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# Review of the Pathways Involved in the Osteogenic Differentiation of Adipose-Derived Stem Cells

*Derek B. Asserson, BS, Hakan Orbay, MD, PhD, and David E. Sahar, MD*

**Abstract:** Grafts and prosthetic materials used for the repair of bone defects are often accompanied by comorbidity and rejection. Therefore, there is an immense need for novel approaches to combating the issues surrounding such defects. Because of their accessibility, substantial proportion, and osteogenic differentiation potential, adipose-derived stem cells (ASCs) make for an ideal source of bone tissue in regenerative medicine. However, efficient induction of ASCs toward an osteoblastic lineage in vivo is met with challenges, and many signaling pathways must come together to secure osteoblastogenesis. Among them are bone morphogenic protein, wingless-related integration site protein, Notch, Hedgehog, fibroblast growth factor, vascular endothelial growth factor, and extracellular regulated-signal kinase. The goal of this literature review is to conglomerate the present research on these pathways to formulate a better understanding of how ASCs are most effectively transformed into bone in the context of tissue engineering.

**Key Words:** Adipose-derived stem cells, bone regeneration, osteogenic differentiation, tissue engineering

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In 2001, 12,700 bone grafts were performed to repair craniofacial defects at a cost of over $549 million in children alone.[1] Another 1.3–1.5 million grafts were constructed in 2010 to fix defects of the cranium, sternum, ribs, and extremities.[2] It is said that bone grafting is also required in 1 out of every 4 dental implants.[3] This reach seems to be a transnational occurrence, as graft materials are approved all over the world, with some countries placing great importance on the procedures in training programs.[2,4]

Current treatment methods using autologous bone, allogenic transplants, and prosthetic materials all carry significant drawbacks, such as donor-site morbidity, immune rejection, and extrusion and infection, while failing to meet the enormous and ever-increasing demand for reconstruction of skeletal deficiencies.[5] This gap has provided impetus to develop regenerative medicine that is being relied on more often to deal with disease and trauma. Engineering bone tissue was only made possible with the discovery and utilization of stem cells that are capable of self-renewal and differentiation into a bevy of lineages.[6] The implementation of stem cells for the purpose of bone regeneration potentially eliminates the aforementioned problems associated with autologous and allogenic grafts.[5]

Because of their high differentiation potential and low morbidity during harvesting, bone marrow stem cells (BMSCs) have been considered the gold standard in bone tissue engineering.[7–9] However, low cellular yields, as well as extremely painful extraction, have pushed research in a novel direction, one that places fat at the core of regenerative medicine.[10–13] Adipose-derived stem cells (ASCs) have similar transcription profiles to BMSCs for genes induced in stem-cell phenotypes, and are identified for their ability to differentiate into a vast array of lineages, including adipogenic, chondrogenic, myogenic, neurogenic, and osteogenic forms.[14,15] Roughly 5000 fibroblast colony-forming units (CFU-F) are obtained per gram of adipose tissue, and ASCs constitute 2% of the nucleated cells in processed lipoaspirate.[16] Compared with only 100 to 1000 CFU-F per milliliter of bone marrow, adipose tissue makes for an excellent source of mesenchymal stem cells (MSCs).[16]

Secondary to ASC harvesting, scaffolds are employed to ensure proper behavior of the stem cells by generating controlled niches and delivering appropriate biomaterials.[17–19] Optimal bone regeneration is achieved with certain physical and biological characteristics of the scaffold, especially cell attachment sites, in which the ideal osteogenic scaffold is a porous, biodegradable, three-dimensional structure.[20–23] The culture medium of ASCs also largely helps determine differentiation potential toward certain lineages.[24]

Although several papers demonstrate the osteogenic differentiation of ASCs, the molecular mechanisms by which the ASCs differentiate into osteoprogenitor cells are not as easily understood and no systematic review of them exists in the literature. This review serves to elucidate those mechanisms through research endeavors that have successfully turned ASCs into bone. The pertinent papers are classified according to main signaling pathways in Table 1.

## METHODS

A search of the PubMed/Medline database was performed with the search terms "adipose-derived stem cells" OR "ASCs" AND "osteogenic differentiation." Initial results yielded 256 papers, which was reduced to 179 after title screening. When subsequently screened for content, 14 backgrounds (some of which were not used) and 29 experimental papers were left. An additional 62 investigations found via connected articles were incorporated to further substantiate the literature review.

## RESULTS

Osteoblastogenesis or osteoblast differentiation during development is controlled by a complex network at both the transcriptional level and extracellular signaling pathways.[5] The key players in the transcriptional control of osteoblast differentiation are Runt-related family 2 (RUNX2), formerly called Cbfa1,[25–29] and the late

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**TABLE 1.** Classification of Signaling Pathways by Paper

| Author | Year | Signaling Pathway |
|---|---|---|
| Arnsford et al | 2009 | Wnt |
| Bandyopadhyay et al | 2006 | BMP |
| Behr et al | 2012 | VEGF |
| Bergwitz et al | 2001 | Wnt |
| Boyden et al | 2002 | Wnt |
| Burke et al | 1998 | FGF |
| Cadigan et al | 1997 | Wnt |
| Chang et al | 2007 | Wnt |
| Chen et al | 2004 | BMP |
| Chillakuri et al | 2012 | Notch |
| Cowan et al | 2004 | BMP |
| Daluiski et al | 2001 | BMP |
| Deregowski et al | 2006 | Notch |
| Ding et al | 2014 | VEGF |
| Dragoo et al | 2003 | BMP |
| Dragoo et al | 2005 | BMP |
| Eswarakumar et al | 2002 | FGF |
| Fan et al | 2016 | Notch |
| Fan et al | 2013 | BMP |
| Fan et al | 2014 | BMP |
| Fan et al | 2016 | BMP |
| Fischer et al | 2002 | Wnt |
| Gilboa et al | 2000 | BMP |
| Gong et al | 2001 | Wnt |
| Grottkau et al | 2013 | BMP, Wnt, Hedgehog |
| Heldin et al | 1997 | BMP |
| Hilton et al | 2008 | Notch |
| Hung et al | 2015 | ERK |
| Jacob et al | 2006 | FGF |
| Kadesch | 2004 | Notch |
| Kokabu et al | 2012 | BMP |
| Kwan et al | 2011 | FGF |
| Levi et al | 2010 | BMP |
| Levi et al | 2011 | BMP |
| Li et al | 2016 | VEGF |
| Lin et al | 2008 | BMP |
| Liu et al | 2002 | FGF |
| Long et al | 2013 | Hedgehog |
| Lu et al | 2012 | BMP |
| Lu et al | 2013 | BMP |
| Marie | 2003 | FGF |
| Marie | 2012 | FGF |
| Mie et al | 2000 | BMP |
| Montero et al | 2000 | FGF |
| Nakamura et al | 1997 | Hedgehog |
| Ohbayashi et al | 2002 | FGF |
| Quarto et al | 2006 | FGF |
| Quarto et al | 2008 | FGF |
| Rice | 2008 | BMP, Wnt |
| Santos et al | 2010 | VEGF |
| Sarkar et al | 2001 | FGF |
| Schmuhl et al | 2014 | ERK |
| Spinella-Jaegle et al | 2001 | Hedgehog |
| St-Jacques et al | 1999 | Hedgehog |
| Stevens et al | 2010 | Wnt |
| Tu et al | 2007 | Wnt |
| Vanhatupa et al | 2015 | BMP |
| Westendorf et al | 2004 | Wnt |
| Wodarz et al | 1998 | Wnt |
| Yuasa et al | 2002 | Hedgehog |

transcription factor osterix (OSX).[30,31] Extracellular signaling pathways converge on these transcription factors to orchestrate and regulate osteoblastogenesis. The pathways are bone morphogenic protein (BMP), wingless-related integration site protein (Wnt), Notch, Hedgehog, fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and extracellular regulated-signal kinase (ERK) (Fig. 1). The same signaling pathways play a role in the direction of putative adult stem cells to an osteoblastic lineage, demonstrated by the bone markers alkaline phosphatase (ALP), type I collagen, osteopontin (OPN), and osteocalcin (OCN), as well as matrix mineralization,[32] for the purpose of tissue engineering.[33,34]

## Bone Morphogenic Protein

The BMPs belong to the transcription growth factor beta superfamily.[35,36] This group is perhaps the most well-known of the cytokines involved in osteogenesis, and as such, the majority of studies related to ASCs and bone formation has revolved around the BMP pathway.

The BMPs operate through signaling mothers against decapentaplegic (Smad) 1 phosphorylation.[35,37] The phosphorylated Smads promote osteogenic differentiation by forming a complex with Smad4.[34] The BMPs also increase the transcription of RUNX2.[38] Subtypes BMP2 and BMP4 are particularly important, governing crucial steps of differentiation by managing the transition from RUNX2- to OSX-positive cells.[39–41] The ASCs increase bone regeneration significantly when loaded onto a scaffold with BMP2 or when transfected with the BMP2 gene.[42–47] On the contrary, BMP3 is thought to have a negative regulatory effect on osteogenesis[48] through interaction with the BMP receptor type II to inhibit BMP2 and BMP4 signaling.[49]

Downregulation of the osteogenic inhibitor Noggin in ASCs triggered more appreciable mandibular regeneration in rats, and this effect became more pronounced when Noggin$^{-/-}$ ASCs were supplemented with BMP2.[50–52] Furthermore, tumor necrosis factor alpha (TNF-α), a major inflammatory factor peaking after bone fracture, promoted BMP2 establishment in human primary osteoblasts and inhibited osteogenic differentiation when the pathway was contacted with Noggin.[53] Preconditioning with TNF-α augmented proliferation, mobilization, and osteogenic differentiation of ASCs and upregulated BMP2, upon silencing of BMP2 by siRNA.[54]

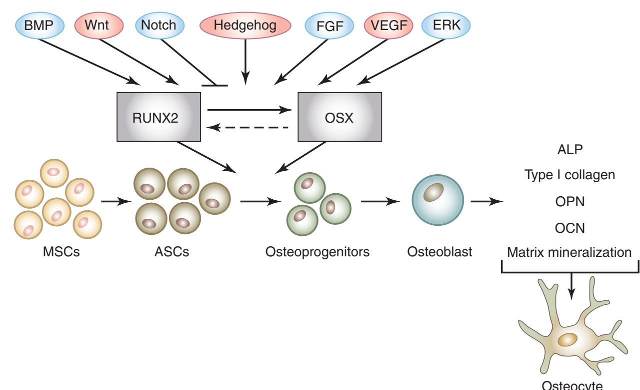


**FIGURE 1.** Schematic of osteogenic differentiation induced by adipose-derived stem cells. ALP, alkaline phosphatase; ASC, adipose-derived stem cell; BMP, bone morphogenic protein; ERK, extracellular regulated-signal kinase; FGF, fibroblast growth factor; MSC, mesenchymal stem cell; OCN, osteocalcin; OPN, osteopontin; OSX, osterix; RUNX2, runt-related family 2; VEGF, vascular endothelial growth factor; Wnt, wingless-related integration site protein.


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## Wingless-Related Integration Site Protein

The Wnt family consists of a large number of secreted glycol proteins that are involved in embryonic development, tissue induction, and axis polarity.[55,56] Wnt ligands bind to frizzled receptors at the cell surface and to LRP5 and LRP6 coreceptors.[34] LRP5 gain of function results in increased bone mass,[57] whereas mutations in the gene cause osteoporosis-pseudoglioma syndrome.[58]

Canonical Wnt signaling results in stabilization and translocation of β-catenin to the nucleus, where it binds to T-cell factor/lymphoid enhancer factor (TCF/Lef) transcription factors.[38] β-catenin-TCF/Lef complexes activate transcription of multiple Wnt-responsive genes, including those implicated in proliferation, osteoblast differentiation, and osteogenesis.[59–61]

Noncanonical Wnt10b is identified for its unique requirement for maintenance of mesenchymal progenitor activity in adult bone after it was distinguished as the only Wnt ligand linking to mesenchymal progenitor function in humans and mice.[62] Some studies revealed that noncanonical Wnt signaling, namely that of Wnt5a with Ror2 and RhoA as counterparts, plus *N*-cadherin-mediated β-catenin signaling, are necessary for mechanically induced osteogenesis.[63] Furthermore, Wnt4 may have potentiality in improving bone regeneration and repair of craniofacial defects.[64] Wnt3a and Wnt7b, also of the noncanonical variety, signal through G-proteins to activate phosphatidylinositol signaling and PKCδ, the latter of which is necessary for osteoblastogenesis.[65]

## Notch

The Notch network is known to be part of an evolutionarily conserved mechanism that balances proliferation and differentiation of stem cells.[66] Such action begins with the Jagged Delta protein's attachment to the receptor[34] prior to cleavage by γ-secretase to release the Notch intracellular domain.[67]

Notch inhibition in the embryonic limb leads to increased bone mass and a reciprocal decrease in bone marrow mesenchymal progenitors, thereby suggesting Notch's function in suppressing osteogenic differentiation and maintaining a sizable progenitor pool.[68] Notch is thought to act by quelling RUNX2 transcriptional activity by induction of the transcription factors HEY1 and HEYL, which physically interact with RUNX2.[69]

The miR-34a was found to upregulate *RUNX2* by inhibiting retinoblastoma binding protein 2 via the *NOTCH1/CYCLIN D1* coregulatory network.[70] Another report, however, observed that Notch-1 overexpression inhibited osteogenesis by interrupting canonical Wnt signaling, but failed to do so with interrupted BMP.[71]

## Hedgehog

Pattern arrangement of bone tissue is greatly influenced by Hedgehog signaling, albeit little work on it has been executed.[34] In particular, Indian Hedgehog (IHH) plays a decisive job in endochondral development, as *Ihh*$^{-/-}$ mice lack osteoblasts within the endochondral tissue, but not in intramembranous tissue.[72] Without IHH, mesenchymal progenitors in the perichondrium of the cartilaginous anlagen do not express *Runx2* and hence fail to undergo differentiation.[73] The IHH receptor Smoothened (Smo) has been deemed necessary to build trabecular bone.[72]

On the contrary to IHH, Sonic Hedgehog (Shh) induces ALP expression[74,75] and augments selectivity in the differentiation of MSCs into the osteoblast lineage.[76]

## Fibroblast Growth Factor

The FGF2, FGF9, FGF18, and their corresponding receptors, FGFR1, FGFR2, and FGFR3, are all linked to skeletal development of the long bones and calvarium.[77,78] Embryos of genotype *Fgf18*$^{-/-}$ had defects in osteoblast maturation despite normal *Runx2* expression.[79,80] In contrast, FGFR1 activity has a tendency to launch osteogenic differentiation at an early stage, only to inhibit mineralization capabilities in mature osteoblasts.[81] Gain of function mutations in FGFR1, FGFR2, and FGFR3 caused craniosynostosis, while implantation of beads soaked with FGF2 and FGF4 around sutures caused osteogenesis and later suture closure.[82,83]

FGF2 serves as the quintessential example of how FGF is connected to bone formation.[84] Although the promotion of bone repair via ASC mediation had been previously reported in vivo, FGF2 was additionally found to inhibit terminal osteogenic differentiation by antagonizing the retinoic acid-mediated upregulation of BMP receptor type IB.[85–87] This paradox can be explained when one considers the principal function of FGF2, to promote the proliferation and expansion of osteoprogenitor cells to maximize the osteoprogenitor pool for future differentiation.[85] Still, other discussions are rooted in the fact that FGF2 can activate different signaling pathways, including ERK, PI3K, and PKC, of which stage and context would resolve the proliferation-versus-differentiation debate.[84,88] Given the complexity of FGF, as well as other signaling pathways, the ability to guide a specific one in the context of ASC-based therapy for bone formation would be a sweeping step in regenerative medicine.[5]

## Vascular Endothelial Growth Factor

Neurovascularization and angiogenesis are paramount to proper bone foundation.[89] It has been exhibited that VEGFA had a more potent effect in precipitating ASC-mediated calvarial regeneration than either BMP2 or FGF2, through a combination of osteogenesis and angiogenesis.[90]

Dimethyloxaloglycine was tested for a dose-dependent effect by levels of *RUNX2*, *OCN*, and *ALP* expression, plus VEGF, which is produced as a product of *HIF-1α* overexpression.[91] The ASCs expressing Runx2 in combination with a vascularized flap led to more effective bone repair than either facet acting alone, VEGF and collagen type I were indicators.[92]

## Extracellular Signal-Regulated Kinase

Another pathway that deals with osteogenic differentiation is ERK, which is sometimes referred to as mitogen-activating protein kinase, and where platelet-derived growth factor (PDGF) comes into play.[93] In 1 study, in the presence of PDGF-BB, ASCs, not BMSCs, heightened osteogenic differentiation by increasing Runx2 and OCN output.[94]

## Other Pathways Involved in Osteogenic Differentiation

Erythropoietin has been reported to give rise to osteogenesis by inhibiting PPARγ, while IGF1 does the same through TAZ, and when joined, resulted in even higher levels of *Runx2*, *OPN*, *OCN*, *ALP*, and matrix mineralization.[95] The ASCs magnofected with *Bcl2*, an inhibitor of apoptosis, prompted greater ALP, extracellular matrix mineralization, and expression of *Ocn*, *Opn*, and *Runx2* than nucleofected cells.[96] miR-135 was shown to positively regulate osteogenic differentiation based on bone markers and extracellular matrix decomposition through a potentially new miR135/Hoxa2/Runx2 pathway.[97] The role of the transcription factor δFosB has been explored, and the results obtained pointed to increased bone mass and decreased adipocyte origination upon overexpression.[98,99] A final biomolecule that has been scrutinized is growth and differentiation factor 5, which appears to be more effective than BMP2 in inducing onset of osteogenic differentiation while simultaneously prompting vascularization through VEGF.[99,100]



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## DISCUSSION

Taken together, these limited numbers of studies have assisted in mapping out the signaling pathways that ASCs go through to become bone. Most work has looked at the function that the BMP course fulfills, followed by those of Wnt, Notch, Hedgehog, FGF, VEGF, and ERK. By differing scaffold type and culture medium, molecular composition, mechanical stress, chemical amalgamation, or bioengineering, the effectiveness of osteogenic differentiation can be evaluated. Activity of ALP, emergence of collagen type I, expression of *OCN* and *OPN*, and mineralization through calcium precipitation were quantified in most cases to analyze potential.

It has become increasingly clear that with proper control of outside inputs, ASCs make an excellent choice for bone tissue engineering in clinical practice. The ideal mixture of favorable inducers is not as straightforward, but the depth of research insists that multiple manipulation strategies exist, as long as the stem cells are exposed to at least one inducer of osteogenesis early on. Additionally, there is a trend in combining inducers as to achieve optimal outcomes.

New methods are necessitated to not only reduce the culture period and quantity of growth factors, but also to enhance the efficiency of osteogenesis and thus bone regeneration. One approach is delivery of cytokines incorporating these molecules into scaffolds as basic as liposomes and microspheres.[17] This makes the growth factor retainable at the site of interest for an extended period while maintaining its biologic activity. Engineered ASCs with gene transfection by virus vectors have evolved to be an attractive option to ameliorate bone repair, especially in large bone defects.[34] As bone formation by implantation of ASCs must be preceded by the in vitro osteogenic differentiation of these cells, it is compulsory to design procedures that ensure a well-characterized and consistent cell population following the differentiation process.[34]

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