Evidence for Lgr6 as a Novel Marker of Osteoblastic Progenitors in Mice

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ABSTRACT
Bone marrow–derived mesenchymal stem cells are an important source of osteoblasts critical for both bone homeostasis and repair. The ability to isolate, or specifically target, mesenchymal stem cells committed to the osteogenic lineage is necessary for orthopedic translational therapy efforts; however the precise molecular signature of these cells remains elusive. Previously, we identified a population of osteoprogenitor cells expressing the Wnt signaling agonist Lgr6, which contributes to the development and regeneration of the mouse digit tip bone. In our present study we build upon this data and investigate the expression of Lgr6 more broadly in the skeleton. We find that Lgr6, and closely related Lgr4, are expressed in mouse primary calvarial cells, bone marrow cells, and bone marrow–derived mesenchymal stem cells. In addition, our data demonstrates that Lgr4 expression is modestly increased throughout the differentiation and mineralization of mesenchymal stem cells. In contrast, we find Lgr6 expression to be strikingly increased upon osteogenic induction and subsequently decreased upon differentiation and mineralization. These findings provide evidence for Lgr6 as a novel marker of osteoprogenitor cells in bone marrow, which could prove useful for isolation of this population toward future research and clinical applications. © 2018 American Society for Bone and Mineral Research

KEY WORDS: MOLECULAR PATHWAYS-REMODELING; WNT SIGNALING; OSTEOBLASTS; STROMAL STEM CELLS; BONE REMODELING

Introduction

Bone marrow is dynamically remodeled throughout life, which allows for innate maintenance of bone mass, as well as skeletal repair following injury. During remodeling, bone resorption and formation is mediated by osteoclasts and osteoblasts, respectively. Osteoclasts are of hematopoietic origin, whereby the bone forming osteoblasts originate from multipotent mesenchymal stem cells. Broadly speaking, mesenchymal stem cells are found throughout the body and can give rise to many cell types, including osteoblasts, chondrocytes, myoblasts, and adipocytes. In the context of bone, mesenchymal stem cells that are competent to give rise to osteoblasts have been found to reside in the bone marrow, periosteum, and endosteum. Moreover, in defined conditions, mesenchymal stem cells isolated from adipose tissue, muscle, and synovium have also been found to give rise to osteoblasts. It remains to be fully understood which resident mesenchymal stem cell population(s) contribute to bone remodeling and/or regeneration in vivo under varying physiological scenarios, and what the defining molecular profile of these osteoblastic progenitors (here loosely defined as mesenchymal stem cells committed to the osteoblastic lineage) are. Research focused on these points can facilitate translational efforts to isolate or pharmacologically stimulate these cells for clinical therapies. Research to determine factors critical for mesenchymal stem cells to differentiate down the osteoblastic lineage has revealed several molecular markers that are experimentally useful for studying the process. For example, STRO-1 is a well-characterized cell surface marker expressed on multipotent mesenchymal stem cells. Studies have shown that cells expressing STRO-1 are multipotent and STRO-1/alkaline phosphatase (ALP)–positive cells are intermediate preosteoblasts and express Runx2, a characteristic transcription factor expressed in preosteoblasts. As osteoblast differentiation ensues, the transcription factor Sp7 (Osterix) is expressed, and as osteoblasts mature they express ALP and Osteocalcin. Recently, several additional cell surface markers have been identified to mark osteoprogenitors in human cells. ALCAM/CD166 and HOP-26/CD63 were identified in undifferentiated mesenchymal cells with an osteoprogenitor phenotype. Moreover, cells co-expressing HOP-26, CD166, and CD49a can form fibroblastic colony-forming unit (CFU-F) colonies. Sca-1–expressing and MCAM/CD146–expressing cells were shown be competent for self-renewal, and MCAM/CD146 also plays a role in progression of osteogenic differentiation. Collectively, all of these factors can serve as robust markers of cells at various stages of osteogenesis; however, we do not yet have a marker specific to a pure population of osteoblastic progenitor cells (mesenchymal stem cells that are specifically committed to the osteogenic lineage). Identification of a marker to this committed lineage would ideally be a cell surface marker to facilitate isolation of live cells for downstream cell-based therapies. Because of their inherent osteogenic potential and proliferative capacity, osteoblastic progenitor cells are of considerable
medical interest, especially in the context of nonhealing fractures or bone mass disorders, such as osteoporosis. Presently, there are several pharmacological therapies developed and/or in clinical trials, targeted at building bone by indirectly stimulating osteoblasts, such as recombinant parathyroid hormone, parathyroid hormone related peptide, and anti-sclerostin monoclonal antibody. Although effective, these therapies have reported side effects, underscoring a need for additional research focused on molecularly stimulating endogenous mesenchymal stem cells toward osteogenesis. Beyond their role in bone mass maintenance, mesenchymal stem cells have emerged as a substrate for cell-based therapies for fracture and other bone-related disorders. Decades of research has demonstrated that transplanted mesenchymal stem cells can incorporate into the bones, cartilage, and other tissues of the host. Importantly, these cells have been found to be competent to rescue metabolic and genetic low bone mass phenotypes. For example, autologous bone marrow-derived mesenchymal stem cell transplantation has been shown to increase bone mass in ovariectomized rabbits and goats. In a mouse model of osteogenesis imperfecta, bone marrow-derived mesenchymal stem cell injection into the femoral cavity stimulated new bone formation, with transplanted cells incorporated into newly formed bone. Similarly, bone marrow transplantation in pediatric patients with osteogenesis imperfecta led to increased bone formation, demonstrating that this population of cells has the capacity to migrate to and contribute to bone in humans. Moreover, autologous bone marrow transplantation can be used for treatment of critical fractures, though the outcomes are varied likely due to inherent variability in mesenchymal stem cell composition and potency of patient samples. Collectively, much remains to be learned about the origin, differentiation potential, and molecular regulation of mesenchymal stem cells, but research focused on isolating and/or specifically stimulating a population committed to an osteogenic fate has the potential to transform the treatment options for a spectrum of bone disorders including both osteoporosis and nonhealing fractures. In our previous study, we demonstrated that Lgr6-expressing cells contribute to the osteoblastic lineage in the regenerating mouse digit tip bone, and that Lgr6 is genetically necessary for regeneration of this bone following amputation. This finding is intriguing specifically in the context that Lgr6 has been defined as an agonist of Wnt signaling, which is necessary for embryonic bone development and osteoblastogenesis of mesenchymal stem cells. Moreover, Lgr6 has been found to be a marker of adult stem cells in a number of tissues, such as the epidermis and sebaceous gland. Thus, given our previous findings, Lgr6 is a strong candidate molecular marker to define a stem cell population in the osteogenic lineage as well. This hypothesis is consistent with recent studies implicating a role for family member Lgr4 and its receptor ligands RSPO1 and RSPO2 in osteogenesis and regulation of bone mass. However, although Lgr4 is closely related to Lgr6 in structure and function, its expression is not restricted to adult stem cells, because it has also been found to be expressed within proliferating/differentiating tissues. Taken together, Lgr6 is a promising candidate to study in the context of mesenchymal stem cells and osteoblastogenesis. In this study, we use mouse primary bone cell cultures and osteogenic differentiation assays to determine the molecular dynamics of both Lgr4 and Lgr6 expression during osteoblastogenesis. Our data finds that both Lgr4 and Lgr6 are expressed throughout osteogenesis of calvarial cells, adherent bone marrow cells, and bone-marrow-derived mesenchymal stem cells. However, expression of Lgr6, and not Lgr4, is strongly increased upon osteoinduction and subsequently decreased upon differentiation, making it a novel marker of osteoprogenitor cells and a molecularly relevant target for future study.

Materials and Methods

Mice

All mouse breeding and experimentation was done with the approval of the Brigham and Women’s Hospital IACUC. All primary experimental tissues and cells were derived from wild-type FVB/NJ female mice (JAX #001800; The Jackson Laboratory, Bar Harbor, ME, USA) from a specific-pathogen-free maintenance colony in-house.

Osteoblast culture and differentiation

To generate mouse calvarial osteoblasts, calvaria were dissected and pooled from four postnatal day 1 mouse pups. Following published protocols, cleaned and minced calvaria were subjected to five sequential dispase/collagenase digestions. Cells pooled from the second to fifth digestions were cultured (α-MEM supplemented with 10% FBS, 2mM L-glutamine, and 1% penicillin/streptomycin). Cells were grown for 3 days in proliferative media (α-MEM supplemented with 10% FBS, 2mM L-glutamine, and 1% penicillin/streptomycin) and then differentiated for 18 days (or harvested at an earlier experimental time point) in osteoblast differentiation media (α-MEM supplemented with 10% FBS, 2mM L-glutamine, 10mM β-glycerophosphate, 50 μg/mL ascorbic acid, 1% penicillin/streptomycin), which was changed every 48 hours. Bone marrow cells were isolated from the femurs of 8-week-old female mice. Following standard protocols, femoral diaphyses were flushed out via syringe with a 22G needle, and total bone marrow was collected and plated directly in osteogenic media (α-MEM supplemented with 10% FBS, 2mM L-glutamine, 10mM β-glycerophosphate, 50 μg/mL ascorbic acid, 100nM dexamethasone, and 1% penicillin/streptomycin). The following day, nonadherent cells were removed with a media change. Adherent bone marrow cells were differentiated for 21 days (or harvested at an earlier experimental time point) with a change of media every 48 hours. Separately, bone mesenchymal stem cells were derived from the proliferating, adherent cells from 8-week-old wild-type femurs, isolated as described for bone marrow cells. As cells reached confluency, they were trypsinized and passaged; eighth passage cells were used in this study. For osteogenic differentiation, mesenchymal stem cells were cultured in osteogenic media for 21 days (or harvested at an earlier experimental time point). For BMP2-mediated osteoblast differentiation experiment, calvarial and mesenchymal stem cells were cultured in BMP2 osteogenic media (α-MEM supplemented with 10% FBS, 2mM L-glutamine, 10mM β-glycerophosphate, 50 μg/mL ascorbic acid, 1% penicillin/streptomycin, and 200 ng/mL recombinant BMP2 [R&D Systems, Minneapolis, MN, USA]).

ALP and Alizarin red assays

To determine ALP activity, cells were plated in triplicate and osteogenic differentiation was induced. Calvarial cells were harvested 3, 7, and 21 days postinduction, bone marrow cells were harvested 0, 7, 11, 14, and 18 days postinduction, and
were calculated with respect to individual experiment, then data from Lgr4 and Lgr6 experimental replicates, fold changes potential clonal selection in any one experiment. When pooling analysis of heterogeneous cell populations, unbiased by these replicates for Lgr4/Lgr6 allowed for a stringent collective quantification with 10% ammonium hydroxide and used for colorimetric quantification.

Alizarin red staining was used to quantify mineralization and calcium deposition. To these ends, proliferative osteoblasts were plated in triplicate and osteogenic differentiation was induced. Calvarial cells were harvested 3, 7, and 21 days postinduction and bone marrow cells and mesenchymal stem cells were harvested 0, 7, 14, and 18 days postinduction. Following standard protocols,[42] cells were fixed (4% paraformaldehyde [PFA]) and stained with 40mM Alizarin red-S. Cells were harvested in 10% acetic acid, incubated at 90°C for 5 min followed by centrifugation; resultant supernatant was mixed with 10% ammonium hydroxide and used for colorimetric quantification at 405 nm. Experiments were repeated at least once, to assure consistent ossification trends.

RNA isolation and gene expression analyses

For multitissue Lgr4 and Lgr6 expression analyses, back skin, calvaria, femurs, and femoral bone marrow cells were isolated from three 8-week-old female wild-type mice. All tissues were manually pulverized in liquid nitrogen, and total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) purification following manufacturer’s protocols. For gene expression analyses throughout osteogenesis of calvarial cells, bone marrow cells, and mesenchymal stem cells, cells were induced to differentiate, and harvested postinduction on the days noted in the Osteoblast culture and differentiation methods section. Total RNA was isolated from each sample by TRIzol reagent purification. First-strand cDNA pools were synthesized for all RNA samples using SuperScript III reverse transcriptase primed with random hexamers (Invitrogen).

Gene expression analyses

Semiquantitative PCR was performed for Lgr4, Lgr6, Osteopontin, Runx2, Collagen I, and GAPDH on differentiating calvarial cell, bone marrow cell, and mesenchymal stem cell cDNA using previously published primers and conditions,[41] and newly designed primers for Lgr4 and Lgr6 as follows: Lgr4.F 5’ GTCTTAAACCTCCAGACAAACT, Lgr4.R 5’ GGCTGGGAAGGGCTT- TAAA, Lgr6.F 5’ ATGGTCAGTTGCTGAGTATG, and Lgr6.R 5’ GTCAACAGTAGCTTGATGAG.

Quantitative PCR was performed with the QuantStudio 5 Real-Time PCR 384-well system (Applied Biosystems, Foster City, CA, USA). Primers for osteogenic genes (Collagen I, Runx2, Sp7, Osteocalcin, and Osteopontin) and HPRT controls were designed with the Roche Design Center (Supporting Table 1). Lgr4 and Lgr6 used established TaqMan gene expression assays with TBP intra-well controls (Mm00554385, Mm01291336, and Mm01277042 [Thermo Fisher Scientific, Waltham, MA, USA]). Delta threshold cycle (ΔCt) was calculated for each well and using in-plate technical triplicates, mean relative expression was calculated using the 2-ΔΔCt method.[43] Biological triplicates were performed for all experiments (Supporting Figs. 1–4); pooling of these replicates for Lgr4/Lgr6 allowed for a stringent collective analysis of heterogeneous cell populations, unbiased by potential clonal selection in any one experiment. When pooling data from Lgr4 and Lgr6 experimental replicates, fold changes were calculated with respect to individual experiment, then pooled for combined analysis. Data was statistically analyzed by one-way ANOVA followed by multiple unpaired t test comparisons with Bonferroni correction, with the exception of the bone marrow cell fold-change analysis for which Holm correction was used. All statistical analysis was done using the R statistical package version 3.4.1.[44] By convention, we set our statistical significance thresholds to: *p < 0.05, **p < 0.01, and ***p < 0.001.

Protein expression analysis

Mouse primary calvarial and mesenchymal stem cells were cultured and differentiated as described in the Osteoblast culture and differentiation section. Cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer, and 30 μg of whole cell lysate protein was run on 4-20% Tris-glycine gels. Following transfer and blocking, blots were incubated at 4°C overnight with primary antibodies to Lgr6 (Protechtech 17658-1-AP [Rosemont, IL, USA] [1:1000]), Sp7 (Abcam ab22552 [Cambridge, MA, USA] [1:1000]), or GAPDH (Enzo ADI-CSA-335-E [Farmingdale, NY, USA] [1:5000]) followed by the incubation with horseradish peroxidase (HRP)-labeled secondary antibodies at room temperature for 90 min (Jackson ImmunoResearch 111-035-003 [West Grove, PA, USA] or Invitrogen G21040 [1:10000]).

Results

Dynamic expression of Lgr6 in osteogenic mouse calvarial cells

Building on our finding that Lgr6 is expressed in cells of the osteogenic lineage within the mouse digit tip,[28] we looked for Lgr6 expression more broadly throughout the skeleton in osteogenic cells. To these ends, we isolated RNA from adult mouse bone marrow, bone, calvaria, as well as skin to be used as a positive control.[32,45] We first analyzed the expression of Lgr4 in these tissues, because Lgr4 is a more broadly expressed paralog to Lgr6 and has already been implicated in osteogenesis.[33,34,46] We found expression of Lgr4 in all tissues examined, and expression levels in femur and calvaria were comparable and not statistically different from each other (Fig. 1A, Supporting Fig. 1A). We turned our analysis to Lgr6 and found it to also be expressed in all of the tissues tested, though Lgr6 expression was 3.7 x more abundant in calvaria as compared to femur samples (p = 1.2e-06) (Fig. 1A, Supporting Fig. 1B). Consistently low levels of both Lgr4 and Lgr6 were detected in bone marrow cells (Fig. 1A, Supporting Fig. 1); however, limited conclusions can be drawn from this finding due to the presumed heterogeneity of cell types and resultant transcripts in these samples.

Development and regeneration of both the mouse calvaria and digit tip rely on intramembranous ossification, thus our finding that Lgr6 expression is increased in calvaria, is consistent with our previous data in mouse digit tip.[28] To further explore the expression of Lgr6 in calvaria, we undertook mouse primary calvarial cell differentiation assays to determine whether the expression is specific to preosteoblasts, or whether it is also expressed during differentiation of these cells. For these experiments, we harvested cells on days 3, 7, and 21 of osteogenic culture. Based on expression of osteogenic markers and ALP/Alizarin red assays (Fig. 1D, Supporting Fig. 5A), we correlated these days with the osteogenic stages of proliferation (day 3), differentiation (day 7), and mineralization (day 21). We first analyzed Lgr4, and found it to be expressed during all stages of calvarial cell osteogenesis, though this expression is modestly decreased during mineralization (day 3 versus day 21, fold-
Decreased expression of Lgr6, but not Lgr4, upon osteogenic differentiation of adherent bone marrow cells

The decreased expression of Lgr6 upon differentiation of calvarial cells led us to explore the dynamics of Lgr4 and Lgr6 expression during in vitro osteogenesis of bone marrow cells derived from long bones, which could have broader translational implications. Because primary bone marrow cells are a highly heterogeneous mix of cells, including mesenchymal stem cells, expression analysis within this population offers insight into Lgr4 and Lgr6 expression prior to the proliferative osteoblastic state found in calvaria. In these experiments, cells were harvested on days 0, 7–9, 11, 14, and 17–18 of osteogenic culture. Quantitative PCR for osteogenic markers and ALP/Alizarin red assays (Fig. 2C, Supporting Fig. 5B) provided staging references for our bone marrow cell osteogenic culture allowing us to loosely define stages as follows: proliferation (days 0 to 8), differentiation (days 8 to 11), and mineralization (days 11 to 18). As expected based on our skeletal tissue analyses (Fig. 1A), neither Lgr4 nor Lgr6 was significantly expressed in freshly harvested and plated bone marrow cells (day 0), likely due to the heterogeneity of the samples (Fig. 2A, B; Supporting Fig. 3). Beyond day 0, Lgr4 was expressed at all stages of bone marrow cell differentiation with no significant difference in expression levels (Fig. 2A). Lgr6 was also expressed at all osteogenic stages beyond day 0; however, this expression was significantly less abundant upon the transition to differentiation (day 9 versus day 11, fold-change \(-9.1 \times\), \(p = 0.047\) with Holm correction) (Fig. 2B). Although Lgr6 expression was significantly reduced in each experimental replicate, in data set 2 this differential expression was most profound between days 7 and 9 (fold-change \(-7.1 \times\), \(p = 5.6e^{-12}\) with Bonferroni correction), indicating that perhaps this set of cells differentiated faster than experimental sets 1 and 3 (Supporting Fig. 3). Moreover, unlike our findings for Lgr6, although Lgr4 is expressed throughout bone marrow cell osteogenic differentiation, no significant dynamic expression trend is found (Fig. 2A, Supporting Fig. 3). These expression trends were validated by both semiquantitative PCR and Western blot (Supporting Figs. 6A and 7A).

Increased Lgr6 expression in mesenchymal stem cell derived osteoprogenitors

Although we were able to determine the expression profiles for Lgr4 and Lgr6 during in vitro bone marrow cell differentiation, this assay did not permit reliable assessment of relative expression levels prior to induction of osteogenesis (day 0), because of the inherent heterogeneity of the samples. To determine the extent to which Lgr4 and Lgr6 are expressed in progenitors cells prior to osteogenic induction, we analyzed
RNA isolated from eighth passage bone marrow–derived mesenchymal stem cells. We found that both Lgr4 and Lgr6 were expressed on day 0, prior to osteogenic induction, revealing that both of these genes are markers of mesenchymal stem cells (Fig. 3). To determine if Lgr4 or Lgr6 were dynamically expressed as these cells become committed to osteoblasts and differentiate, we used an in vitro osteogenesis assay of mesenchymal stem cells and collected cells on days 0, 1, 3, 7, 11, 14, and 18. As in our previous osteogenesis experiments, qPCR for Collagen1, Runx2, Sp7, Osteocalcin, and Osteopontin, in combination with ALP activity and Alizarin red staining, was used to broadly group these time points into three stages: proliferation (days 0 to 3), differentiation (days 3 to 11), and mineralization (days 11 to 18) (Fig. 3C, Supporting Fig. 5C). We first analyzed Lgr4 expression levels and found no significant change in expression following osteoinduction (day 1) (Fig. 3A; Supporting Fig. 4A, B). As cells transitioned to differentiation on day 3, we detected a modest increase in Lgr4 expression which persisted throughout differentiation and mineralization (day 1 versus day 3, fold-change 1.5×, p = 0.006) (Fig. 3A). In contrast to Lgr4, Lgr6 expression was significantly more abundant following osteoinduction on day 1 (day 0 versus day 1, fold-change 4.3×, p = 0.016), a finding that was not readily appreciable from the primary bone marrow cell culture assays (Fig. 3B; Supporting Fig. 4C, D). In addition, confirming our findings from both the calvarial cell and bone marrow cell osteogenic assays, Lgr6 levels were decreased as the cells transitioned from proliferation to differentiation (day 1 versus day 3, fold-change 5.5×, p = 3.0e-15), and this decrease was maintained throughout differentiation and mineralization (Fig. 3B; Supporting Fig. 4C, D). These expression trends were all validated by both semiquantitative PCR and Western blot (Supporting Figs. 6C and 7B).

To further confirm our findings, we used recombinant BMP2 to induce osteoblast differentiation in lieu of dexamethasone in the media. We cultured mesenchymal stem cells with 200 ng/mL BMP2 in parallel with cells cultured in dexamethasone differentiation media. Induced differentiation was confirmed for both media groups by Runx2 and Osteocalcin expression levels (data not shown). Analysis of Lgr4 expression revealed an increase in Lgr4 expression on day 3 in the presence of BMP2 (day 1 versus day 3, fold-change 2.0×, p = 0.005) (Supporting Fig. 8A). This increase is comparable to the dynamics of Lgr4 in our original dexamethasone induced experiments (Fig. 3C), though the dexamethasone induced increase in Lgr4 expression in this experiment was below our statistical significance cut-off. Analysis of Lgr6 expression following BMP2 induction also recapitulated our findings with dexamethasone whereby expression was significantly decreased upon differentiation (day 1 versus day 3, fold-change 2.7×, p = 3.8e-04) (Supporting Fig. 8B). Collectively, these BMP2 induction assays indicate that our correlation of Lgr6 decreased expression with osteoblast differentiation is not a function of the osteoinduction agent.

**Discussion**

In this study, we find that Lgr4 and Lgr6 are expressed in mouse calvarial cells as well as bone marrow–derived mesenchymal stem cells. Using an in vitro osteogenic differentiation assay of mouse mesenchymal stem cells, we find increased Lgr4 expression during differentiation and mineralization. This data is consistent with published reports demonstrating the
necessity of Lgr4, and associated R-spondin ligands, for the differentiation of osteoblasts.\textsuperscript{33,35,47,48} In contrast, our experiments reveal a dynamic expression profile for Lgr6 during osteogenesis, which is distinct from that of Lgr4. We find that prior to osteoinduction, like Lgr4, Lgr6 is expressed at low levels. Following osteoinduction, prior to significant expression of Collagen I, Runx2, or ALP, Lgr6 expression is increased 4.3-fold. Moreover, as osteogenic differentiation proceeds, and Lgr4 expression increases, Lgr6 expression is strongly reduced back to preinduction levels (Lgr6\textsubscript{low}). These findings indicate that Lgr6\textsubscript{low} expression, much like Lgr4, could be used as a broad marker for cells in the osteogenic lineage. Additionally, the spike of Lgr6 expression (Lgr6\textsubscript{high}) following osteoinduction can be used as a novel marker of mesenchymal stem cells committed to the osteogenic lineage.

Our hypothesis that Lgr6\textsubscript{high} cells specifically mark a stem/progenitor population, is supported by extensive research on the paralogous gene, Lgr5, in the intestinal epithelium. In the intestinal crypt, single Lgr5\textsubscript{high} cells are progenitors and competent to give rise to intestinal crypt organoids, whereby Lgr5\textsubscript{low} cells are not.\textsuperscript{49} Lineage analyses demonstrate that as Lgr5\textsubscript{high} progenitor cells differentiate, they give rise to Lgr5\textsubscript{low} progeny that move into the rapidly proliferating “transit-amplifying zone,” where Lgr4 is highly expressed.\textsuperscript{50} Intriguingly, the data in our present study support a similar osteoprogenitor/progeny model in the bone marrow. The timing of increased Lgr6 expression (Lgr6\textsubscript{high}) in bone marrow derived mesenchymal stem cells is coincident with osteoinduction, thus we hypothesize that these cells are committed osteoprogenitors. The timing of decreased Lgr6 expression (Lgr6\textsubscript{low}) is coincident with differentiation markers and Lgr4 upregulation, thus we hypothesize these cells are osteoblastic Lgr6 progeny, whose proliferation and differentiation are supported by Lgr4 expression.

Moving forward, research focused on understanding both the identity and function of Lgr6-expressing bone marrow derived mesenchymal stem cells is needed. Although our data demonstrate that Lgr6 is dynamically expressed in this population in mice, it remains to be determined whether this is a rare or common cell type, and whether these findings extend to human bone marrow. In addition, it will be important to delineate whether Lgr6 is solely a marker for this population, or whether it is molecularly necessary in these cells to potentiate Wnt signaling to support bone turnover and/or repair. The expression data detailed in this report provide an important jumping off point to delve into these questions more deeply.

**Disclosures**

The authors state that they have no conflicts of interest.

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Authors’ roles: VK designed and performed experiments, acquired and interpreted the data, and drafted the manuscript. JAL designed the experiments, interpreted the data, and revised the manuscript. Both authors have critically reviewed this manuscript and approve it for submission.
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