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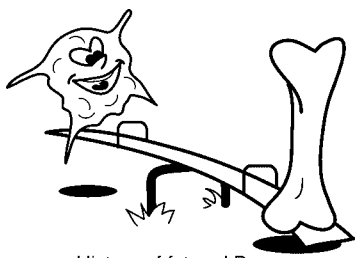
Playing With Bone and Fat

Jeffrey M. Gimble,[1]* Sanjin Zvonic,[1] Z. Elizabeth Floyd,[1] Moustapha Kassem,[2] and Mark E. Nuttall[3]

[1]Stem Cell Laboratory, Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, Louisiana
[2]Department of Endocrinology, Odense University Hospital, Odense, Denmark
[3]Centocor, Johnson & Johnson, Philadelphia, Pennsylvania

Abstract The relationship between bone and fat formation within the bone marrow microenvironment is complex and remains an area of active investigation. Classical in vitro and in vivo studies strongly support an inverse relationship between the commitment of bone marrow-derived mesenchymal stem cells or stromal cells to the adipoctye and osteoblast lineage pathways. In this review, we focus on the recent literature exploring the mechanisms underlying these differentiation events and discuss their implications relevant to osteoporosis and regenerative medicine. J. Cell. Biochem. 98: 251–266, 2006. © 2006 Wiley-Liss, Inc.

Key words: adipoctye; bone; fat; mesenchymal stem cell; osteoblast



History of fat and Bone

Research from a number of fields documents the close relationship existing between bone and fat formation. Studies suggest that the marrow cavity is like a playground "see-saw" that can swing back and forth between bone and fat formation. For over a decade, the field has been dominated by the hypothesis that an inverse relationship exists between adipocytes and osteoblasts within the marrow cavity [Beresford et al., 1992; Dorheim et al., 1993]. In vitro studies using bone marrow-derived mesenchymal stem/stromal cells found that agents inducing adipocyte differentiation [Beresford et al., 1992; Dorheim et al., 1993]. Likewise, agents inducing osteoblast differentiation inhibited adipogenesis [Gimble et al., 1995]. These findings were consistent with classic pathological [Custer and Ahfeldt, 1932; Vost, 1963; Hartsock et al., 1965] and epidemiological studies [Meunier et al., 1971] linking increased marrow adiposity with aging, bone loss, and osteoporosis. We [Gimble, 1990; Gimble et al., 1996a; Nuttall and Gimble, 2000, 2004; Gimble and Nuttall, 2004] and others [Tavassoli, 1984; Weiss and Sakai, 1984; Chan and Duque, 2002; Kirkland et al., 2002] have reviewed the literature relating to this subject in past years. While a wealth of data supports this model, recent findings have begun to present a strong challenge to this paradigm. The current update focuses on the most recent

Abbreviations used: ASC, adipose-derived stem cells; BMP, bone morphogenetic protein; C/EBP, CAAT/enhancer binding protein; CTGF, connective tissue growth factor; Cyr61, cysteine rich protein 61; DKK, dickkopf; ER, estrogen receptor; ERR, estrogen-related receptor; GSK3 β, glycogen synthase kinase 3 β; HIV, human immunodeficiency virus; HSC, hematopoietic stem cell; LRP, low density lipoprotein receptor-related protein; LXR, oxysterol receptor; MSC, mesenchymal stem cells; NOV, nephroblastoma over expressed; PPAR, peroxisome proliferator activated receptor; RXR, retinoid X receptor; SAM-P/6, senescence accelerated mice-P/6; SREBP, steroid response element binding protein; TAZ, transcriptional co-activator with PDZ binding motif; TGFβ, transforming growth factor β; vaspin, visceral adipose tissue-derived serine protease inhibitor; WISP, Wnt induced secreted protein.

*Correspondence to: Jeffrey M. Gimble, MD, PhD, Stem Cell Laboratory, Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, LA 70808.
E-mail: gimblejm@pbrc.edu.

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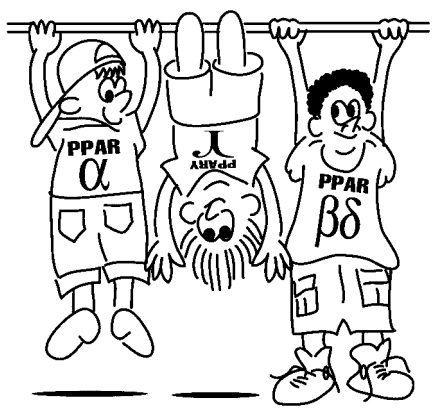
findings describing the interdependency between adipogenesis and osteogenesis.

THE BIG KIDS ON THE PLAYGROUND: NUCLEAR HORMONE RECEPTORS

The nuclear hormone receptor family of transcriptional regulatory proteins is activated by a range of ligands, including the classical "steroid hormones," naturally occurring metabolites, synthetic chemicals, and as yet to be identified endogenous compounds (orphan receptors). Members of the nuclear hormone receptor family control critical adipogenic and osteogenic steps. With the development of novel synthetic ligands with agonist, partial-agonist, or antagonist properties and gene-specific mutant mice, it is now possible to do both loss and gain of function studies to dissect these processes.

PPARs—Phat Pharm(a)



The peroxisome proliferator activated receptor γ (PPARγ) plays a central role in initiating adipogenesis, in bone marrow and other stromal-like cells in vitro [Gimble et al., 1996b]. Thiazolidinedione PPARγ ligands such as rosiglitazone (Avandia®) and pioglitazone (Actos®) play a prominent role in the treatment of type 2 diabetic patients. In vitro analyses demonstrate that various PPARγ ligands (rosiglitazone, 9,10 dihydroxyoctadecenoic acid, 15-deoxy12,14-PGJ2) not only induce murine bone marrow stromal cell adipogenesis but also inhibit osteogenesis [Lecka-Czernik et al., 2002]. Indeed, there is evidence that the adipogenic and osteogenic processes can be separated through the use of partial agonists. For example, the PPARγ ligands GW0072 and netoglitazone, both members of the thiazolidinedione family, can block osteoblast differentiation without inducing adipogenesis in murine cell lines [Lecka-Czernik et al., 2002; Lazarenko et al., 2005]. Development of a thiazolidinedione with bone sparing and insulin sensitizing properties but without adipogenic activities would be of potential improvement over existing therapeutic compounds. There is new evidence regarding the nature of endogenous or in vivo PPARγ ligands [Tzameli et al., 2004; Schopfer et al., 2005]. Although long chain fatty acids have been known to activate PPARγ at micromolar levels [Tzameli et al., 2004], there is now evidence that nitric oxide derivatives of linoleic acid are potent adipogenic agonists at levels of 133 nM, well within the physiological range [Schopfer et al., 2005]. It remains to be determined if these putative endogenous PPARγ ligands inhibit osteogenesis.

A number of groups have extended their analyses of individual PPARγ ligands to in vivo models. Under these conditions, not all PPARγ ligands exhibit the same effects and this may reflect differences between their receptor binding affinity and conformation of the receptor/ligand complex. For example, long-term treatment of mice with the thiazolidinedione troglitazone increased bone marrow adipocyte content without reducing bone mass and trabecular volume [Tornvig et al., 2001]. In contrast, treatment of mice with rosiglitazone, a thiazolidinedione with higher affinity for PPARγ, decreased bone mineral content, bone formation rates, and trabecular bone volume while increasing adipogenesis [Rzonca et al., 2004; Ali et al., 2005]. Similar results were observed when ovariectomized rats received rosiglitazone [Sottile et al., 2004]. In part, rosiglitazone inhibited bone formation through a suppression of the osteogenic transcription factors, Runx2/Cbfa1, osterix, and Dlx5 [Rzonca et al., 2004; Ali et al., 2005; Lazarenko et al., 2005]. Consistent with this is the observation that netoglitazone, a thiazolidinedione with less osteogenic inhibitory function relative to rosiglitazone, induced adipocyte genes such as aP2/FABP4 without reducing levels of Runx2 or Dlx5 [Lazarenko et al., 2005]. An inter-play between PPARγ and Runx2 may underlie the association between advancing age with increased marrow adipogenesis and increased bone loss [Moerman et al., 2004]. Similar age-related changes occur in muscle satellite cells [Taylor-Jones et al., 2002]. The muscle satellite cells resemble

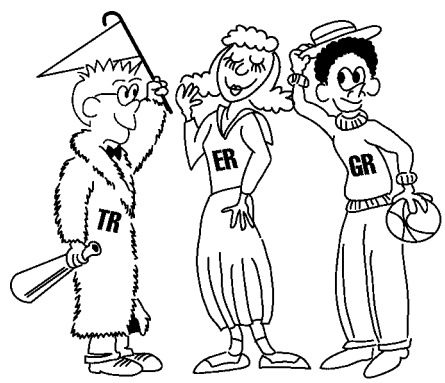
mesenchymal stem cells, acquiring adipocyte characteristics and activating PPARγ [Taylor-Jones et al., 2002]. Therefore, this process could be common to multiple adult or somatic stem cells as they age.

New findings suggest that a shared co-activator protein, known as the transcriptional co-activator with PDZ binding motif (TAZ), accounts for such a link between Runx2 and PPARγ [Hong et al., 2005]. In murine cell models, the TAZ protein co-activated Runx2 and osteogenesis while suppressing PPARγ and adipogenesis [Hong et al., 2005]. Thus, additional components of the transcriptional complex may influence a cell's lineage commitment.

Mutations of the *PPARγ* gene are associated with an altered balance between bone and fat formation in the marrow. Independent groups have examined PPARγ deficient murine models. A congenital mutation in the PPARγ2 locus is found in PPARγ hypomorphic mice [Cock et al., 2004]. These mice, which are "lipodystrophic," show reduced PPARγ1 and PPARγ2 mRNA levels in the residual white adipose tissue depots [Cock et al., 2004]. At the same time, the mice display enhanced bone formation to the point where the volume of the marrow cavity is so compromised that hematopoiesis moves to extramedullary sites, such as the spleen [Cock et al., 2004]. In some respects, this resembles the osteosclerosis observed in mice exposed to supraphysiologic concentrations of estradiol, the estrogen receptor (ER) ligand [Medina and Kincade, 1994]. Targeted mutations in the *PPARγ* gene resulted in homozygous animals where death occurred at embryonic day 10.5–11.5 [Akune et al., 2004]. Subsequent in vitro studies of PPARγ$^{-/-}$ embryonic stem cells demonstrated that the cells spontaneously displayed osteogenic function and failed to respond to adipogenic stimuli [Akune et al., 2004]. In the young, viable heterozygous mice (PPARγ$^{+/-}$), increased bone mass in vivo was associated with an ~twofold increase in osteoblast formation and an ~twofold decrease in adipocyte formation by bone marrow mesenchymal stem cells (MSCs) in vitro [Akune et al., 2004]. Consistent with this observation, the PPARγ$^{+/-}$ phenotype was associated with elevated mRNA levels of the osteogenic genes Runx2, osterix, and Lrp5 [Kawaguchi et al., 2005].

These findings indicate that PPARγ is a hypothetical drug target for age-related bone loss intervention [Duque, 2003; Moerman et al., 2004]. Other PPAR proteins may prove to be complementary targets. In studies using cells derived from PPARβ/γ$^{-/-}$ deficient mice, the absence of the PPARβ/γ protein reduced the adipogenic response to thiazolidinedione [Matsusue et al., 2004]. Moreover, in cells from wild-type controls, the further additon of a PPARβ/γ ligand, L165041, modestly increased adipocyte differentiation [Matsusue et al., 2004]. Thus, simultaneous manipulation of multiple PPAR pathways, relying on exogenous or endogenous ligands, may provide a novel approach to bone loss prevention and therapy [Schopfer et al., 2005].

Classical Steroid Hormones (ER, TR, VDR)—Old School



Old School

New experimental tools, such as gene microarrays, are being used to document the relationship of classical steroid hormones to bone and fat formation in marrow. One study has examined the skeletal phenotype of mice deficient in both thyroid receptors α and β (TRα and TRβ) using a combination of gene microarrays and quantitative real time PCR [Kindblom et al., 2005]. The TRα$^{-/-}$/TRβ$^{-/-}$ mice exhibited increased mRNA levels for adipocyte specific genes and a >200% increase in bone marrow adipocyte numbers based on histomorphometry [Kindblom et al., 2005]. The authors suggest that the increased amount of fat in bone marrow could be caused by a reduction in the activity of the GH/IGF-1 axis, although it cannot be excluded that T3 itself exerts direct effects on adipocyte/osteoblast differentiation independent of the GH/IGF-1 axis. These changes correlated with reduced trabecular and total bone mineral density [Kindblom et al., 2005]. The inbred SAM-P/6 murine strain provides a

model of accelerated senescence characterized by osteopenia and increased bone marrow fat mass [Kajkenova et al., 1997]. Recent studies found that 1,25 $(OH)_2$ vitamin D_3 treatment inhibited adipogenesis and enhanced osteogenesis in the SAM-P/6 mice [Duque et al., 2004a]. This correlated with a 50% reduction in PPARγ mRNA and protein levels [Duque et al., 2004a] as well as a decrease in oil red O positively stained cell numbers [Duque et al., 2004b]. Gene microarray analyses demonstrated a coordinated induction of osteoblastogenic genes and a reduction of adipogenic genes following 1,25 $(OH)_2$ vitamin D_3 [Duque et al., 2004b]. The 1,25 $(OH)_2$ vitamin D_3 treatment effected not only bone formation but also bone resorption based on circulating biomarkers of bone turnover [Duque et al., 2005].

The effects of estrogen on bone and adipose tissue formation have long been recognized in rat and canine ovariectomy models [Martin et al., 1990; Martin and Zissimos, 1991]. In humans, changes in estrogen status due to advancing age and menopause have been correlated with increased levels of cytokines IL-6 and IL-11, both associated with bone loss [Cheleuitte et al., 1998]. In vitro studies using murine bone marrow MSCs have found that estrogen reciprocally promotes osteoblastogenesis while inhibiting adipogenesis [Dang et al., 2002; Okazaki et al., 2002]. This process can be mediated by either ERs α or β, based on transfection approaches [Okazaki et al., 2002]. Indeed, there is evidence that the homologous orphan receptor, estrogen related receptors (ERR) α and β may also contribute to MSC lineage commitment [Bonnelye et al., 2001, 2002; Bonnelye and Aubin, 2002, 2005]. It is interesting to speculate whether the increase in adipogenesis subsequent to menopause is due to a relief of repression or an induction of the adipogenic phenotype. In vitro culture systems, albeit artificial, may argue that the default 'switch' is adipogenesis and that this process is inhibited normally in vivo prior to estrogen depletion.

In vitro studies using murine bone marrow MSCs have found that the soy phytoestrogen diadzein exhibits a dose-dependent biphasic action on osteoblastogenesis [Dang and Lowik, 2004]. This reflects a balance between the ER and PPARγ levels [Dang and Lowik, 2004]. The phytoestrogens activated ER and downregulated PPARγ transcriptional activity in transfection experiments [Dang and Lowik, 2004]. Similar studies in human MSCs have found that the soy isoflavone genistein induced osteogenesis based on Runx2/Cbfa1, alkaline phosphatase, and transforming growth factorβ (TGFβ1) mRNA levels and decreased adipogenesis based on C/EBPα, PPARγ, lipoprotein lipase, and adipsin mRNA levels [Heim et al., 2004].

Overall, these recent findings involving classical steroid receptors support the inverse relationship between adipogenic and osteogenic differentiation in the bone marrow microenvironment. This is mediated, in part, through cross-talk between the pathways activated by steroid receptors, the PPARs, and other cytokines and paracrine factors.

LXR, Cholesterol, and Oxysterols—Problem Children in the Junk Food Nation

Junk Food

The *LXR* gene was originally identified as an "orphan receptor" based on its heterodimerization with the 9-*cis* retinoic acid receptor retinoid X receptor (RXR) [Willy et al., 1995]. Follow-up studies rapidly identified oxysterols, cholesterol, and bile acid metabolites as its endogenous ligands [Janowski et al., 1996]. It is likely that oxidized lipids are in close contast with osteoblasts. Lipoproteins and lipids accumulate in bone and undergo oxidation, and bone contains a significant number of blood vessels, with cellular constituents of bone localized in close proximity to the interwoven vascular beds. We speculate that the LXR proteins, as receptors for cholesterol-derived ligands, may mediate osteogenic mechanisms. This is supported by studies in murine models have demonstrated that the statin compounds, which inhibit HMG CoA reductase and cholesterol synthesis, improve bone formation rates [Mundy et al., 1999]. In murine bone marrow-derived MC-3T3E1 cells, simvastatin enhanced mineralization and the expression of osteoblast and

vasculogenic gene markers [Maeda et al., 2001, 2003, 2004]. These actions were blocked by intermediate compounds in the cholesterol synthetic pathway [Maeda et al., 2004]. In contrast, mevastatin treatment of the murine bone marrow-derived M2-10B4 cells inhibited osteoblast differentiation and mineralization [Parhami et al., 2002]. Simultaneous addition of some, but not all, cholesterol synthetic intermediates reversed this action [Parhami et al., 2002]. Further studies in this cell line have demonstrated that specific oxysterols, such as 20S and 22R hydroxycholesterol, enhanced osteogenesis, both alone and in synergy with bone morphogenetic protein (BMP2) [Kha et al., 2004]. The same oxysterols inhibited adipogenesis induced by the PPARγ ligand troglitazone [Kha et al., 2004]. While some findings provide a hypothetical link between hyperlipidemia and osteoporosis [Parhami, 2003], the data relating to the osteogenic effect of putative LXR ligands differs between murine MSC models.

Independent studies in murine 3T3-L1 cells link LXR to adipogenesis. A microarray analysis identified LXR as an mRNA induced during adipocyte differentiation [Ross et al., 2002]. This can be explained by the fact that the LXR promoter is activated by PPARγ [Juvet et al., 2003; Hummasti et al., 2004]. While an LXR ligand alone had no effect on 3T3-L1 cells differentiation, inappropriate overexpression of LXR in the presence of ligand inhibited adipogenesis via the Wnt signaling pathway [Ross et al., 2002]. Homozygous $LXR^{-/-}$ mice have smaller adipose tissue depots compared to their wild-type littermates, suggesting that LXR regulates lipid storage [Juvet et al., 2003]. Indeed, there is evidence that LXR activates the PPARγ promoter and enhances adipogenesis in 3T3-L1 cells [Seo et al., 2004]. In summary, while there is evidence-linking LXR to adipocyte and osteoblast differentiation and function, the results of independent experiments are not entirely consistent. Further studies will be required to clarify the nature of this relationship.

OUTSIDE-IN SIGNALING

Multiple transmembrane signaling pathways regulate adipocyte and osteoblast differentiation and function. These pathways function independently and/or as part of a network. This section covers recent findings relating to the pathways listed in Table I.

TABLE I. Signal Pathways

Leptin
Wnt
TGFβ/BMP
CCN
Delta-like kinase (Dlk)/Pref-1

Leptin, a secreted "adipokine," modulates adipocyte and osteoblast metabolism and function. Evidence from multiple sources suggests that the route of leptin delivery determines the nature of its effect. In vitro studies by Thomas et al. [1999] found that leptin blocked human bone marrow MSC adipogenesis while enhancing osteogenesis. Subsequent, in vivo studies examined the effect of intra-peritoneally administered leptin in a rat tail suspension model of bone loss [Martin et al., 2005]. Here, leptin reduced bone loss by inhibiting osteoclast-mediated resorptive activity and prevented the increase of marrow adipose volume [Martin et al., 2005]. Independent studies have examined the effect of leptin administration on leptin-deficient *ob*/*ob* mice [Ducy et al., 2000; Takeda et al., 2002; Hamrick et al., 2004, 2005]. When leptin was infused into the cerebral ventricles, it stimulated bone loss in both *ob*/*ob* and wild-type mice [Ducy et al., 2000]; these actions were mediated through sympathetic outputs and could be blocked with β-adrenergic antagonists [Takeda et al., 2002]. In contrast, subcutaneous infusion of leptin to *ob*/*ob* mice led to increased bone mineral content and decreased bone marrow adipose tissue volume in the long bones of the hindlimb [Hamrick et al., 2005]. Indeed, Hamrick et al. [2004] and Khosla [2002] suggest that leptin is an osteogenic factor in animals that are leptin deficient, but when circulating leptin levels return to normal, additional leptin may no longer have a positive effect on osteogenesis and therefore may act as a rheostat of this cellular process.

There is some discrepancy in the literature concerning the skeletal phenotype of the *ob*/*ob* mice [Ducy et al., 2000; Hamrick et al., 2004]. While one group found that leptin deficiency promotes greater bone mass [Ducy et al., 2000], a second group reported that leptin deficiency had variable effects, increasing bone mineral density in the vertebral bodies while decreasing it in the long bones of the hindlimb [Hamrick et al., 2004]. Consequently, the role of leptin and

its mechanism of action in regulating the balance between adipocytes and osteoblasts remain controversial.

The Wnt signaling pathway encompasses multiple ligands, antagonists, receptors, co-receptors, and transcriptional mediators, such as β-catenin [Logan and Nusse, 2004]. Specific elements of the Wnt signaling pathway have been found to inhibit adipogenesis [Ross et al., 2000, 2002; Bennett et al., 2002] while promoting osteogenesis [Gong et al., 2001; Boyden et al., 2002; Kato et al., 2002; Little et al., 2002; Bennett et al., 2005]. Wnt inhibition of adipogenesis is mediated via β-catenin, which interferes with PPARγ transcriptional activation of downstream targets [Liu and Farmer, 2004]. The β-catenin function can be modulated by glycogen synthase kinase 3 β (GSK3 β) inhibitors, suggesting that this class of drugs could be used to direct MSC differentiation [Liu and Farmer, 2004]. Loss of function mutations or deletion of the Wnt co-receptor, LDL receptor-related protein 5 (LRP5), caused bone loss and osteopenia in murine models and patients [Gong et al., 2001; Kato et al., 2002]. In contrast, an inherited point mutation in the *LRP5* gene has been associated with enhanced bone formation and bone density in patients [Boyden et al., 2002; Little et al., 2002] and has been mimicked in a transgenic murine model [Babij et al., 2003]. Like LXR, LRP5 is associated with cholesterol metabolism since deficient mice are hypercholesterolemic [Fujino et al., 2003]. Mice deficient in the Wnt antagonist Dickkopf 2 (*DKK2*) gene displayed reduced bone marrow MSC osteoblast differentiation in vitro and osteopenia in vivo [Li et al., 2005]. The temporal relationship of DKK2 expression relative to the Wnt protein Wnt7B influences MSC lineage commitment [Li et al., 2005]. In vitro studies determined that forced expression of DKK2 prior to Wnt7B blocked osteoblast function, while expression subsequent to Wnt7B enhanced mineralization [Li et al., 2005].

The Wnt pathway works in co-ordination with other transmembrane signals. Following exposure to TGF-β, human bone marrow MSCs increased their expression of various Wnt receptors and ligands [Zhou et al., 2004]. This correlated with an inhibition of adipogenesis and an enhanced chondrogenesis [Zhou et al., 2004]. Independent studies have shown that TGF-β treatment inhibited the induction of the adipogenic transcription factors C/EBPα, C/EBPβ, and PPARγ during skeletal unloading in rodent models [Ahdjoudj et al., 2002, 2005]. These results suggest that similar networking occurs between the Wnt pathway and other TGF-β related cytokines such as the BMPs. Another pathway to consider is the CNN family, which includes connective tissue growth factor (CTGF), cysteine rich protein 61 (Cyr61), nephroblastoma over expressed (NOV), and the Wnt induced secreted proteins 1–3 (WISP1-3) [Jiang et al., 2004]. These secreted proteins bind to integrins and other extracellular matrix proteins and regulate cell migration, proliferation, and differentiation. The expression of CNNs in human bone marrow MSCs are differentially regulated during adipogenesis, chondrogenesis, and osteogenesis [Schutze et al., 2005]. It remains to be determined if the CNNs are downstream targets of the Wnt pathway regulating MSC proliferation and lineage selection.

The protein Pref-1, a member of the epidermal growth factor family also known as delta-like kinase 1 (dlk1) influences adipogenesis and osteogenesis. Pref-1 was initially identified as a cleavable surface protein highly expressed on pre-adipocytes and downregulated during adipogenesis [Smas and Sul, 1993; Smas et al., 1997]. The cleaved Pref-1 protein acts to inhibit adipogenesis [Smas et al., 1997]. Targeted deletion of Pref-1 generated deficient mice with increased levels of peripheral adipose tissue and retarded skeletal growth [Moon et al., 2002]. Since the *Pref-1* gene is imprinted, the pattern of its inheritance influences the phenotype of heterozygote deficient pups [Moon et al., 2002]. In vitro analysis in human bone marrow MSCs has determined that Pref-1 overexpression blocks both adipogenesis and osteogenesis [Abdallah et al., 2004]. This finding is consistent with the hypothesis that Pref-1 maintains MSCs in a multipotent state [Abdallah et al., 2004]. Pref-1 provides a novel example of a signal transduction pathway that is under epigenetic control. It will be exciting to follow developments in the Pref-1 story as new technologies emerge targeting epigenetic phenomena, such as CpG island genomic microarrays.

CONTROLLED SUBSTANCE ABUSE: ALCOHOL

While total caloric intake controls adipose tissue development, the type of nutrients is also a factor influencing the volume and location of


Alcohol

adipose tissue deposition. This holds true in the bone marrow where in vitro studies using human MSCs have shown that alcohol exposure directly stimulates adipogenesis by inducing PPARγ expression [Wezeman and Gong, 2004]. At the same time, alcohol inhibited human MSC mineralization and alkaline phosphatase expression [Gong and Wezeman, 2004]. In vivo studies using rats fed an alcohol-enriched liquid diet determined that alcohol exposure increased the triglyceride content of the femoral marrow [Wezeman and Gong, 2001]. This correlated with an increased marrow cavity volume, decreased cortical bone thickness, and, in the case of male rats, an elevation of estrogen levels [Wezeman and Gong, 2001]. These experimental observations were consistent with clinical studies of alcoholics, who displayed increased marrow adiposity and osteopenia [Wezeman and Gong, 2004]. These studies raise additional questions, specifically: (a) is there a specific alcohol receptor functioning within MSCs and, (b) if so, does this mediate the effects of alcohol on bone and fat metabolism?

PROTEASE INHIBITORS

The advent of protease inhibitors for the human immunodeficiency virus (HIV) treatment has uncovered new aspects of adipose and bone metabolism. Patients receiving this class of drugs exhibit selective lipodystrophy and decreased bone mass [Jain and Lenhard, 2002]. In rat calvarial cultures, a subset of HIV protease inhibitors enhanced osteoclast activity and bone resorption [Jain and Lenhard, 2002]. Likewise, in vitro studies using human MSCs showed that a subset of HIV protease inhibitors inhibited biomarkers of adipocyte and osteoblast differentiation [Jain and Lenhard, 2002]. It remains to be determined if naturally occurring protease inhibitors mediate similar functions. A potential candidate is visceral adipose tissue-derived serine protease inhibitor (Vaspin), initially identified as an adipocytokine in obese rats [Hida et al., 2005]. Vaspin administration to obese rats improved insulin sensitivity and reversed the induction of adipose genes induced by a high fat/high sucrose diet [Hida et al., 2005]. Studies are indicated to determine if vaspin or related endogenous protease inhibitors influence bone marrow MSC differentiation and function, in vitro and in vivo.

CONTROLLING THE TURF


Claiming/Real Estate

The signals directing MSC differentiation have a structural as well as biochemical basis [McBeath et al., 2004]. Investigators have manipulated the shape of human MSC in vitro by culturing the cells on surfaces prepared with adhesion molecule coatings at different densities [McBeath et al., 2004]. When bound to high-density surfaces, the MSCs took on a flattened appearance and underwent osteogenesis; on a low-density surface, the MSCs rounded up and underwent adipogenesis [McBeath et al., 2004]. These same cellular differentiation events were achieved by modulation of the RhoA, an actin/myosin-dependent GTPase. This seminal study demonstrates that an alteration of an MSC's biophysical and mechanical properties can cause distinct differentiation outcomes [McBeath et al., 2004]. RhoA and other biomechanically-linked pathways may provide novel targets for drug discovery.

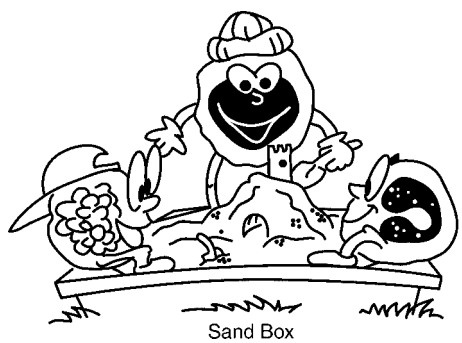
TAKE A DEEP BREATH FOR AEROBIC EXERCISES: HYPOXIA AND ANTIOXIDANT

Like any other cell, bone marrow MSCs require oxygen to maintain metabolic activities.

While the actual oxygen tension in the marrow cavity is less than that in highly perfused organs such as the lung or heart, it is higher than putative "hypoxic" sites such as articular cartilage. In vitro studies indicate that oxygen levels directly influence bone marrow MSC adipogenesis [Fink et al., 2004; Zhou et al., 2005]. Low ambient oxygen (2%) or the hypoxia mimetic compound deferoxamine blocked induction of adipocyte differentiation in human and murine MSCs [Zhou et al., 2005]. Based on studies using cells derived from Smad3$^{-/-}$ mice, the TGFβ mediated the inhibitory effects of hypoxia. Likewise, studies using hypoxia inducible factor-1$\alpha^{-/-}$ deficient mice have determined that the HIF-1α transcription factor contributes to hypoxic inhibition of adipogenesis [Yun et al., 2002]. Cells lacking HIF-1α undergo adipogenesis even under hypoxic conditions [Yun et al., 2002]. Similarly, murine MSCs deficient in redox enzyme, superoxide dismutase 2, displayed spontaneous adipogenesis, and an enhanced response to adipogenic agonists [Lechpammer et al., 2005]. Nevertheless, independent studies on human bone marrow-derived MSCs suggest that the adipogenic effects of oxygen may be dose or species dependent [Fink et al., 2004]. In the presence of 1% oxygen and the absence of adipogenic agents, human MSCs spontaneously accumulated lipid inclusions; however, this occurred without a concurrent induction of the adipogenic transcription factors PPARγ and steroid response element binding protein (SREBP1c) and their downstream targets, lipoprotein lipase, and aP2/fatty acid binding protein 4 [Fink et al., 2004]. Together, these findings indicate that oxygen tension and cellular redox pathways influence bone marrow adipogenesis. While it remains to be determined if this directly correlates with reciprocal changes in osteogenesis, the existing data lends itself to such a conclusion.

Unlike bone, the vascular supply to the cartilage is limited. It is widely assumed that cells of the cartilage are hypoxic and that limitations in the oxygen supply regulate the energetic state of the maturing cells. There are multiple publications indicating that hypoxia enhances chondrogenesis, both in vitro using MSCs and in vivo using HIF-1 $\alpha^{-/-}$ deficient mice [Lennon et al., 2001; Schipani et al., 2001; Robins et al., 2005; Wang et al., 2005]. Various reports lend strong support to the view that chondrocytes are very well adapted to low oxygen tensions; thus, under hypoxic conditions, there is a high level of expression of both HIF and AP-1, and energy conservation appears to be near maximum [Rajpurohit et al., 1996]. Therefore, it is feasible that hypoxic inhibition of MSC adipogenesis promotes chondrogenesis and endochondral bone formation.

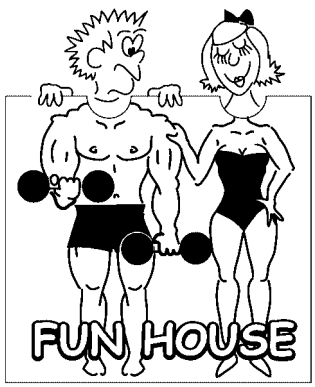
AT THE BOTTOM OF THE SANDBOX: HEMATOPOIESIS AND PLAYING WITH BLOOD CELLS



Adipogenesis and osteogenesis in the bone marrow occur in concert with the equally complex process of hematopoiesis [Gimble, 1990]. There has been renewed attention to the concept of the hematopoietic stem cell (HSC) "niche" within the bone marrow microenvironment [Kincade et al., 1989; Taichman and Emerson, 1994; Calvi et al., 2003; Zhang et al., 2003]. Evidence from in vivo and in vitro murine studies indicates that osteoblasts, under the regulation of PTH, notch ligand, and BMP, support HSC proliferation in vivo [Calvi et al., 2003; Zhang et al., 2003]. There is also evidence that cells once identified as "stromal" cells and

now characterized as multipotent MSCs are responsible for creating the HSC "niche" [Kincade et al., 1989]. Anatomical mapping studies indicate that multipotent MSCs with adipogenic, chondrogenic, and osteogenic properties co-localize to the hematopoietic sites in the developing murine embryo [Mendes et al., 2005]. The MSCs appear in the aorta-gonadal mesonephros and bone marrow prior to the time when each site becomes hematopoietic [Mendes et al., 2005]. Indeed, human bone marrow adipocytes supported $CD34^+$ HSC differentiation along the lymphoid and myeloid pathways [Corre et al., 2004], consistent with findings using comparable murine cells [Gimble et al., 1990, 1992]. Moreover, in $Smad3^{-/-}$ deficient mice, disruption of the TGFβ signaling pathway enhanced both hematopoiesis and adipogenesis [Epperly et al., 2005]. These results continue to reinforce the fact that bone marrow adipogenesis and osteogenesis cannot be divorced from hematopoiesis.

TRANSDIFFERENTIATION POTENTIAL OF EXTRAMEDULLARY ADIPOSE TISSUE CELLS: PLASTIC IN A MATERIAL WORLD



An important question to consider is whether MSCs can transit between two "terminally" differentiated states? There is in vitro and in vivo evidence to support this hypothesis. Cloned human bone marrow MSCs are capable of multiple lineage differentiation (adipocyte, chondrocyte, osteoblast) [Nuttall et al., 1998; Pittenger et al., 1999; Wolf et al., 2003]. Human MSCs are capable of expressing both adipogenic and osteogenic biomarkers simultaneously, consistent with a plasticity model [Ponce et al., 2005]. Moreover, single cell-derived human MSC clones can sequentially differentiate into adipocytes, dedifferentiate, and subsequently transdifferentiate into osteoblasts in vitro [Song and Tuan, 2004]. This plasticity has important therapeutic implications because it means that there is not an early commitment to a particular lineage differentiation. Even though cells may be differentiating (or indeed differentiated) down a particular lineage, therapeutic intervention may cause these same cells to transdifferentiate. Interestingly, it has been reported that even though osteoblasts were actively secreting osteocalcin, a phenotypic marker of a late stage differentiated osteoblast, they were still able to undergo adipogenesis when the culture conditions were modified [Nuttall et al., 1998]. The reports from Ponce et al. [2005], Oreffo et al. [2005b], and Nuttall et al. [1998] argue that perhaps cells can maintain an intermediate phenotype which is able to express a hybrid of characteristics of both adipocytes and osteoblasts. It is tempting to speculate that such a cell is capable and/or susceptible to perturbations in the local environment allowing 'phenotypic drift' when required. Indeed, the potentiality of the bone marrow MSCs may extend to additional lineage pathways. Consistent with this hypothesis is the fact that Oct-4 is expressed in BMSC's derived from rhesus monkeys [Izadpanah et al., 2005]. Evidence suggests that Oct-4 expression is confined to cells that share a toti/pluripotent phenotype [Pesce and Scholar, 2001].

Adipose-derived stem cells (ASCs) isolated from extramedullary fat depots of multiple species (human, rhesus monkey, murine) display multipotent properties, differentiating along the adipocyte, osteoblast, and other pathways [Halvorsen et al., 2001a,b; Zuk et al., 2001, 2002; Hattori et al., 2004; Hicok et al., 2004; Justesen et al., 2004b; Guilak et al., 2005; Izadpanah et al., 2005; Rodriguez et al., 2005a,b]. Comparison of human bone marrow-derived MSCs and ASCs indicate that while they share common functionality in terms of differentiation, they differ based on microarray analysis of gene expression [Lee et al., 2004]. Whether or not ASCs and MSCs are distinct cell populations remains a controversial topic that merits further investigation. Regardless of the outcome of this discussion, each cell type represents a potential tool for future regenerative medical applications [Gimble, 2003; Gimble and Guilak, 2003; Rodriguez et al., 2005].

BETTER LIVING THROUGH RECOMBINATION: TRANSGENIC MURINE MODELS

As the number of transgenic or gene deficient mice has increased, phenotypes reflecting altered bone marrow adipogenesis and/or osteogenesis have been encountered more frequently (Table II). Several models display a reciprocal relationship between bone marrow adipogenesis and osteogenesis. These include mice transgenic for Δ-Fos B protein, a component of the AP-1 transcription factor, and a congenic strain exhibiting reduced expression of IGF-1 [Kveiborg et al., 2004; Rosen et al., 2004]. A similar phenotype is observed in wild-type mice made acutely diabetic by streptozotocin treatment [Botolin et al., 2005]. Mice transgenic for a dominant negative form of the *N*-cadherin adhesion molecule exhibited reduced osteogenesis and increased peripheral adipose tissue [Castro et al., 2004]. Although marrow adipose tissue content was not directly examined, these findings appear to support a reciprocal relationship model. In contrast, transgenic expression of the truncated form of the adipogenic transcription factor C/EBPβ, also known as liver inhibitory protein, reduced both adipogenesis and osteogenesis simultaneously [Harrison et al., 2005; Hata et al., 2005]. Likewise, while mice deficient in the glucocorticoid metabolic enzyme 11-β hydroxysteroid dehydrogenase displayed reduced adipocyte numbers, their bone histomorphometry was normal [Justesen et al., 2004a]. These are just some of the relevant genetic murine models, and it is likely that more will appear to shed further insights (and possibly confusion) on the mechanisms regulating bone marrow MSC commitment.

COMING OF AGE

Pioneering histological studies have firmly established the direct relationship between advancing age, increased adipocyte numbers, and decreased bone formation [Custer and Ahfeldt, 1932; Vost, 1963; Hartsock et al., 1965]. Osteoporosis exacerbates this phenomenon by increasing the proportion of adipocytes in the marrow cavity [Meunier et al., 1971] and recent studies provide further confirmatory evidence [Verma et al., 2002]. In vitro studies using isolated human MSCs cultured in three-dimensional sponges demonstrate that the expression of osteogenic markers as well as the osteoclast antagonist, osteoprotegerin, decreased with advancing age [Makhluf et al., 2000; Mueller and Glowacki, 2001]. These findings have potential implications with respect to the treatment of age-related bone loss [Ahdjoudj et al., 2004]. These data argue that intrinsic changes to the cells in the aged skeleton contribute to their altered differentiation status and that changes in circulating systemic factors do not account exclusively for this phenomenon.

INTO THE FUTURE

The literature relating to the bone/fat relationship continues to expand, reflecting the growing appreciation of this issue at the basic science and clinical levels. While there remains significant support for the inverse relationship

TABLE II. Genetic Modulation of Marrow Phenotype

	Marrow phenotypes			
Gene target	Adipogenesis	Osteogenesis	Other	Reference
Δ-Fos B Tg	↓	↑		Kveiborg et al. [2004]
11 β $HD^{-/-}$	↓	Normal		Justesen et al. [2004]
IGF-1 reduced congenic	↑	↓		Rosen et al. [2004]
Dom. Neg *N*-cadherin Tg	Not examined	↓	↑ body fat	Castro et al. [2004]
C/EBP β (LIP) Tg	↓	↓		Harrison et al. [2005]; Hata et al. [2005]

Dom Neg, dominant negative transgene; HD, hydroxysteroid dehydrogenase; IGF-1, insulin-like growth factor-1; LIP, liver inhibitory protein; Tg, transgene.

model, we need to consider alternative explanations and paradigms. The bone marrow MSC may consider multiple differentiation pathways during its lifetime and, indeed, may dedifferentiate and transdifferentiate in response to changes in the microenvironment. One word of caution as this field develops is that the characterization of cell phenotype based on 'cell-specific markers' does not necessarily provide definitive evidence of functionality. This has been argued by Oreffo et al. [2005] who suggest that caution is required in interpretation of cell plasticity. Nevertheless, modulation of cell phenotype opens opportunities for preventive and interventional therapies to address the growing problem of osteoporosis in the aging population. The MSCs provide an in vitro model for drug discovery and can be used to screen small molecule combinatorial libraries for agents that block adipogenesis and enhance osteogenesis. In addition, genomic, metabolomic, and proteomic approaches centered on the MSC may uncover novel molecules or genes relating to differentiation. These tools may identify alternative metabolic pathways as targets for intervention. While we have routinely used chemical agents to treat chronic medical diseases, alternative modalities may prove feasible. It may be possible to use MSCs and other adult stem cells as therapeutic entities in their own right. The MSCs, together with biocompatible matrices, can be used to accelerate repair processes in orthopedic procedures [Bruder et al., 1998]. Using genetic engineering approaches, it will be possible to use MSCs to target specific cytokines, growth factors, or other proteins directly to the marrow microenvironment for regenerative purposes [Justesen et al., 2001; Ballas et al., 2002].

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