–195.

23 Karanu FN, Murdoch B, Miyabayashi T et al. Human homologues of Delta-1 and Delta-4 function as mitogenic regulators of primitive human hematopoietic cells. Blood 2001;97:1960–1967.

24 Varnum-Finney B, Brashem-Stein C, Bernstein ID. Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. Blood 2003;101:1784–1789.

25 Histology Forms and Protocols. Available at http://www.urmc.rochester.edu/musculoskeletal-research/core-services/histology/protocols.cfm. Accessed October 16, 2014.

26 Kurooka H, Kuroda K, Honjo T. Roles of the ankyrin repeats and C-terminal region of the mouse notch1 intracellular region. Nucleic Acids Res 1998;26:5448–5455.

27 Hernigou P, Poignard A, Beaualjean F et al. Percutaneous autologous bone-marrow grafting for nonunions: Influence of the number and concentration of progenitor cells. J Bone Joint Surg Am 2005;87:1301–1317.

28 Dominici M, Le Blanc K, Mueller I et al. Minimal criteria for defining multipotent mesenchymal stromal cells: The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315–317.

29 Modder UI, Roforth MM, Nicks KM et al. Characterization of mesenchymal progenitor cells isolated from human bone marrow by negative selection. Bone 2012;50:804–810.

30 Phinney DG, Prockop DJ. Concise review: Mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair: Current views. Stem Cells 2007;25:2896–2902.

31 Tsutsumi S, Shimazu A, Miyazaki K et al. Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. Biochem Biophys Res Commun 2001;288:413–419.

32 Hoffman MD, Benoit DS. Agonism of Wnt-β-catenin signalling promotes mesenchymal stem cell (MSC) expansion. J Tissue Eng Regen Med 2013; [Epub ahead of print].

33 Farre J, Roure S, Prat-Vidal C et al. FGF-4 increases in vitro expansion rate of human adult bone marrow-derived mesenchymal stem cells. Growth Factors 2007;25:71–76.

34 Baksh D, Tuan RS. Canonical and non-canonical Wnts differentially affect the development potential of primary isolate of human bone marrow mesenchymal stem cells. J Cell Physiol 2007;212:817–826.

35 Dahlberg A, Delaney C, Bernstein ID. Ex vivo expansion of human hematopoietic stem and progenitor cells. Blood 2011;117:6083–6090.

36 Dallas MH, Varnum-Finney B, Delaney C et al. Density of the Notch ligand Delta1 determines generation of B and T cell precursors from hematopoietic stem cells. J Exp Med 2005;201:1361–1366.

37 Delaney C, Heimfeld S, Brashem-Stein C et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nat Med 2010;16:232–236.

38 Delaney C, Varnum-Finney B, Aoyama K et al. Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells. Blood 2005;106:2693–2699.

39 Ohishi K, Varnum-Finney B, Bernstein ID. Delta-1 enhances marrow and thymus repopulating ability of human CD34+CD38− cord blood cells. J Clin Invest 2002;110:1165–1174.

40 Calvi LM, Adams GB, Weibrecht KW et al. Osteoblastic cells regulate the hematopoietic stem cell niche. Nature 2003;425:841–846.

41 Isidor B, Lindenbaum P, Pichon O et al. Truncating mutations in the last exon of NOTCH2 cause a rare skeletal disorder with osteoporosis. Nat Genet 2011;43:306–308.

42 Simpson MA, Irving MD, Aslimaz E et al. Mutations in NOTCH2 cause Hajdu-Cheney syndrome, a disorder of severe and progressive bone loss. Nat Genet 2011;43:303–305.

43 King AW, Xiao SM, Cherry S et al. Association of JAG1 with bone mineral density and osteoporotic fractures: A genome-wide association study and follow-up replication studies. Am J Hum Genet 2010;86:229–239.

44 McCright B, Lozier J, Gridley T. A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. Development 2002;129:1075–1082.

45 Go MJ, Takenaka C, Ohgushi H. Forced expression of Sox2 or Nanog in human bone marrow derived mesenchymal stem cells maintains their expansion and differentiation capabilities. Exp Cell Res 2008;34:1147–1154.

46 Greco SJ, Liu K, Rameshwar P. Functional similarities among genes regulated by OCT4 in human mesenchymal and embryonic stem cells. Stem Cells 2007;25:3143–3154.

47 Liu TM, Wu YN, Guo XM et al. Effects of ectopic Nanog and OCT4 overexpression on mesenchymal stem cells. Stem Cells Dev 2009;18:1013–1022.

48 Yoon DS, Kim YH, Jung HS et al. Importance of Sox2 in maintenance of cell proliferation and multipotency of mesenchymal stem cells in low-density culture. Cell Proli 2011;44:424–440.

49 Tso J, Chen S, Yang T et al. Osteosclerosis owing to Notch gain of function is solely Rbpj-dependent. J Bone Miner Res 2010;25:2175–2183.

50 Engin F, Yao Z, Yang T et al. Dimorphic effects of Notch signaling in bone homeostasis. Nat Med 2008;14:299–305.

51 Zhu F, Sweetwyne MT, Hankenson KD. PKCδ is required for Jagged-1 induction of human mesenchymal stem cell osteogenic differentiation. Stem Cells 2013;31:1181–1192.