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Multi-lineage differentiation of mesenchymal stem cells – To Wnt, or not Wnt

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Abstract
Mesenchymal stem cells (MSCs) are multipotent precursor cells originating from several adult connective tissues. MSCs possess the ability to self-renew and differentiate into several lineages, and are recognized by the expression of unique cell surface markers. Several lines of evidence suggest that various signal transduction pathways and their interplay regulate MSC differentiation. To that end, a critical player in regulating MSC differentiation is a group of proteins encoded by the Wnt gene family, which was previously known for influencing various stages of embryonic development and cell fate determination. As MSCs have gained significant clinical attention for their potential applications in regenerative medicine, it is imperative to unravel the mechanisms by which molecular regulators control differentiation of MSCs for designing cell-based therapeutics. It is rather coincidental that the functional outcome(s) of Wnt-induced signals share similarities with cellular redox-mediated networks from the standpoint of MSC biology. Furthermore, there is evidence for a crosstalk between Wnt and redox signalling, which begs the question whether Wnt-mediated differentiation signals involve the intermediary role of reactive oxygen species. In this review, we summarize the impact of Wnt signalling on multi-lineage differentiation of MSCs, and attempt to unravel the intricate interplay between Wnt and redox signals.

Keywords
Mesenchymal stem cells; Differentiation; Wnt; Reactive oxygen species; Cellular fate

Keywords
ADSCs Adipose tissue-derived MSCs, APC Adenomatous polyposis coli, BIO 6-bromo-indirubin-3'-oxime, BMP Bone morphogenetic protein, C/EBPα CCAAT/Enhancer binding protein alpha, C/EBPβ CCAAT/enhancer binding protein beta, CaMKII Calcium/calmodulin-dependent protein kinase II, CK1α Casein kinase 1 alpha, CRD Cysteine-rich domain, CREB cAMP response element binding protein, Dkk Dickkopfs, Dvl Dishevelled, DZNep 3-deazaneplanocin A, EZH2 Enhancer of Zeste Homology 2, Fz Frizzled, GPX Glutathione peroxidase, GSK-3β Glycogen synthase kinase-3 beta, H_2O_2 Hydrogen peroxide, IGF Insulin-like growth factor, JNK c-jun N-terminal kinase, KLHL12 Kelch-like 12, LiCl Lithium chloride, LRP5/6 Lipoprotein receptor-related protein 5/6, MSCs Mesenchymal stem cells, NAC

N-acetylcysteine, NELL-1 Nel-like protein, NO Nitric oxide, Nox NADPH oxidase, NRX Nucleoredoxin, PCP Planar cell polarity, PKC Protein kinase C, PPARγ Peroxisome proliferator-activated protein gamma, PRXs Peroxiredoxins, ROS Reactive oxygen species, sFRPs Secreted frizzled-related proteins, SOD Superoxide dismutase, TCF/LEF T cell factor/Lymphoid-enhancing factor, WIFI Wnt inhibitory factor 1, YAP1 Yes-associated protein 1

Introduction

Clinical significance of mesenchymal stem cells and their differentiation

There has been a recent surge in interest in the clinical use of mesenchymal stem cells (MSCs) in regenerative medicine, tissue repair, and other cell-based therapies [1]. MSCs have been isolated from various sources such as bone marrow [2], adipose tissue [3], umbilical cord tissue [4], periodontal ligament [5], synovial membrane [6], menstrual fluid [7], and dental pulp [8]. Although bone marrow was the earliest identified source of MSCs [9], the utility of marrow-derived MSCs was limited due to several factors such as invasive surgery and subsequent patient discomfort. Hence, sources of MSCs other than the bone marrow, such as described above, have been aggressively explored in recent years.

MSCs are capable of extensive self-renewal in an undifferentiated state, but upon appropriate and specific stimuli are capable of multi-potent differentiation towards various cell lineages. Although the preference is for differentiation to mesenchymal lineages, there is strong experimental evidence for differentiation to all three germ layers - mesoderm, ectoderm, and endoderm [5, 10-13]. As such, a better understanding of the process of MSC differentiation at the molecular level is essential if MSCs are to achieve the potential for their application in regenerative medicine. This demands an understanding of the signal transduction pathways regulating MSC differentiation, amongst which Wnt signalling is one of the key players. Hence, in this review, we highlight how the Wnt signalling network impacts MSCs and influences their fate. Our primary focus will be the interaction between the canonical Wnt signalling pathway and MSC differentiation. Further, we will describe the effect of reactive oxygen species (ROS) on MSC differentiation and the crosstalk between ROS and Wnt to influence MSC differentiation.

1.2. Wnt signalling

Wnt signalling is evolutionarily conserved from nematodes to mammals, regulating various stages of embryonic development as well as tissue homeostasis in adulthood [14, 15]. Wnt signalling affects major cellular events such as proliferation, survival, apoptosis, angiogenesis and cell polarity. It is also involved in regulating self-renewal and differentiation of stem cells in adult tissues, and regulating the regenerative processes in response to disease, trauma, and ageing. The Wnt molecules are secreted glycoproteins composed of 350-400 amino acid residues, of which 23-24 are highly conserved cysteine residues (molecular weight: 39kDa-46kDa) [14]. The difference in mode of signalling subsequent to the generation of the Wnt signal classifies the Wnt signalling network into two main branches, depending on the intracellular response it triggers. One is the canonical pathway mediated by β-catenin, while another is the non-canonical signalling pathway that involves protein kinase C (PKC) and c-jun N-terminal kinases (JNK).

1.2.1. Canonical Wnt signalling

The canonical Wnt signalling pathway is β-catenin mediated. Here, the Wnt ligand forms a ternary complex with a Frizzled (Fz) receptor and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptor to activate the intracellular Dishevelled (Dvl) protein. Dvl in turn phosphorylates and inhibits glycogen synthase kinase-3β (GSK-3β) of the destruction complex. The destruction complex is composed of the proteins, axin, adenomatous polyposis

coli (APC), casein kinase 1α (CK1α), and GSK-3β, and is responsible for the phosphorylation and subsequent degradation of β-catenin in the absence of Wnt signalling. In the presence of a Wnt stimulus the complex remains inactive, leading to accumulation of the unphosphorylated β-catenin in the cytoplasm and consequent translocation to the nucleus. In the nucleus, β-catenin replaces the co-repressors and binds to the transcriptional factor complex composed of T cell factor/Lymphoid-enhancing factor (TCF/LEF), thereby triggering the transcription of the Wnt target genes.

1.2.2. Non-canonical Wnt signalling

Non-canonical Wnt signalling is independent of β-catenin and involves several distinct pathways. One of the non-canonical pathways impacts on cell migration by regulating the activation of Calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC), thereby resulting in the release of intracellular calcium [16, 17]. This association with cellular Ca^{2+} mobilization has led to the description 'Wnt/Ca^{2+} signalling pathway'. A second, non-canonical pathway controlling cell polarity and cytoskeletal organization is referred to as the Wnt/planar cell polarity (PCP) signalling pathway. It acts by activating Rho-GTPases and Jun N-terminal kinases (JNKs) [16, 17]. The molecular events occurring in these non-canonical pathways are generally less well defined than the canonical pathway.

1.2.3. Regulators of the Wnt signalling pathway

Among the various molecules that modulate the Wnt signalling pathway [18] are extracellular Wnt antagonists such as secreted Frizzled-related proteins (sFRPs), Dickkopfs (Dkks), and Wnt Inhibitory Factor1 (WIF1) [19, 20]. Briefly, sFRPs are the first identified secreted antagonists of the canonical and non-canonical Wnt signalling pathways and**,** in humans**,** they are 5 in number (sFRP1-5) [20-24]. Among them, the major Wnt antagonist sFRP4 was first isolated by our group by the use of a rat cDNA library [21]. By inactivating Wnt, sFRPs prevent the accumulation of active unphosphorylated β-catenin in the cytoplasm, thereby suppressing the activity of Wnt target genes [19, 25].

The Fz receptors possess an extracellular cysteine-rich domain (CRD) composed of 120 amino acids [15], which shares structural homology to the CRD in the N-terminal region of sFRPs [26]; sFRPs lack the transmembrane domain present in Fz receptors. sFRP anatgonizes Wnt signalling by interfering with the Wnt-Fz interaction through direct binding to the Wnt or to the Fz receptor, thereby forming a non-functional complex. While the latter mechanism (sFRP-Fz binding) is facilitated by the CRD of the sFRPs [27]**,** the former mechanism (sFRP-Wnt binding) can occur either via the CRD [28] or the domains in the C-terminal region of sFRPs [29]. In addition, the biphasic regulatory potential of sFRPs has been elucidated by evidence of Wnt binding to the CRD in the N-terminal region of sFRPs and inhibiting β-catenin accumulation; while Wnt binding to the domain in the C-terminal region of sFRP allows the CRD at the N-terminus to interact with Fz, hence facilitating Wnt signalling [29]. Other Wnt anatagonists such as the Dickkopf (Dkk) family includes 4 members (Dkk 1-4), which inhibit only the canonical Wnt signalling by binding to the LRP5/6 co-receptor [19]. In addition to these naturally occurring extracellular Wnt antagonists, there are pharmacologically derived antagonists such as XAV939 [23] and iCRT3 [24]. In this review we focus on how these different Wnt regulators play a role in Wnt signal transduction and influence the cell fate determination of MSCs.

2. Role of Wnt signalling pathways in differentiation of mesenchymal stem cells

2.1. Role of Wnt signalling in adipogenic differentiation

Evidence from the last decade indicates an important role for Wnt signalling in adipogenic differentiation from precursor cells. [30]. An inhibitory effect on adipogenesis by Wnt-

activating molecules such as Wnt 10b, GSK-3β inhibitors such as lithium chloride (LiCl) [31], and CHIR 99021 [32] has been demonstrated using 3T3 pre-adipocytes. Wnt4 and Wnt5a, both non-canonical Wnt ligands, have been shown to possess a promoting effect on 3T3 cell adipogenesis through the PKC-CamKII pathway. Additionally, the authors refer to antagonism of the canonical Wnt signalling pathway by Wnt5a [33].
In MSCs, the inhibitory effect on adipogenesis has been demonstrated using the GSK-3β inhibitor (Wnt activator) - 6-bromo-indirubin-3'-oxime (BIO) [34]. Wnt activation (by silencing the Wnt antagonists sFRP4 and Dkk1) was performed in adipose MSCs, which resulted in significant down-regulation of adipogenic differentiation with regard to lipid accumulation as well as adipogenic markers [35]. It has also been reported that a short 48 hour treatment with sFRP1 and sFRP4 upregulated adiponectin secretion in human MSCs [36].
Additionally, during adipogenic differentiation of MSCs, the levels of the Wnt antagonists sFRP4 and Dkk1 were seen to be higher than in the undifferentiated state [35]. Our laboratory has demonstrated that addition of exogenous sFRP4 promoted adipogenic differentiation of MSCs [37]. This *in vitro* evidence indicates the presence and significance of the suppressed state of Wnt signalling during adipogenesis. The importance of Wnt antagonist levels has been teased out by a few clinical studies. In one of them, sFRP4 levels were seen to be directly proportional to impaired glucose and triglyceride metabolism [38], and in another study sFRP4 was proposed as a biomarker for predicting type II diabetes mellitus [39]. Overall, the activation of the Wnt signalling pathway inhibits adipogenic differentiation in MSCs, while the presence of Wnt antagonists promotes a contrary effect on adipogenesis, which could be considered when developing new strategies to manage obesity and diabetes.

2.2. Wnt signalling regulates osteogenic differentiation

Growing evidence suggests a regulatory role for Wnt signalling in bone development and homeostasis. In MSCs, the *in vitro* potential of Wnt signalling on osteogenic differentiation has been controversial, with both stimulatory and inhibitory effects being reported. It has been shown that the canonical Wnt signalling pathway activates differentiation of MSCs into osteoblasts [40]. The disruption of Wnt signalling by a functional mutation or targeted destruction of LRP5 in mice has been shown to promote osteoporosis and a low bone mass phenotype [41, 42], while its over-expression leads to a high bone mass syndrome [43]. In another study, the supportive role of Wnt proteins towards osteogenesis was shown by preventing apoptosis of osteoblast progenitors and differentiated osteoblasts in the osteoblast cell line OB-6 and pre-osteoblast cell line MC3T3-E1 [44]. The Wnt ligand Wnt10b was shown to prevent bone loss occurring due to oestrogen deficiency and aging in mice [45]. It was reported that Wnt signalling favoured osteogenesis at the expense of adipogenesis [46, 47]. It was also demonstrated that suppression of PPARγ and C/EBPα, and the simultaneous enhancement of the expression of osteoblastogenic transcription factors such as RunX2, Dlx5, and Osterix occurred in mouse mesenchymal cells [45, 46].
With regard to the effect of Wnt antagonism on osteogenesis, it has been reported to be mostly inhibitory. In mice over-expressing the Wnt antagonist sFRP4 in osteoblasts, there was a reduction observed in bone formation, leading to a low bone mass [48]. This inhibitory effect of Wnt antagonists on osteogenesis was also demonstrated by an *in vitro* study where sFRP4 inhibited osteogenic differentiation in MSCs from periodontal tissue [49]. Among other Wnt antagonists, sFRP1 over-expression was found to also inhibit *in vivo* bone formation [50] and increase the rate of osteoblast and osteocyte cell death [48]. The lack of sFRP1 reduced apoptosis and accelerated osteoblast proliferation and differentiation in human osteoblasts and MSCs [51], and also enhanced trabecular bone formation and

improved fracture healing [51-53], indicating the inhibitory effect of Wnt antagonists on osteogenesis.

Many other signalling pathways, such as the Hippo signalling pathway, interact with Wnt. Hippo signalling involves Yes-associated protein 1 (YAP1), which upregulates Dkk1 and, in turn, supresses canonical Wnt signalling to inhibit the osteogenic potential of MSCs [54]. The stimulatory effect of Wnt has been shown in human MSCs [55] and also in mouse MSCs [56], where an increased expression of the osteogenic transcription factor RunX2 was observed [56]. Overall, these studies indicate that canonical Wnt activation promotes osteogenesis, while Wnt antagonists inhibit osteogenesis from MSCs.

Other studies have reported an inhibition of osteogenesis by the canonical Wnt pathway in human bone marrow MSCs [57-59]. One group reported that sFRP3 promoted osteoblast differentiation [60]. A recent study explained this phenomenon in further detail by showing that sFRP3 antagonises the non-canonical Wnt pathway through Wnt5a binding, which in turn blocks Wnt5a's inhibitory effect on the canonical Wnt pathway, and hence promotes osteogenesis [49]. This mechanistic approach adds further evidence to the role of canonical Wnt signalling in promoting osteogenic differentiation. All these studies indicate the extensive crosstalk contributed by the Wnt signalling network during osteogenic differentiation, which should be considered when devising possible new therapeutic interventions for bone-related diseases. Moreover, the available evidence reinforces the reciprocal relationship that exists between bone and fat development in response to an activated canonical Wnt pathway.

2.3. Role of Wnt signalling in chondrogenic differentiation

Wnt signalling is one among the main regulators of chondrogenesis. The promoting role of Wnt signalling in chondrogenesis has been demonstrated by a study showing that sFRP1-deficient mice demonstrated enhanced chondrocyte maturation in both *in vivo* and *in vitro* conditions [61]. A functional role for Wnt5a, a non-canonical Wnt ligand, in the chondrogenesis of MSCs derived from the chicken wing bud has also been demonstrated [62]. The level of Wnt5a was up-regulated after treatment with transforming growth factor β (TGFβ), which promoted chondrogenesis. This was further supported by the upregulated pre-cartilage condensation and chondrogenesis in *in vitro* micromass cultures in MSCs transfected with Wnt5a [62]. The mode of action underlying this could be the blunting of canonical Wnt signalling by the non-canonical Wnt ligand Wnt5a, and hence promoting chondrogenesis. An increased expression of Wnt3a was associated with the chondrogenic differentiation of high density C3H10T1/2 murine mesenchymal cells [63], and the overexpression of Wnt3a was also demonstrated to promote this process in the same cell line [64].

On the other hand, it has been reported that continuous treatment with Wnt1 protein for 21 days reduced the expression of chondrogenic-specific markers, while treatment with the Wnt antagonist Dkk1 increased the expression of chondrogenic markers such as collagen II, Sox9, and aggrecan in human adipose-derived MSCs [65]. These results are corroborated by studies demonstrating that both sFRP1 and Dkk1 enhanced glycosaminoglycan synthesis, Sox9, and type II collagen expression in early chondrogenesis of human MSCs [66], and these Wnt antagonists also exhibited a chondrogenesis-promoting effect in long-term pellet cultures [67].

2.4. Role of Wnt signalling in cardiogenic differentiation

Previous studies in chick and frog embryos demonstrated that Wnt antagonism induced cardiac development while an activated canonical signalling pathway inhibited the differentiation [68, 69]. Later, it was found that stimulation of canonical Wnt signalling (by use of LiCl) increased the bioavailability of β-catenin, and hence improved myogenic differentiation in MSCs derived from aged cardiac patients [70]. The non-canonical Wnt

ligand, Wnt11, promoted cardiomyocyte differentiation in MSCs transduced with Wnt11 by upregulating GATA-4. The authors postulated that the mechanism involved Wnt11-mediated activation of the non-canonical PKC or JNK pathway [71]. Contrastingly, MSCs overexpressing sFRP2 have been shown to have reduced apoptosis rates and improved engraftment and cardiac function in mice [72]. Exogenous addition of sFRP2 inhibited type I procollagen maturation, which is important in scar formation post-myocardial infarction [73], and therapeutic concentrations of sFRP2 significantly reduced fibrosis and improved cardiac function in rats [73]. However, in another study, a reduced infarct size was observed in sFRP2 null mice, thus the authors referred to the known biphasic effect of sFRP2 to explain their findings [74]. Overall, an activated Wnt signalling pathway contributes to enhanced cardiogenic differentiation in MSCs and this phenomenon should be considered when devising MSC-based therapies for myocardial infarction.

2.5. Wnt signalling inhibits hepatogenic differentiation

The Wnt pathway has been demonstrated to work synergistically with the MAP/ERK signalling pathway to support hepatic progenitor cell proliferation [75]. A study on hepatic progenitor cells of the mouse embryo demonstrated that inhibition of Wnt signalling (accomplished using the Wnt antagonist sFRP3) prevented the differentiation of progenitor cells into the hepatic lineage [76].

Hepatic differentiation in the mouse has been shown to be tightly regulated, with the distinct expression of Wnt antagonists such as sFRPs and Dkks in embryonic liver tissues [76]. In humans, hepatic differentiation is differentially regulated by the Wnt signalling pathway, depending on the stem cell source. Wnt signalling was shown to be down-regulated during the hepatic differentiation of adipose tissue-derived MSCs (ADSCs), while it was activated in human embryonic stem cells (hESCs) [77]. The distinct response seen in hESCs was also observed when Wnt3a pre-treatment facilitated the differentiation of hESCs towards hepatic endoderm [78].

Similar to the effect seen on ADSCs [77], during hepatic differentiation of umbilical cord tissue-derived MSCs, there was a downregulation of canonical Wnt signalling. Hence, suppression of Wnt signalling in turn promoted hepatic differentiation [79]. It has also been demonstrated that application of Frizzled 8 siRNA on MSCs, in which Wnt signalling was reduced, resulting in enhanced hepatic differentiation in human bone marrow-derived MSCs [80]. Overall, these studies indicate that enhanced hepatic differentiation is observed in human MSCs following down-regulation of Wnt signalling.

2.6. Wnt signalling promotes myogenic differentiation

The effect of different Wnt molecules on myogenic differentiation has been well documented in embryonic development. In MSCs, the Wnt molecules- Wnt1, Wnt3, Wnt7a, Wnt7b, Wnt4, and Wnt11 had a promoting effect on gene expression of at least one myogenic marker. Among these Wnt proteins, Wnt11 was the most potent activating factor demonstrating gene expression of all myogenic factors-Myf5, MyoD, myogenin, and myogenin regulating factor (Mrf4) [81]. The promoting effect of Wnt3a in myogenic differentiation has also been reported in rat bone marrow MSCs [82]. The GSK-3β inhibition by LiCl and consequent Wnt activation resulted in an increased bioavailability of β-catenin and enhanced the myogenic differentiation potential in aged MSCs [70]. Another study demonstrated that hypoxia-preconditioned MSCs possessed better engraftment potential into ischaemic tissue, and improved skeletal and vascular muscle regeneration when compared to normal MSCs. They found that this enhanced effect was brought about by a Wnt4-dependent pathway, and that hypoxia-preconditioned MSCs had an increased number of Wnt4 transcripts in the ischaemic area compared to the normal MSCs [83]. Altogether, it suggests that activated Wnt signalling enhances the myogenic differentiation potential of MSCs.

2.7. Wnt signalling positively influences neurogenic differentiation

It has been demonstrated that Wnt3a protein promotes the differentiation of neural stem cells into neuronal lineages while inhibiting their self-renewal [84]. In cortical mouse neural precursor cells, Wnt7a also enhanced neuronal differentiation [85]. Further, in MSCs, the activating role of canonical Wnt signalling has been determined using specific neuronal markers such as neuron-specific enolase and nestin. These markers were up-regulated when Wnt signalling was activated using Wnt3a and down-regulated when Wnt signalling was blocked by siRNA for β-catenin [86]. It was also found that Wnt7a enhanced neuronal differentiation in human MSCs via both canonical and non-canonical signalling pathways [87]. Further, the Wnt antagonists Dkk1 and sFRP4 reversed Wnt7a's promoting effect [87]. Re-activation of the Wnt signalling pathway reversed the age-related decline in neurogenic differentiation in MSCs derived from dental pulp [88]. Further investigation into the mechanism of Wnt-induced up-regulation of neurogenic differentiation in MSCs has revealed the role of T-cell leukaemia 3 (Tlx3) in interacting with the TCF3/4 of the canonical Wnt signalling pathway. In this study, it was shown that Wnt1 upregulates Tlx3, and a forced expression of Tlx3 improved neurogenic differentiation [89]. Wnt5a promoted neurogenic differentiation in human ADSCs, binding to Fz3 and Fz5, and signalling through the Wnt5a-JNK pathway [90]. In summary, activation of Wnt signalling plays a key role in promoting the differentiation of MSCs towards a neuronal fate.
The overall impact exerted by the activation of the Wnt signalling pathway on the differentiation of human MSCs has been schematically represented in Fig. 1.

3. Link between wnt/β-catenin signalling and reactive oxygen species

Recent findings have demonstrated that there is a close association between reactive oxygen species (ROS) and activation of the Wnt/β-catenin pathway. β-catenin is translocated to the nucleus after the inhibition of the protein complex comprised of Axin, APC, GSK-3β, and CK1α [91]. This inhibition is assisted by Dishevelled (Dvl), a protein present in the cytoplasm, which inhibits GSK-3β-mediated phosphorylation of β-catenin, allowing its translocation [92]. Dvl and a thioredoxin-like protein, nucleoredoxin (NRX), have been shown to directly interact and inhibit Wnt/β-catenin signalling and this association is attenuated by hydrogen peroxide (H_2O_2), suggesting that Wnt/β-catenin signalling may be modulated by oxidative stress [93]. Wnt signalling is carefully balanced by the competition between NRX and a Kelch-like 12 (KLHL12) protein, both associated with the PDZ-domain of Dvl. In the absence of a Wnt ligand, the binding of NRX to Dvl promotes the inactive state of Dvl, thereby preventing aberrant activation of the pathway until a sufficient Wnt stimulus occurs. Upon Wnt binding, Dvl frees itself from NRX, activates downstream signalling, and is then ubiquitylated by KLHL12, thus preventing persistent activation of the Wnt signalling pathway [94]. Recently it was also demonstrated that inhibition of Ca^{2+}-mediated ROS production from the mitochondria attenuated the Dvl/NRX association, which led to a decrease in Wnt/β-catenin activation and inhibited the ability of neuronal cells to differentiate *in vitro* [95]. Although a full understanding of ROS activation of the Wnt/β-catenin pathway has yet to be elucidated, it is known that Wnt antagonists also play a role in the modulation of ROS. It has been shown that sFRP4 can increase superoxide and H_2O_2 levels while decreasing catalase activity in endothelial cells, and thus inhibiting angiogenesis [25]. Further research into the mechanisms of ROS activation of the Wnt/β-catenin pathway has the potential to provide possible treatment options for many Wnt-associated diseases.

3.1. The source and generation of reactive oxygen species

There are many potential sources of ROS within the cell; the primary source of ROS is from the mitochondria where free-radical superoxide anion (O_2^-) is generated via the electron transport chain, primarily from complex I [96] and III by single electron reduction of molecular oxygen [97]. Superoxide (O_2^-) is converted to H_2O_2 (which has a much longer half-

life) through ROS scavengers such as superoxide dismutase (SOD) in the cytosol (SOD1/CuZn-SOD), mitochondrial matrix (SOD2/MnSOD), and in extracellular compartments (SOD3) [98].

Another important source of ROS is from the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complexes, which together are known as NOX enzymes [99]. Oxygen is converted to superoxide through pyridine nucleotide-mediated reduction by NOX enzymes at specific membrane-associated sites [100], thus generating superoxide for release into phagolysosomes (in phagocytic immune cells) and either cytosolic or extracellular release. The superoxide anion can also react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$) [101], which has been shown to induce apoptosis through caspase activation [102].

Hydrogen peroxide is classed as a reactive oxidising chemical that can be converted to water and oxygen in the peroxisome and cytosol through enzymatic action by catalase. Furthermore, it is reduced to H_2O through the enzymatic action of antioxidant glutathione peroxidases (GPx), which catalyse the reaction $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$ and by peroxiredoxins (Prxs) in the cytosol, Prxs (reduced) + $H_2O_2 \rightarrow$ Prx (oxidised) + $2H_2O$ [103].

Other intracellular sources of ROS include the endoplasmic reticulum as well as other enzyme systems such as xanthine oxidase, lipoxygenase, cyclooxygenase, cytochrome P450, and monooxygenase [99]. ROS, together with the peroxisomes and mitochondria, serve many functions including cellular proliferation [104], migration [105], differentiation [100], survival [106-108], and cell signalling [109]. ROS play a crucial role in cellular homeostasis and a variety of processes within all cell types. Interestingly, the study of ROS within MSCs has gathered pace recently due to its complex nature, the involvement of a variety of cell signalling pathways, including Wnt, a myriad of protein interactions, and its involvement in mesenchymal differentiation.

3.2. Mesenchymal stem cells and reactive oxygen species

MSCs exhibit relatively low levels of ROS, possibly due to their increased levels of glutathione, SOD1/SOD2, catalase, and GPx [110]. It has also been shown that varying levels of ROS control different signalling pathways within MSCs, which are responsible for many cellular functions and cellular differentiation. These pathways include Wnt, Forkhead box (FOXO), Hedgehog [111], Nel-like protein 1 (NELL-1), insulin-like growth factor (IGF), and bone morphogenetic protein (BMP). Various, highly defined levels of ROS activate these aforementioned signalling pathways to regulate MSC differentiation into chondrocytes [112], adipocytes [113], and osteocytes [114]. Although the role of redox regulation in MSCs has yet to be fully elucidated, other stem cells such as haematopoietic stem cells and progenitor cells have been described in terms of stem cell development, function, and survival in this regard [115]. In this review we have aimed to comprehensively summarise the redox regulation of MSC differentiation.

4. Reactive oxygen species-mediated differentiation of mesenchymal stem cells

4.1. Adipogenic differentiation

MSCs differentiate to an adipogenic lineage through the activation of several transcription factors including CCAAT/enhancer binding protein (C/EBP)β, which induces C/EBPα and PPARγ [116]. It has now been shown that adipogenic differentiation may be mediated by NADPH oxidase 4 (Nox4) via the cAMP response element-binding protein (CREB) [113]. It has been shown that CREB over-expression in pre-adipocytes promotes the expression of C/EBPα and RXRα [117]; while concurrently, it has been demonstrated that antioxidants such as N-acetylcysteine (NAC) or apocyanin will suppress CREB [113]. There is further evidence for ROS-mediated adipogenic differentiation in MSCs by Prx1 over-expression inhibiting lipid accumulation, as well as antioxidant (NAC) inhibition of C/EBPα and PPARγ [113]. Superoxide generating complex III has also been implicated in the transcriptional

activation of PPARγ [118], while H_2O_2 increases adipogenesis [119] and diminishes the expression of adipo-cytokines [100]. This evidence suggests that increasing intracellular levels of ROS are involved in signalling cascades responsible for the differentiation of MSCs into adipocytes.

4.2. Osteogenic differentiation

There have been numerous signalling pathways implicated in osteogenic differentiation in MSCs including Wnt, FOXO, and Hedgehog; all of which have been shown to be suppressed by high levels of ROS [100]. The Wnt signalling pathway is a positive regulator of osteogenesis through the translocation of β-catenin into the nucleus, where it binds TCF/LEF, allowing the activation of Wnt target genes such as *Axin2* as well as increasing the expression of Run2x, Dlx5, and Osterix, which are critical osteogenic transcription factors [120]. Wnt signalling has been shown to decrease the expression of C/EBPα and PPARγ, and thus favours osteogenesis rather than adipogenesis [31]; although high levels of ROS, specifically H_2O_2 (which has been shown to inhibit Wnt/β-catenin signalling), would then promote a more favourable adipogenic environment [121].

The FOXO signalling pathway is also mediated by ROS, where high levels induce phosphorylation and drive its translocation into the nucleus where it is activated. FOXO has been shown to suppress osteogenic differentiation [122] while concurrently promoting adipocyte differentiation [123]. Increased levels of H_2O_2 stimulate FOXO transcription, although it also promotes the association of FOXO with β-catenin, which leads to an inhibition of Wnt target genes essential for osteogenic differentiation [124]. Overall, higher levels of oxidative stress antagonize the Wnt/β-catenin pathway, leading to attenuation of osteogenesis while stimulating FOXO-mediated adipogenic differentiation. It is noteworthy that a shift to adipogenic lineage in MSCs has been reported in patients with osteoporosis. This has been linked to the increased expression of the histone H3 methylating protein Enhancer of Zeste homology 2 (EZH2) which silences the Wnt genes, and hence pharmacological inhibition of EZH2 with 3-deazaneplanocin A (DZNep) reverted the cells to osteogenic lineage [125]. These data argue in favour of targeting EZH2 as a potential strategy against osteoporosis. Alternatively, the use of anti-oxidants, such as N-acetyl cysteine (NAC), or SOD mimetics might also serve to tailor the redox environment for osteogenic differentiation.

The interaction between ROS (in the form of H_2O_2) and the Wnt signalling pathway, and the resultant effect on MSC differentiation into adipocytes and osteoblasts is demonstrated in Fig. 2.

4.3. Chondrogenic differentiation

Recent studies have demonstrated that ROS is required for chondrogenic differentiation, primarily through NADPH oxidase 2 and 4 (Nox2 and Nox4) [112]. This was demonstrated by inhibition of chondrogenic differentiation gene markers Sox9, Col2a1, and Col10a1 by the introduction of the ROS scavenger NAC; and so, provide proof that ROS is necessary for chondrogenic differentiation [112]. Although less is known in regards to chondrogenic differentiation mediated by ROS in MSCs, this is an attractive area of study and may have wider implications in the field of regenerative medicine.

5. Conclusions

In this review we have exemplified the differential impact that Wnt signalling exerts on MSC differentiation pathways. Summarising the broad spectrum of the influence of Wnt signalling on MSC differentiation, we have shown that the outcomes of activated Wnt are inhibition of adipogenic and hepatogenic differentiation. On the other hand, a different outcome of activated Wnt signalling favours neurogenic and myogenic differentiation. Wnt signalling exerts mixed effects on osteogenic, chondrogenic, and cardiogenic differentiation, where it

can either promote or inhibit the process; and this could be dependent on many factors such as cell type and interaction with other signal transduction pathways, etc. The exact mechanism underlying the molecular regulation of differentiation processes remains to be unravelled.

The role of ROS in MSC differentiation has also yet to be completely elucidated, although research has shown that the level of H_2O_2 tends to favour differentiation into adipocytes, chondrocytes, or osteocytes. ROS-mediated differentiation into the other possible MSC lineages such as neuronal cells, hepatocytes, and myocytes has yet to be investigated in detail. MSC differentiation occurs through the interplay of different pathways including the FOXO and Wnt/β-catenin signalling pathways. It has been demonstrated that these pathways can regulate ROS levels, and concomitantly ROS levels can influence the activity of these pathways. ROS signalling has been implicated in osteogenic differentiation, where high levels of H_2O_2 inhibit Wnt/β-catenin signalling and favour osteogenesis, and low levels of H_2O_2 activate Wnt/β-catenin signalling to promote adipogenesis. Further studies into the mechanisms of ROS-mediated differentiation are important for a complete understanding of MSC differentiation.

MSCs have gained a lot of clinical interest, considering their potential for various tissue engineering and reconstructive surgery applications, due to their extensive self-renewal and multipotent differentiation capabilities. Hence the mechanism underlying these differentiation processes, tightly regulated by signal transduction networks such as the Wnt signalling pathway and ROS, needs to be further examined. A profound knowledge about these processes will in turn aid in the development of novel strategies in regenerative medicine. Recent evidence points to a critical role of tissue hypoxia on the proliferation and differentiation of multipotent MSCs [126,127]. To that end, manipulation of the cellular redox milieu could have potential therapeutic implications, whereby the use of anti-oxidants could serve to switch MSCs from adipogenic to osteogenic differentiation; however, if the desired outcome is to obtain adipogenic differentiation, one could be tempted to employ agonists of the nuclear PPARγ receptor. Furthermore, hypoxia was shown to enhance MSC viability and proliferation as well as differentiation into adipocytes and osteocytes [127], which could have potential implications in regenerative medicine.

Conflict of interests

The authors report no conflict of interests.

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Fig. 1 Schematic illustration of the effect of Wnt signalling on the various MSC differentiation pathways.
Fig. 2 Schematic demonstrating the effect of ROS (depicted here as H_2O_2) in blocking Wnt/β-catenin signalling and promoting differentiation of MSCs into adipocytes and osteoblasts.