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Review Article

Regulatory pathways associated with bone loss and bone marrow adiposity caused by aging, chemotherapy, glucocorticoid therapy and radiotherapy

Kristen R Georgiou[1], Susanta K Hui[2], Cory J Xian[1]

[1]*Sansom Institute for Health Research, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, SA 5001, Australia;* [2]*Department of Therapeutic Radiology, College of Medicine, University of Minnesota, Minneapolis, MN 55455, USA*

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Abstract: The bone marrow is a complex environment that houses haematopoietic and mesenchymal cell populations and regulates bone turnover throughout life. The high proliferative capacity of these cell populations however, makes them susceptible to damage and injury, altering the steady-state of the bone marrow environment. Following cancer chemotherapy, irradiation and long-term glucocorticoid use, reduced bone and increased fat formation of marrow stromal progenitor cells results in a fatty marrow cavity, reduced bone mass and increased fracture risk. These bone and marrow defects are also observed in age-related complications such as estrogen deficiency and increased oxidative stress. Although the underlying mechanisms are yet to be clarified, recent investigations have suggested a switch in lineage commitment of bone marrow mesenchymal stem cells down the adipogenic lineage at the expense of osteogenic differentiation following such stress or injury. The Wnt/β-catenin signalling pathway is however has been recognized the key mechanism regulating stromal commitment, and its involvement in the osteogenic and adipogenic lineage commitment switch under the damaging conditions has been of great interest. This article reviews the effects of various types of stress or injury on the commitment to the adipogenic and osteogenic lineages of bone marrow stromal progenitor cells, and summarizes the roles of the Wnt/β-catenin and associated signalling pathways in the lineage commitment, switch, and recovery after damage, and as a therapeutic target.

Keywords: Bone marrow, mesenchymal stem cells, osteogenesis, adipogenesis, ageing, oxidative stress, chemotherapy, radiotherapy, glucocorticoids, Wnt/β-catenin signalling

Introduction

The bone marrow (BM) is a complex microenvironment, which houses two stem cell types and their progeny; mesenchymal or bone marrow stromal stem cells (MSCs or BMSCs) and haematopoietic stem cells (HSCs). A regulated balance of proliferation and differentiation maintains a steady-state functioning marrow including bone formation from MSCs and haematopoiesis from HSCs. Stress-induced conditions, such as those observed following medical treatments or with ageing, cause disruptions to the homeostatic balance of cell populations within the bone marrow. Associated with intensive and long-term cancer chemotherapy or radiotherapy is the depletion of haematopoietic populations and altered differentiation of both haematopoietic and stromal precursors. Following short-term damaging treatments and lower dosage of radiation or chemotherapy, disruption to the bone marrow microenvironment is typically a transient event and recovery ensues, reestablishing the bone marrow and its cell populations to steady-state conditions. However, following repeated insults or with increasing age, stem cell populations that enable re-establishment of the bone marrow diminish and recovery becomes less efficient. Such changes to the marrow are typically associated with bone loss and a fatty marrow or adipocyte-rich phenotype, increasing fracture risk and long-term bone defects. Thus it is of great interest to further elucidate the mechanisms that allow recovery of the bone and bone marrow following damaging conditions, in order

to improve or hasten the recovery capacity. This review aims to illustrate the altered cellular composition of the bone marrow cavity following damaging therapies and with age, focusing on the mechanisms that underlie the damage and subsequent recovery observed in both clinic and animal studies.

Bone marrow mesenchymal stem cells, osteogenesis and adipogenesis

Commitment to a particular lineage is largely dependent on transcription factors and the interplay of complex signalling mechanisms in the microenvironment. Close interactions between these two cell types support both haematopoiesis and appropriate commitment of multipotent MSCs. MSCs represent only a very small population (up to 0.01% from a BM aspirate) [1, 2]. Although the true identity of this multipotent stem cell-type remains elusive, more specific identification techniques have been employed to investigate this further [3, 4]. BMSCs have the capacity to differentiate into multiple cell lineages under specific signals including osteoblasts, adipocytes, chondrocytes and myocytes [5].

Osteogenesis and osteogenic transcription factors

Along the osteogenic cell lineage differentiation path, BMSCs are firstly committed to become highly proliferative osteoprogenitor cells which then develop into pre-osteoblasts and further differentiate into mature osteoblasts [6] (**Figure 1**). Osteogenesis is a complex process, initiated and regulated by transcription factors runt-related transcription factor 2 (Runx2) and osterix (Osx), whose expression is in turn stimulated by β-catenin and homeobox protein MSX2 and repressed by adipogenic transcriptional factor peroxisome proliferator activated receptor-gamma (PPAR-γ) [7, 8]. Activity of Runx2 (also known as core binding factor-alpha 1, cbfα1) is dependent upon core binding factor-beta (Cbfβ), which together enhance DNA binding activity [9]. In the early stages of differentiation, osteoblasts synthesise an extracellular matrix consisting predominantly of type I collagen and go on to express alkaline phosphatase (ALP), which are representative of the osteoblast phenotype. The complex of Runx2 and Cbfβ along with Osx activates target genes responsible for osteoblast maturation, expressing bone proteins such as bone sialoprotein and osteocalcin, resulting in mineralisation of bone [7]. At the end stage, osteoblasts actively synthesise and mineralise the bone matrix [10] and eventually become bone lining cells or osteocytes embedded within bone matrix which regulate remodelling and maintenance of the trabecular bone structure and strength [11]. There are a great many contributing factors to the commitment and differentiation process of BMSCs; of particular note are the Wnt/β-catenin and Hedgehog signalling pathways, both of which are discussed in more detail below.

Adipogenesis and adipogenic transcription factors and regulators

Recently, there has been a great deal of interest surrounding the potential switch in lineage commitment between osteoblasts and adipocytes, whereby conditions such as aging, osteoporosis, glucocorticoid and cancer treatments result in bone loss and exhibit an adipocyte-rich bone marrow [12-15]. As adipocytes and osteoblasts share the MSC as a precursor, it has been proposed that differentiation down the adipogenic program is preferential to osteogenesis under such conditions. Adipogenesis is a highly regulated process in which a cascade of transcription factors enables formation of mature adipocytes [8] (**Figure 1**). Transcription factors PPAR-γ and CCAAT-enhancer binding protein-alpha (C/EBPα) form a complex that goes on to coordinate the expression of adipogenic genes, enabling adipocyte commitment and terminal differentiation, with the induction of glycerol-3 phosphate dehydrogenase, hormone-sensitive lipase, fatty acid synthase, fatty acid binding proteins (FABPs), perilipin, and the secretion of adipokines such as leptin, adiponectin, adipsin, tumor necrosis factor (TNF-α) and retinol binding protein 4 [8].

Haematopoietic component of marrow environment

Contributing to the regulation of stromal cell commitment and differentiation are HSCs of the bone marrow, localised to a specialised structural environment or niche along the endosteal surface, at the border between the bone and bone marrow [16, 17]. Numbers of HSCs are maintained by allowing regulated self-renewal, continued anti-apoptotic properties and quiescence [18-20]. Quiescence and self-

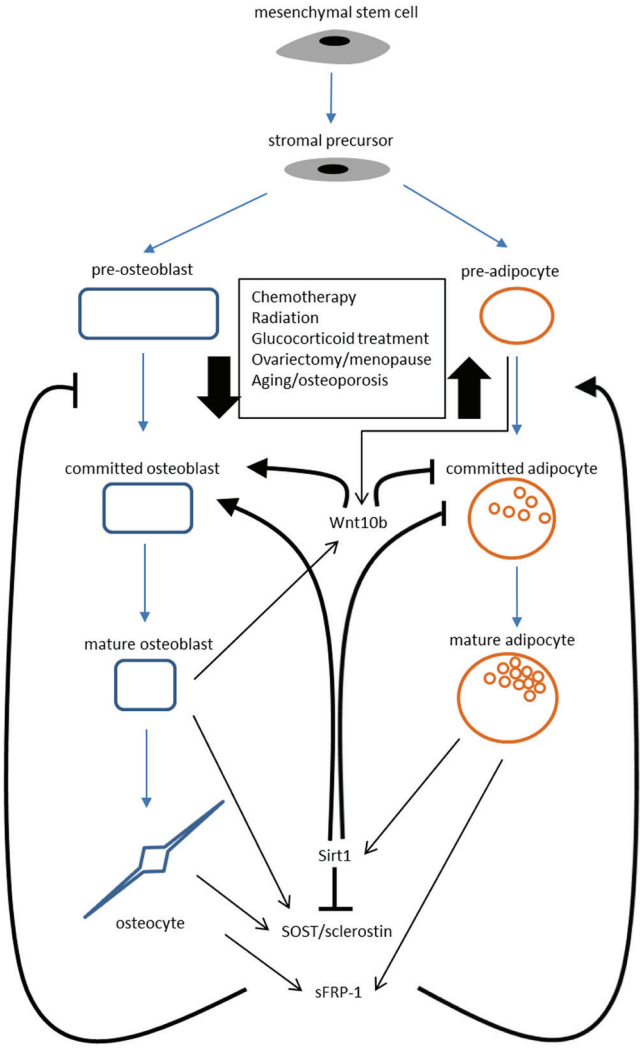

Figure 1. A brief overview of regulation of the balance between stromal progenitor cell commitment down the osteogenic and adipogenic lineages. Damaging conditions induced by treatment regimens and age-related changes promote adipogenesis at the expense of osteogenesis, potentially by increasing expression of Wnt/β-catenin antagonists. Adipogenic differentiation can be suppressed by Wnt10b and Sirtuin-1 and osteogenic commitment and differentiation promoted.

renewal are thought to be essential to protect HSCs from stress such as that induced by chemotherapy and to sustain long-term haematopoiesis and recover or re-establish the marrow microenvironment upon need [19, 21]. At the onset of cell division, one daughter cell will move towards the non-niche microenvironment to initiate differentiation, whilst the other remains in the niche as a self-renewing stem cell [20]. Previous studies have demonstrated that establishment of the stem cell niche in the bone marrow is largely due to interaction between HSCs and osteoblasts [21-24], as a lack of osteoblastic maturation results in a lack of bone marrow throughout the entire skeleton [22]. These HSC-niche interactions are capable of maintaining the bone marrow microenvironment by mediating the balance between HSC quiescence, self-renewal and differentiation [16], in turn contributing to stromal cell commitment and differentiation.

An increased fatty marrow cavity present under different conditions as discussed below has downstream effects on the regulation of a steady-state functioning bone marrow. Adipocytes have been illustrated to have a negative effect on haematopoiesis, whereby an adipocyte-rich marrow reduces not only haematopoietic progenitor cell number, but also affects cell cycling at the HSC level [25]. In order to confirm such an effect of adipocytes on haematopoietic function, mice unable to form adipocytes had significantly improved capacity for marrow haematopoietic recovery and function following lethal irradiation and subsequent marrow transplantation [25]. Similarly, a co-culture system of $CD34^+$ human haematopoietic precursors and adipocytes indicated a dampened capacity for granulopoiesis as measured by the number of mature granulocytes formed [26]. Investigations such as these illustrate the importance of maintaining cell populations of the bone marrow microenvironment, yet the underlying molecular regulators of the changes to bone marrow cell lineage commitment and differentiation remain to be fully elucidated.

Wnt signalling pathway in regulation of osteogenesis vs adipogenesis

As described above, regulation of the balance between adipocyte and osteoblast differentiation is largely unknown and a more fluid understanding of transcriptional regulation of the proposed adipocyte/osteoblast switch is

required. It has been demonstrated that osteogenesis, induced by factors such as bone morphogenetic protein or BMP-2 and Wnt ligands, is regulated by both canonical and non-canonical pathways of Wnt signalling. While the canonical Wnt/β-catenin pathway does so by inducing osteogenesis transcription factors but inhibiting expression of adipogenesis transcription factor PPAR-γ as described below, the non-canonical Wnt pathway which is less understood, controls activation of a histone methyltransferase histone-lysine N-methyltransferase SETDB1 that represses PPAR-γ transactivation through histone H3K9 methylation of target genes [27].

Known to be a key regulator of osteogenesis and stromal commitment is the Wnt/β-catenin signalling pathway or the canonical Wnt signalling pathway. This pathway has been shown to be essential for the regulation of osteogenic differentiation, while suppressing excess adipogenesis. Briefly, the Wnt/β-catenin signalling pathway is activated upon a Wnt ligand binding to the frizzled (Fzd), lipoprotein-related protein 5/6 (LRP5/6) co-receptor complex, which recruits an intracellular molecule disheveled (Dsh), inhibiting the action of a complex that acts to phosphorylate and tag β-catenin for ubiquitin-mediated degradation. When this complex, known as the destruction complex, consisting of intracellular signalling molecules is inhibited, β-catenin accumulates in the cytoplasm and translocates to the nucleus to enable target gene transcription [28].

There are a number of canonical Wnt ligands (including Wnt1, Wnt3a, Wnt7b, and Wnt10b) that activate the Wnt/β-catenin signalling pathway. Of particular note in regulating the osteogenesis and adipogenesis balance is Wnt10b, which has been identified to stimulate commitment to the osteoblast lineage and in turn inhibit adipocyte formation [28, 29] (**Figure 1**). Wnt10b is expressed by pre-adipocytes and stromal vascular cells and represses adipogenesis by maintaining pre-adipocytes in an undifferentiated state. It does so by suppressing PPAR-γ and C/EBPα expression, causing inhibition of terminal adipogenic differentiation and inducing Runx2, Dlx5 and Osx to promote osteogenesis [28-30]. The regulatory role of Wnt10b has been recently investigated, where its addition to the culture medium of a pre-adipocyte cell line dampened adipogenic differentiation and enhanced osteogenic differentiation in a stromal cell line (ST2) [31]. This was illustrated to be in a β-catenin-dependent fashion, whereby β-catenin knockdown in the pre-adipocyte cell line removed the anti-adipogenic effect induced by the addition of Wnt10b [31]. The importance of Wnt10b and the Wnt/β-catenin signalling pathway in the regulation of adipogenic commitment and differentiation has been further characterized in recent studies [31-33]. Interestingly one of these studies implicated C/EBPβ in the inhibition of Wnt10b over the course of adipogenic differentiation by directly binding to the Wnt10b promoter, thus increasing adipogenesis and providing a link between Wnt signalling and adipogenic commitment and differentiation [33].

There are a number of secreted Wnt antagonists that bind to Wnt ligands or the co-receptors to prevent Wnt/β-catenin signalling activation, in which case the destruction complex is activated and β-catenin is degraded, preventing or dampening target gene transcription. Notable antagonists (**Figure 1**) include secreted frizzled-related protein-1 (sFRP-1), sclerostin, dickkopf-1 (Dkk-1) and Wnt inhibitor factor-1 (Wif-1) [28]. As osteoporosis can be identified as increased marrow adiposity and bone loss, deregulation of the Wnt/β-catenin signalling pathway by the above antagonists has been investigated. Interestingly, osteoporotic women have higher serum Dkk-1 than controls, thought to be influencing Wnt/β-catenin signaling and thus the regulation of bone formation [34].

The Wnt antagonist sFRP-1 has also been suggested as a player in deregulated osteoblast and adipocyte commitment and differentiation. It has been shown to be secreted by pre-adipocytes and acts to negatively regulate osteogenic differentiation potential *in vitro* [35, 36]. However, it has also been shown to increase in expression over the course of differentiation from pre-osteoblast to pre-osteocyte [37]. sFRP-1 expression declined in mature osteocytes, indicating that aside from pre-adipocytes, osteoblasts and pre-osteocytes also largely contribute to sFRP-1 production and regulation of Wnt/β-catenin signalling. Furthermore, a correlation was found between the increased sFRP-1-induced antagonism of Wnt/β-catenin signalling and increased apoptosis, when sFRP-1 was stably over-expressed

in the human osteoblast and pre-osteocyte cell lines [37].

Hedgehog signaling and interaction with Wnt pathway in the regulation of stromal commitment

Members of the hedgehog (Hh) family of secreted proteins, represented by Desert (Dhh), Indian (Ihh) and Sonic (Shh) hedgehogs, play a vital role in developmental programming, with Shh playing the most widespread developmental role [38]. The role of hedgehog signalling in the commitment of MSCs down both the chondrogenic and osteogenic lineages has been well studied. It has been established that Ihh plays a vital role in initiating the osteogenic program of osteoprogenitors [39]. In brief, Shh undergoes post-translational modification before the N-terminal active form (Shh-N) is released from secreting cells. The Shh signal is enabled by the transmembrane receptor Patched (Ptch), which releases its inhibition of Smoothened (Smo), which is a transmembrane protein much like the frizzled/LRP5/6 receptor family. Thus in the absence of an Hh ligand, Smo is inhibited by Ptch, however whether this is a physical inhibition or otherwise, remains unclear [40]. Upon Ptch releasing Smo, it is able to exert downstream effects via the Gli family of DNA-binding proteins mediating the Hh signal. Previous investigations have found the mouse mesenchymal-like cell line (CH10T1/2) enters the osteogenic program after stimulation with exogenously added Shh, increasing ALP activity [38]. In a further study on the effects of Hh signalling on the commitment of CH10T1/2 cells down either the osteogenic or adipogenic program, the presence of Shh-N increased ALP activity and reduced adipogenic differentiation. This finding was also confirmed by a reduction in mRNA expression of genes associated with early and late adipogenic commitment and differentiation, including C/EBPα, PPAR-γ, FABP and leptin in the Shh-N exposed cells [40]. Furthermore, Gli1 was illustrated to play a role in the Shh-N inhibition of adipogenesis in this cell type [40].

Transcription of the Wnt inhibitor sFRP-1 is regulated by Gli1 and Gli2 and as a consequence of increased Hh signaling in mouse embryonic fibroblasts, elevated expression of sFRP-1 reduced Wnt1-mediated β-catenin accumulation in the cytosol [41]. These findings provide a molecular link between Hh and Wnt signaling pathways, further demonstrating an extensive signaling network that regulates bone and bone marrow maintenance throughout life.

Sirtuins in regulating adipogenic and osteogenic differentiation

Another player in the regulation of the proposed adipocyte/osteoblast switch is the nuclear NAD-dependent protein deacetylase, Sirtuin 1 (Sirt1). Sirt1 has been shown upon activation to decrease the differentiation of pre-adipocytes to adipocytes, reducing mature adipocyte number, whilst increasing the expression of osteogenic and osteoblast markers including ALP, collagen-1a1, osteocalcin, Runx2, Interleukin-6 (IL-6), Osteoprotegerin (OPG) and receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) in primary bone marrow cultures [42-44], suggesting Sirt1 inhibits adipogenesis whilst promoting osteogenesis. In order to investigate the mechanisms by which Sirt1 regulates bone formation and adipogenesis, a recent study has demonstrated female $Sirt1^{+/-}$ mice to have reduced bone volume as well as reduced *ex vivo* mineralization potential concurrent to an increase in *ex vivo* adipogenic differentiation potential [45]. Furthermore, Sirt1 repressed the expression of SOST, which encodes for sclerostin, a negative regulator of Wnt/β-catenin signaling and bone formation. In effect Sirt1 directly and negatively regulated SOST gene expression by binding the SOST promoter [45]. In a recent investigation on how Sirt1 expression is regulated, C/EBPα was found to bind directly to the Sirt1 promoter in 3T3-L1 adipocytes, up-regulating Sirt1 transcription. This was further confirmed by Sirt1 protein expression dose-dependently increasing with C/EBPα protein addition to cultures, as well as Sirt1 and C/EBPα protein expression concomitantly increasing over 3T3-L1 cell differentiation [46]. This study illustrates a potential mechanism for Sirt1 regulation of excess adipogenesis.

Bone loss and marrow adiposity

Bone loss and marrow adiposity associated with aging and estrogen deficiency

An osteoporotic phenotype has been well characterized, aside from a reduction in bone formation and an increase in bone resorption, a

largely fatty marrow cavity is also apparent. When assessed using magnetic resonance imaging, elderly osteoporotic and osteopenic males and females were found to have increased vertebral marrow fat compared to those with normal bone density [47-50]. This clearly illustrates the reciprocal relationship between reduced bone density and increased marrow adiposity.

Age is the key risk factor for osteoporosis. In age-related osteoporosis, there is an increase in adipogenic differentiation capacity but a reduction in osteoblast differentiation of bone marrow stromal precursor cells [13, 14, 51-53]. Furthermore, MSCs from osteoporotic patients had increased adipogenic potential and increased levels of active PPAR-γ protein when compared to control human MSCs [54]. This was found to be associated in part with a reduced responsiveness of MSCs to leptin, which exerted an anti-adipogenic effect on control cells and is known to induce proliferation and differentiation of MSCs down the osteogenic lineage [54]. One animal study found that aged rats had substantially increased expression of adipogenic transcription factor PPAR-γ when compared with young rats and this was supported by a significant increase in marrow adipocyte number and adipogenic differentiation potential of BMSCs in the aged rats [13].

The contribution of post-menopausal estrogen deficiency to bone loss has been well studied. The estrogen receptor is present on stromal cells, osteoblasts, osteoclasts and macrophages [55]. One major mechanism by which estrogens (E2) regulate bone mass is by responding to E2 via conventional E2 receptors on osteoclast precursors, which regulates their survival, osteoclast formation and activity [56, 57]. In addition, under conditions of low E2 in the case of menopause in aging females or ovariectomy (OVX) in animal models, enhanced osteoclast formation has been demonstrated, which increases bone resorption and results in net bone loss [55, 58]. One early study demonstrated that this was in part a result of an increase in levels of inflammatory cytokines within the bone marrow microenvironment of OVX mice [55].

Recent investigations have also focused on the contribution of adipocytes to altered bone formation in addition to increased action of osteoclasts. An animal study assessing both age and estrogen regulation of bone and fat maintenance observed that old OVX mice display a significant increase in marrow adipogenesis when compared to young OVX mice, which was accompanied by an increase in bone marrow PPAR-γ protein expression, as quantified by immunofluorescence [59]. E2 replacement in both young and old OVX mice dampened this increase in adipogenesis, as well as the spike in PPAR-γ expression [59]. In culture experiments, serum from postmenopausal women induced greater adipogenic differentiation of stromal precursors, however hormone replacement did not reverse the adipogenic differentiation observed *in vitro* [60]. In addition, OVX mice have been illustrated to have an increased adipogenic marrow, accompanied by a reduction in Sirt1 protein expression and an increase in PPAR-γ protein, which were reversed by estrodial administration [59].

The above investigations illustrate an overall disruption to steady-state functioning of the bone marrow microenvironment both independent and dependent on estrogen in ageing and menopause. However, the mechanisms by which these cell populations interact and regulate osteogenesis, adipogenesis and bone resorption in ageing and menopause-related osteoporosis remain to be further elucidated.

Oxidative stress and marrow adiposity

Oxidative stress has also been proposed to contribute to age-related bone loss. Oxidative stress is characterized as an imbalance between antioxidant function and free radical production. As such, a number of antioxidant-producing enzymes including catalase, superoxide dismutase and glutathione peroxidase play an important role in scavenging the reactive oxygen species (ROS) produced under conditions of oxidative stress [61]. Such increases in the production of ROS are caused by various types of injury and with age, influencing bone marrow haematopoietic and stromal cell populations [62, 63].

In OVX rats, one study demonstrated a reduction in glutathione peroxidase and superoxide dismutase as well as an increase in lipid peroxidation in femurs, concluding that oxidative stress may be responsible for the estrogen deficiency-associated bone loss [64]. As osteo-

porosis is associated with increased marrow adiposity as well as bone loss, investigations into whether oxidative stress contributes to mesenchymal lineage commitment is of great interest. An ageing-associated increase in bone marrow adipocytes in mice was found to coincide with a reduction in the antioxidants superoxide dismutase, glutathione and malondialdehyde in bone marrow cells [65]. One recent investigation into the antioxidant fullerol found it to reduce glucocorticoid-induced adipogenic differentiation of the mesenchymal stem cell line D2 by alleviating the impaired ROS status. Fullerol caused a reduction in the adipogenic transcription factor PPAR-γ and increased expression of osteogenic transcription factor Runx2 and bone formation marker osteocalcin [66]. It also increased the expression of antioxidative enzymes when compared to dexamethasone treatment alone, indicating an oxidative stress-induced bone/fat switch [66].

Cells attempt to counteract the adverse effects of ROS via defense mechanisms such as the activation of forkhead box O (FoxO) family of transcription factors. FoxOs promote cell survival by inducing cell cycle arrest and quiescence in response to oxidative stress. There are 4 subtypes, namely FoxO1, FoxO3, FoxO4 and FoxO6; however FoxO3 is the predominant FoxO in bone and bone cells [67, 68]. FoxOs shuttle between the cytoplasm and nucleus depending on phosphorylation at specific sites by kinases, and oxidative stress promotes nuclear retention of FoxOs, activating transcription by promoting such post-translational modification [68]. Interestingly, FoxO-mediated transcription requires binding of β-catenin, which is also essential for TCF/LEF-mediated transcription involved in canonical Wnt signalling [67, 69]. Therefore, due to competition, oxidative stress diverts β-catenin from TCF-mediated transcription to FoxO-mediated transcription, thus reducing the limited pool of active β-catenin for TCF-mediated transcription, required for osteogenesis. This competitive diversion has been demonstrated to be more obvious in aged mice (31 months old) than 4-month young mice, as shown by a stronger stimulation of expression in the aged mice of FoxO target genes (such as Gadd45) than Wnt/β-catenin/TCF signalling target genes (such as axin-2 and OPG) [67].

β-catenin/TCF-mediated transcription allows regulated osteogenic differentiation as well as suppression of PPAR-γ activity and hence adipogenic commitment and differentiation. As described above, oxidative stress causes a diversion of β-catenin from TCF-mediated transcription to FoxO-mediated transcription; however this also prevents the steady-state β-catenin suppression of PPAR-γ, increasing its transcriptional activation. As an example of this, ROS directly increased levels of FoxOs, reducing β-catenin/TCF transcription and increased PPAR-γ expression, reducing osteogenic commitment and increasing osteoblast apoptosis in the calvaria of aging mice [69, 70]. Further to this, PPAR-γ has an additional function in the suppression of β-catenin and Wnt signalling, by causing increased degradation of β-catenin [69, 71]. This relationship between β-catenin and PPAR-γ was shown to be independent to GSK-3B/APC and p53-mediated pathways and found to be a novel ubiquitination-mediated proteosomal degradation pathway [71]. ROS-induced lipid peroxidation of polyunsaturated fatty acids (PUFAs) allows these oxidized PUFAs activate PPAR-γ and hence promote degradation of β-catenin further inhibiting Wnt/β-catenin signalling [69]. An example of the direct relationship between PPAR-γ and β-catenin was illustrated in a study showing that troglitazone-induced PPAR-γ expression in breast cancer and fibroblast cell lines caused inhibition of β-catenin expression [71]. Furthermore, mutations in PPAR-γ helices in a fibroblast cell line reduced its binding to β-catenin, disrupting the ability of PPAR-γ to induce degradation of β-catenin, illustrating a direct relationship between β-catenin and PPAR-γ [72, 73]. The hypothesis that age-related bone loss may result, in part, from the suppressive effects of FoxO on the Wnt/β-catenin signalling pathway has also been supported by the findings that FoxOs promote the transcription of Wnt inhibitors SOST, sFRP-1 and sFRP-2 [74]. Thus taken together, the effects of ROS on FoxO and PPAR-γ provide potential mechanisms for oxidative stress-induced deregulation of mesenchymal lineage commitment away from osteogenic differentiation and toward adipogenesis.

Bone loss and marrow adiposity in multiple myeloma

There are a number of disease states and medical treatments that are associated with altered

bone and bone marrow maintenance. Characteristic of multiple myeloma (MM) are osteolytic lesions accompanied by an increase in osteoclast activity and bone resorption. Increased Wnt antagonists Dkk-1 and sFRP-2 are typically observed in MM patients, being produced in excess by myeloma cells. Such an increase in Dkk-1 in turn results in inhibition of the Wnt/β-catenin signaling pathway and reduced osteoblast differentiation and activity, causing a reduction in bone formation [75]. In MM patients increased levels of serum Dkk-1 and sclerostin (another Wnt antagonist predominantly expressed by osteocytes) have been observed, which together contribute to Wnt/β-catenin antagonism and hence bone loss in MM patients [75]. Interestingly, MM is a disease of ageing, with the average age of diagnosis being 65 years and the incidence further increasing with age [76].

As described above, associated with age is an increase in marrow adipocytes, presenting a mode of potential contribution of adipocytes in the marrow cavity to the MM disease environment in addition to the established role of enhanced osteoclast activity. Indeed there appears to be a functional consequence of adipocytes in the marrow cavity of MM patients, as they secrete cytokines and growth factors such as leptin that contribute to the MM environment [76, 77], whereby both murine and human MM cells were illustrated to express the leptin receptor [76]. However, such involvement of adipocytes is exclusive to the early stages of disease development, as adipocytes are replaced by MM cells as the disease progresses [76]. Further investigations are required to understand how disease- and treatment-induced defects to the bone and bone marrow affect one another and the molecular regulators that may be manipulated for treatment purposes.

Glucocorticoid usage-induced bone loss and marrow adiposity

Long-term glucocorticoid usage is a well-known risk factor for osteoporosis and increased fracture risk [78]. Animal studies have shown glucocorticoid excess to cause an up-regulation in the bone of Wnt antagonists Dkk-1 and sclerostin, while reducing Wnt receptor complex components Frizzled -4 and -7 and downstream transcription factor LEF-1 [79]. Consistently, when primary human osteoblasts were exposed to dexamethasone, ALP activity was reduced along with cytoplasmic and nuclear β-catenin. However, when the Wnt signalling antagonist Dkk-1 was silenced in these osteoblasts by siRNA, the reduction in ALP activity was alleviated [80]. Similarly, *in vitro*, Dkk-1 knockdown resulted in abrogation of glucocorticoid-mediated increases in adipogenic differentiation potential and reduced osteogenesis [81]. These studies illustrate that glucocorticoid-induced alterations to osteogenic differentiation are at least in part a result of alterations to Wnt/β-catenin signalling antagonist Dkk-1. In addition, it has been shown that antagonist sFRP-1 contributes to glucocorticoid-induced disruption of Wnt/β-catenin signalling *in vitro*. For example, supplementation of dexamethasone in MC3T3 osteoblast culture caused a significant reduction in MC3T3 mineralization potential, which was associated with increased sFRP-1 mRNA expression, as well as an increase in intracellular antagonist axin-2, concomitantly causing a reduction in β-catenin (Hayashi et al 2008). Consistently, cultured human BMSCs dose-dependently responded to dexamethasone treatment by increasing adipogenic differentiation as well as increasing adipocyte size [82]. Similarly, in primary rat BMSC cultures, dexamethasone treatment caused a dose-dependent increase in adipogenic differentiation potential with a concomitant increase in adipogenic-associated gene expression [83] yet reduced expression in osteogenic transcription factors Runx2 and Osx [84]. These studies strongly suggest that glucocorticoid usage deregulates Wnt/β-catenin signalling and consequently causes the associated bone loss and marrow adiposity.

Cancer chemotherapy-induced bone loss and marrow adiposity

Similar to the deregulation of osteogenic/adipogenic balance within the marrow of age-related osteoporotic patients, long-term cancer chemotherapy disrupts the complex network of signaling pathways that regulate lineage commitment, differentiation, and balance within the marrow cavity [85, 86]. In the clinic, patients receiving chemotherapy and irradiation treatment for Hodgkins disease, seminoma, prostate and breast cancer have an observed increase in central marrow fat [87, 88].

Understanding the cellular and molecular mechanisms that are responsible for lineage determination and that are deregulated by chemotherapy or other such damaging treatments is key to developing specific and targeted therapies that are currently lacking to prevent cancer chemotherapy-induced bone and bone marrow defects.

When looking at the direct effects on the stromal lineage, exposure of MSCs to chemotherapeutic agents doxorubicin and etoposide *in vitro* resulted in enhanced differentiation potential towards the adipogenic lineage in preference to osteogenic differentiation [89]. In a rat model of methotrexate and 5-FU chemotherapy, a reduction of the trabecular bone volume and an increase in marrow adiposity are apparent over the treatment time-course [15, 90-95]. Furthermore, *ex vivo* culture of methotrexate-treated rats has illustrated an effect on the differentiation capacity of BMSCs toward the adipogenic lineage at the expense of the osteogenic lineage [96]. This switch in lineage commitment was at least partly attributed to deregulation of the Wnt/β-catenin signalling pathway, whereby methotrexate chemotherapy-induced increased marrow adiposity and bone loss were alleviated by an agonist of the Wnt/β-catenin pathway [95]. Interestingly, in the above methotrexate model, expression of both Dkk-1 and sFRP-1 mRNA was found upregulated one day after completion of 5-day methotrexate treatment (day 6) and sFRP-1 mRNA expression was also found to be elevated 4 days after completion of treatment (day 9), illustrating the involvement of Wnt antagonists in deregulation of Wnt signalling in this model [95].

Apart from a direct effect of chemotherapy on the stromal lineage, it also appears to have an indirect effect on the stromal lineage by altering ovarian function. Premenopausal women receiving a cyclophosphamide, methotrexate and 5-fluorouracil (CMF) chemotherapeutic regimen for the treatment of breast cancer were found to have significantly reduced bone mineral density (BMD) most obvious in those who suffered from ovarian dysfunction resulting in cessation of their menstrual cycle (amenorrhea) [88]. This illustrates a clear involvement of estrogen as discussed above, in the maintenance and recovery of BMD following chemotherapy. Glucocorticoid treatment, also used in chemotherapeutic regimens, has been associated with an increase in bone loss and increased marrow fat in cancer patients [97].

Radiotherapy-induced bone loss and marrow adiposity

It is a well-established notion that radiation treatment causes myelosuppression and damage to bone marrow cell populations [98-100]. For example, in a recent study total body irradiation (TBI) resulted in depletion of total femur bone marrow cellularity in treated mice 9 days after TBI and white blood cells number measured in peripheral blood specimens remained low up until day 28 [99]. Studies have found radiotherapy to have damaging effects on haematopoietic and stromal lineages of the bone marrow microenvironment similar to chemotherapy as described above. Patients receiving radiation therapy alone or in combination with chemotherapy experience significant bone marrow cell depletion, bone loss increasing fracture risk and increased marrow fat cellularity [101-103].

A recent investigation using a mouse radiotherapy model has demonstrated early and transient severe hematopoietic depletion and vascular pathology over the course of radiation damage and recovery [104]. Research strongly supports bidirectional co-regulation of bone and marrow components at the microenvironmental level, indicating the importance of evaluating these tissues as an integrated unit in order to further our understanding of the response to radiation injury or physiologic change [105, 106]. As MSCs play a role in hematopoietic recovery after radiation injury [107], they may be strongly influential in hematopoietic recovery following transplantation [108, 109]. In addition, repair of radiation damage may be associated with migration of HSCs as well as MSCs from non-radiated sites [110, 111], further complicating interpretation for site-specific radiation effects and recovery.

Radiation toxicity predominantly affects multipotent MSCs and occurs at unexpectedly low doses, which may in part, contribute to the catabolic pathology of bone tissue [112]. *In vitro* investigations have found the murine mesenchymal-like cell line C3H10T1/2 to have increased apoptosis and cellular senescence following radiation treatment [113]. *In vitro*

studies have also shown that radiation dose-dependently damaged the proliferation and osteogenic/adipogenic differentiation capacity of hMSCs, which was followed by a complete recovery of growth and renewal capacity in the surviving MSCs following long-term culture [114]. However, osteogenic differentiation potential of hMSCs seemed less resistant to irradiation than adipogenic potential of hMSCs [114]. Similarly, upon irradiation, osteoblastic stromal precursors obtained from rat cavaria were found to have significantly reduced osteogenic differentiation potential, and their cell viability was dose-dependently reduced [115]. These studies suggest that radiation can directly reduce the osteogenic potential of MSCs or stromal precursor cells.

The influence of ovarian hormones in the regulation of bone marrow cell damage and recovery in response to radiation treatment has recently been investigated [104]. In this study, there was substantial but incomplete bone marrow recovery 30 days after local irradiation in intact female mice. On the other hand, multiple fold increases in the marrow fat fraction following irradiation were not reflected in equal loss of either bone or hematopoietic cellularity in OVX mice [104]. This indicates that post-irradiation changes among haematopoietic, adipose and osseous tissues were distinctly different depending upon the presence or absence of ovarian hormones. OVX appeared to predispose the MSC population toward an adipogenic phenotype and radiation further enhanced adipogenesis without proportionate effects on the adjacent bone or hematopoietic cellularity [104].

The molecular mechanisms by which radiation affects stromal cell survival, commitment, differentiation and recovery potential are yet to be identified. However potential involvement of TNF-α function and Wnt/β-catenin signalling have been suggested. Recently, it has been shown that irradiated murine stromal cell line cells (CH10T1/2) to have increased TNF-α protein in the conditioned medium. Haematopoietic precursor cell apoptosis was also found increased following radiation over the short-term (3-days) and this increase was concurrent to increased TNF-α mRNA expression [116]. Consistently, cultured marrow mononuclear cells (MNCs) from the treated mice were found to have increased TNF-α protein in the conditioned medium and long-term (3 cycles) radiation treatment in mice was also found to induce bone marrow dysfunction, reducing red blood cells, white blood cell and platelet numbers in the bone marrow. However, in TNF-α knock-out mice, the long-term radiation did not appear to change the haematological parameters [116]. This set of data illustrates the involvement of TNF-α and associated signalling in the damage observed to bone marrow cell populations following radiation treatment. In addition, radiation-induced apoptotic and senescent C3H10T1/2 cells were found to have increased expression of Wnt ligands Wnt3a and Wnt5a, suggesting potential involvement of canonical and non-canonical Wnt/β-catenin signalling in regulating radiation-induced mesenchymal progenitor cell apoptosis and senescence [113].

However, the role of radiation in regulation of bone marrow cell populations, including haematopoietic, osteogenic and adipogenic lineages and the recovery potential of these cell populations remains to be clearly established. Thus a clearer understanding of deregulation of steady-state bone marrow function following radiation damage *in vivo* and *ex vivo* must be gained before appropriate therapeutic targets can be identified.

Therapeutic targets to increase bone formation and reduce adipogenesis

Developing treatments that specifically target regulatory mechanisms in order to improve bone/fat balance is of great interest to alleviate the bone loss observed under conditions such as those described above. The Wnt/β-catenin signalling pathway presents a potential target for future therapies to treat bone loss conditions such as ageing/menopause-induced osteoporosis, damaging therapy-induced bone defects and other such ailments. However, as Wnt/β-catenin signalling is a regulatory pathway involved in proliferation and differentiation, potential over-activation leading to tumorigenesis is potentially also of concern. In order to address this, there have been a number of investigations into targeting the secreted antagonists of the pathway, particularly Dkk-1, sclerostin and to a lesser extent sFRP-1. In mouse models, these antagonists are primarily expressed by osteoblasts or osteocytes [117-119], in which case systemic administration of targeting treatments would not disrupt Wnt sig-

nalling in other organs/cell types, making them attractive as therapeutic targets.

Sclerostosis, which is due to the insufficiency of sclerostin production, is a disease characterized by high bone mass [120]. There have also been consistent animal studies demonstrating that the knockout of the sclerostin gene, SOST, results in high bone mass [121, 122]. These findings have provided the basis for further investigations into the potential therapeutic applications of inhibition of sclerostin under conditions of reduced bone mass [121]. Inhibition of sclerostin using a specific monoclonal antibody (Scl-Ab) was used in an OVX model, whereby 5-week treatment completely reversed the bone loss associated with estrogen deficiency, increasing bone mass and strength to a greater extent than that of the intact animal control [123]. In an aged male rat study, Scl-Ab improved cortical thickness at critical sites such as the fifth lumbar vertebral body, femoral neck and distal femur compared to vehicle, and it dose-dependently increased serum osteocalcin, indicative of greater bone formation at weeks 3 and 5 of treatment compared to the vehicle group [124]. Furthermore, in a non-human primate model, systemic administration of the sclerostin antibody for 10 weeks caused increases in BMD and strength throughout the entire skeleton, including sites known to be susceptible to osteoporosis, such as the lumbar spine, total hip and distal radius [125]. The first clinical study using a humanized Sclerostin monoclonal antibody known as AMG785 was performed on healthy men and postmenopausal women, administering a single IV or SC dose at varying concentrations [126]. Dose-dependent increases in bone formation markers propeptide of type 1 collagen , bone alkaline phosphatase and osteocalcin were observed, as well as an increase in BMD of the lumbar spine and total hip in all cohorts. Interestingly the effect of a single dose of the sclerostin antibody was found to be comparable to that of 6 months of daily parathyroid hormone (PTH) treatment [126]. The above studies have demonstrated that an anti-sclerostin antibody represents a promising therapy for osteoporosis and other such bone loss conditions. However, further investigations into the effects of Scl-Ab treatment on other cell types/tissues and in longer-term treatment models are required.

Apart from targeting sclerostin, investigations into inhibition of other Wnt antagonists such as Dkk-1 and sFRP-1 have also been undertaken. There have been a number of studies conducted in animal models investigating the efficacy of a Dkk-1 neutralizing antibody in increasing bone mass. For example, a Dkk-1 antibody had been established *in vitro* to increase osteogenic differentiation of a mesenchymal-like cell line (CH10T1/2) and the use of this antibody in growing female mice was observed to increase trabecular bone mineral density (BMD) [127]. In a follow-up study, 8 weeks of treatment with this anti-Dkk-1 antibody alleviated OVX-induced bone loss as revealed by increased femoral and lumbar spine BMD, demonstrating its potential use in the treatment of postmenopausal osteopenia or osteoporosis [128]. Investigations into sFRP-1 inhibition have also seen some success. For example, an orally bioavailable small molecule inhibitor of sFRP-1 has been developed, which disrupts the protein-protein interaction between Wnts and sFRP-1, thus allowing more Wnt ligands to bind the LRP5/6 receptor to increase bone formation [129]. *Ex vivo* mouse calvarial cultures with or without this compound found increased osteoblast numbers and areas of new bone formation when examined histologically [129, 130]. Studies such as these illustrate a promising future for therapies targeting the Wnt/β-catenin signalling pathway to promote bone formation.

Current therapies for the treatment of osteoporosis in ageing or postmenopausal women typically include the anti-resorptives such as bisphosphonates. Anti-resorptives act on osteoclasts to dampen their enhanced activity and bone degradation, reducing net bone loss, yet their effectiveness may vary depending on the initial severity of damage [131]. In addition to these properties, studies have found aminobisphosphanates to also have an effect on the stromal lineage, increasing osteogenesis and reducing adipogenesis of BMSCs isolated from OVX rats treated with the agent [132]. These findings were further supported by expression of osteogenesis vs adipogenesis-related genes and histomorphometric assessments, illustrating its potent effects under such conditions in the bone marrow microenvironment [132]. In one study conducted over three years, treatment of postmenopausal women with risendronate or a placebo found a reduction in adipo-

cyte volume, number and diameter, as well as a reduction in PPAR-γ protein expression by adipocytes in the risendronate-treated women when compared to the placebo group [133]. This is further supported by an animal study in which OVX rats were treated with alendronate and *ex vivo* culture of BMSCs from treated rats demonstrated an increase in osteogenic differentiation potential as well as a reduction in adipogenic differentiation when compared to control rats. This was confirmed by a significant increase in mRNA levels of bone-related genes and transcription factor Runx2, concurrent to a reduction in adipogenic transcription factor PPAR-γ [134].

In addition to the anti-resorptives, intermittent treatment with anabolic agent human recombinant intact PTH (PTH 1-84) or human recombinant PTH peptide 1-34 (Teriparatide) is an option for the treatment of severe osteoporosis. It has been well established that PTH regulates bone formation by increasing osteoblast number and activity and reducing osteoblast apoptosis [135-137]. However, whether it plays a role in osteogenic commitment and regulation of adipogenic differentiation is less established. Similar to the findings of the above studies on treatment with bisphosphonates on osteogenic and adipogenic differentiation, Teriparatide addition to the culture medium of human bone marrow stromal cells had an effect on their commitment and differentiation. Intermittent treatment with PTH reduced the adipogenic differentiation capacity but increased ALP positivity of osteogenic cultures [138]. Similarly, treatment with parathyroid hormone-related protein (PTHrP) (1-36) reduced adipogenesis of human MSCs as revealed by reduced expression of adipogenic related genes PPAR-γ and lipoprotein lipase [139].

Another interesting target for treatment against age-associated increased marrow adiposity is Sirt1. As described above, a reduction in Sirt1 expression has been linked with increased adipogenesis and reduced bone formation, further illustrating its role in the regulation of the bone marrow microenvironment. One study found Sirt1 to be a negative regulator of SOST expression by binding to the promoter of SOST, which encodes for the Wnt antagonist sclerostin. BMSCs from Sirt1 deficient ($Sirt1^{+/-}$) mice had an increased adipogenic potential when compared to BMSCs from the wild-type. In addition, when SOST was down-regulated by siRNA in $Sirt1^{+/-}$ osteoblasts, gene expression of osteocalcin and bone sialoprotein was significantly increased, confirming the defects observed in Sirt1 deficient mice was a result of increased SOST [45]. In line with these findings, adipogenic differentiation of pre-adipocytes was dose-dependently reduced with addition of a Sirt1 agonist resveratrol into the culture medium [140]. This reduction in differentiation was found to be attributed to an increase in Sirt1 expression by both pre- and later stage adipocytes when assessed for mRNA expression [140]. To this effect, further knowledge of the regulation of Sirt1 and its action on PPAR-γ-regulated adipogenesis may provide a therapeutic target for the treatment of age or medical treatment-induced fatty marrow and osteoporosis.

Conclusions

This review has focused on the stromal progenitor cell populations of the bone marrow microenvironment and potential regulatory mechanisms that allow their commitment, differentiation and function under the steady-state conditions, as well as damaging conditions and associated mechanisms that cause bone loss and marrow adiposity. Physiological and pharmacological stress or insult, such as age-related estrogen deficiency or ovariectomy, oxidative stress, glucocorticoid use and damaging cancer chemo- or radio-therapies, disrupt the steady-state bone marrow microenvironment and result in deregulation of stromal cell commitment and differentiation, favoring adipogenesis at the expense of osteogenesis. While the mechanisms that are responsible for or contribute to such a significant increase in marrow fat and bone loss remain to be clearly identified, it has been established that there is a network of signalling pathways that act in conjunction to enable an appropriately functioning bone marrow. The Wnt/β-catenin signalling pathway not only promotes bone formation, but inhibits adipogenic differentiation. Wnt10b in particular has been extensively investigated for its role in inhibiting adipogenesis by inhibiting PPAR-γ and C/EBPα. Wnt antagonists, of either the Wnt ligands themselves or of the LRP5/6 co-receptors, including sclerostin, Dkk-1 and sFRP-1, have also been illustrated to

play a role in regulating the lineage commitment and differentiation of stromal precursors. sFRP-1, expressed by pre-adipocytes, osteoblasts and pre-osteocytes, has been found to negatively regulate bone formation and to increase adipogenesis. Interestingly, sFRP-1 expression is regulated by downstream targets of the Hedgehog signalling pathway, transcription factors Gli1 and Gli2, further illustrating the complex link between different signalling pathways that regulate stromal commitment and differentiation under steady-state and damaging conditions.

As described herein, deregulation of components of the Wnt/β-catenin signalling pathway has been shown to occur, contributing to bone loss and marrow adiposity under damaging conditions such as chemotherapy, glucocorticoid use and with ageing, although it remains to be established whether defects in this signalling pathway also contribute to radiation-induced bone/bone marrow damage. So far the Wnt/β-catenin signalling pathway has been identified as a potential target for promoting bone formation and reducing fat formation. For example, recent investigations into the potential use of a sclerostin antibody for the treatment of osteoporosis have established its effect on alleviating estrogen-deficiency-induced bone loss and clinical studies have also shown positive outcomes.

Further studies are required to gain a better understanding of the ways in which the Wnt/β-catenin signalling pathway and other signalling pathways are deregulated following damaging therapies or as a result of ageing or disease states, which may give an indication of its contribution to a damaged bone marrow microenvironment. In order to refine current treatments and develop more specifically targeted therapies to treat disruption to the steady-state bone marrow function, a clearer understanding is required of the complex mechanisms that regulate commitment and differentiation of bone marrow cell populations under steady state and stress/damaged conditions.

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Address correspondence to: Dr. Cory J Xian, Sansom Institute for Health Research, University of South Australia, City East Campus, GPO Box 2471, Adelaide 5001, Australia. Phone: (618) 8302 1944; Fax: (618) 8302 1087; E-mail: cory.xian@unisa.edu.au

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