Regulation of Mesenchymal Stem Cell Differentiation

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David Cook and Paul Genever

Abstract

A population of multipotent stromal cells exists within bone marrow and other adult tissues, which is able to differentiate into different skeletal tissues such as bone, cartilage and fat. These cells are frequently referred to as mesenchymal stem cells (MSCs) and offer significant therapeutic potential, particularly in orthopaedic applications, but may also have broader roles in regenerative medicine, cancer treatment, as anti-inflammatories, immunosuppressives and vehicles for gene/protein therapy. Much attention has focused on understanding MSC biology and the regulation of differentiation to help realise these clinical aspirations. Here we review some of the key molecular determinants of MSC function, with an emphasis on transcription factor control and the cell-cell signalling pathways that regulate MSC differentiation. The source information comes from a range of different models, including isolated human MSC cultures, animal-derived MSC-like cell lines, animal models and skeletal developmental processes to provide a wide-angled overview of the important players in MSC biology and tri-lineage specification.

Keywords

Mesenchymal stem cells • Osteogenesis • Chondrogenesis • Adipogenesis • Transcriptional control

12.1 Introduction

12.1.1 Origins of MSCs

The proposal for the existence of a population of multipotent stromal cells/mesenchymal stem cells (MSCs) was first put forward by Friendenstein and colleagues [1], who reported a population of bone marrow stromal cells capable

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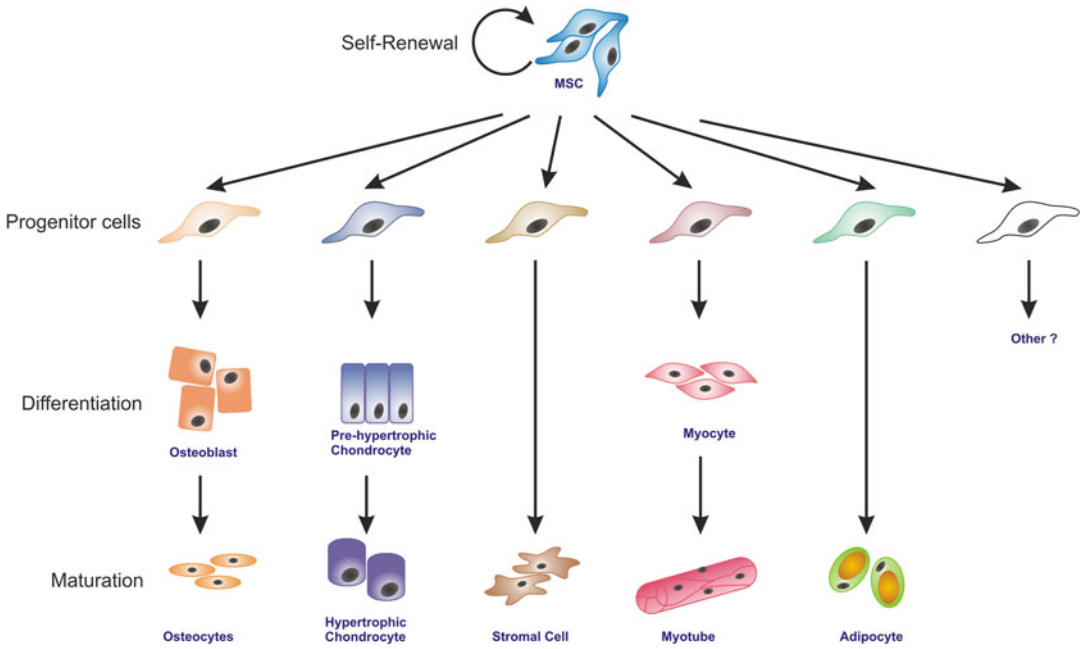

Fig. 12.1 Potency of MSCs. MSCs are a multipotent cell capable of self renewal, and differentiation into multiple mesenchymal lineages, including osteoblasts, osteocytes, chondrocytes and adipocytes. MSCs differentiate through a series of committed progenitor cells, and differentiated stages before final maturation into fully committed terminally differentiated cells (Adapted from Caplan and Bruder [106])

of generating bone following heterotopic transplantation. The same group later showed that these precursors were a subset of fibroblast like cells capable of forming colonies, termed colony-forming unit fibroblasts (CFU-Fs), when selected by adherence to plastic surfaces [2]. Subsequent work showed the ability of these cultured cells derived from a single CFU-F to proliferate in vitro, whist maintaining their ability to differentiate into osteoblasts, adipocytes and chondrocytes [3]. Together, these data are characteristics of two hallmarks of stemness; the ability to self renew, and to differentiate into multiple lineages, consequently these cells came to be commonly known as mesenchymal stem cells (Fig. 12.1).

Since their discovery MSCs have generated a lot of interest in the biomedical field as a source for stem cell therapies, with their relatively simple ex vivo expansion, multilineage capacity and potential for autologous transplantation. Indeed, clinical trials have been performed in patients with osteogenesis imperfecta, where allogeneic bone marrow-derived MSCs were given to patients after bone marrow transplantation. MSC engraftment was shown and a marked increase in patient recovery was detected [4]. The use of MSCs in tissue engineering is also an area of great scientific interest, with multiple groups generating novel scaffolds and delivery procedures for tissue repair. Tissue engineering involves the generation of a biocompatible scaffold on which cells are cultured before implanting into the patient, and in the case of MSCs this requires a thorough understanding of the differentiation process to ensure correct function of the implanted construct.

The study of MSCs in vivo and the isolation of MSC populations has been hindered by the lack of specific cell surface markers for immuno-phenotype identification. Cultured human mesenchymal stem cells do express a panel of cell surface markers, such as CD105, CD73 and CD90, and lack CD45, CD34 and CD14 [5], however these can be donor-, isolation- and passage-dependent and may not represent the true in vivo MSC population. Due to the difficulty in identifying MSCs in vivo,

the majority of work studying the properties of MSCs has been performed using cultured MSCs selected by adherence to culture plastic. However, this generates problems of its own, with different species, isolation techniques, culture conditions and donor sites generating increased complexity in the system. Furthermore, some studies of MSC differentiation have been performed not with primary cells, but with cell lines such as C3H10T1/2 [6, 7] and MC3T3-E1 for osteogenesis, and MC3T3-L1 for adipogenesis, preventing the direct extrapolation of the findings to human MSCs. In addition to the difficulties faced with inter-sample variation, there is the added problem of having highly heterogeneous MSC populations.

MSCs are defined by their ability to adhere to plastic and ability to differentiate into osteoblasts, adipocytes and chondrocytes. MSCs are classically derived from the bone marrow [3], however they have now been isolated from many adult stromal tissues [8], with the more common sources for in vitro differentiation analysis being bone marrow, adipose tissue, and periosteum.

12.1.2 In Vitro Differentiation of MSCs

MSCs have the ability to differentiate into osteoblasts, adipocytes and chondrocytes by definition, and various methods have been developed to mimic these processes in vitro. Osteoblasts develop through a series of phases, initiated by cellular proliferation, followed by extracellular matrix maturation and matrix mineralisation. These changes in cellular activity correlate with a pattern of maturation of the cells from committed osteoprogenitors to pre- and finally terminally differentiated osteoblasts. This process of cell maturation can be induced in vitro by the addition of bone morphogenetic proteins (BMPs), often BMP-2 [9], or the addition of a differentiation cocktail of dexamethasone, ascorbate and β-glycerophosphate [10]. While both these methods are capable of inducing the osteogenic differentiation of MSCs, it is likely that they act through different mechanisms to generate a comparable response. As with osteoblasts, adipocytes mature though a series of increasingly committed cell types, before becoming terminally differentiated adipocytes, expressing adipocyte specific markers such as FABP4 and 5 [11] and forming lipid vesicles. In vitro adipogenesis can be induced in MSCs by the addition of a differentiation cocktail of dexamethasone, isobutylmethylxanthine (IBMX), indomethacin and insulin. Methods to induce the process of chondrogenesis have also been developed in vitro. Chondrogenic differentiation in vivo requires an initial condensation of the MSCs, which is mimicked in vitro by culturing MSCs as micromass pellets. Chondrogenic differentiation can then be induced by the presence of transforming growth factor-β (TGF-β) resulting in the appearance of a chondrocyte-like phenotype characterised by upregulation of cartilage-specific molecules such as collagen type II and IX, aggrecan, versican, biglycan, and decorin [12]. Differentiating chondrocytes mature through a sequence of defined steps, initially the MSCs differentiate into a proliferative non-hypertrophic stage termed chondroblasts. This stage is characterised by a change from collagen type-I to type-II, IX and XI expression and a highly order columnar organisation. This stage is then followed by a hypertrophic stage, marked by the expression of collagen type-X, which is vital for vascular invasion, osteoblast differentiation, and bone formation.

12.2 Transcription Factors in MSC Differentiation

12.2.1 Osteogenesis

A range of transcription factors are known to be involved in the regulation of osteogenesis [13], with two of the more widely studied being Runx2 (Cbfa1) and Osterix. **Runx2** is considered the major transcription factor controlling osteoblast commitment and differentiation. Runx2 is a member of the Runt-domain gene family and is expressed in mesenchymal cells early in skeletal development and throughout osteoblast differentiation with molecular and genetic studies indicating its necessity in osteoblast differentiation of mesenchymal cells [14–16]. Runx2 was identified

as an important transcription factor in osteogenesis by its binding to a cis-element on the osteocalcin gene and its forced expression in osteoblast precursor cells, MC3T3-E1, caused the transcription of the osteoblast specific genes osteocalcin and collagen 1A1. Further research showed that overexpression of Runx2 can induce osteogenesis in vitro and in vivo. This was demonstrated by increased osteoblastic markers, osteopontin and osteocalcin, increased alkaline phosphatase (ALP) expression and mineralisation in vitro, while in vivo studies showed accelerated healing in critical-sized skull defects [17]. Conversely, Runx2 null mice showed a complete absence of ossification, owing to the maturational arrest of osteoblasts [15]. More recent work has also implicated Runx2 in the trans-differentiation of preadipocytes into osteoblasts. Takahashi (2011), demonstrated that over expression of Runx2 in the preadipocyte cell line, 3T3-E1, resulted in a decrease in the adipocyte markers PPARγ2 and C/EBPα and an increase in osteogenic markers such as ALP, osteocalcin and bone sialoprotein 2 (BSP) [18]. This trans-differentiation was further enhanced by the addition of dexamethasone or the overexpression of the mitogen-activated protein kinase phosphatase-1 (MKP-1). The phosphorylation status of Runx2 is also important. Dexamethasone, a synthetic glucocorticoid, acts to enhance the activity of Runx2 by reducing the amount of Runx2 phosphoserine levels via MKP-1 [19]. While others have demonstrated the phosphorylation of Runx2 on tyrosine, theonine and serine residues increases during dexamethasone induced osteogenesis [20].

Osterix (Osx) is another important transcription factor involved in osteoblast commitment, with Osx-deficient mice showing an absence of osteoblasts and defective bone formation [21]. However, Osx appears to act downstream of Runx2 as Osx is not expressed in Runx2 null mice, but Runx2 expression remains in Osx null mice [21]. The studies into the effects of overexpression of Osx are a little less clear, with multiple groups demonstrating that Osx overexpression is sufficient to induce osteogenesis [22, 23], where as Kurata et al. [24] recorded that Osx overexpression was capable of initiating osteogenesis, shown by early marker expression, but failed to generate terminally differentiated osteoblasts [24].

Other transcription factors of interest in relation to osteogenesis are the **Msx/Dlx** family of transcription factors. Dlx and Msx are homeodomain transcription factors homologous to the *Drosophila* Distal-less and muscle specific homeobox genes. Dlx5 and 6 are expressed in very similar patterns throughout almost all of the skeletal elements [25]. Furthermore, overexpression of Dlx5 can accelerate osteoblast differentiation in vitro [26]. Conversely, Dlx5 knockout mice have craniofacial and sensory skeletal defects [27], while double knockouts of Dlx5 and 6 have more severe defects [28], suggesting partial redundancy or compensation between the two transcription factors. Dlx3 is also implicated in osteogeneic differentiation, with expression of Dlx3 in the mouse embryo being associated with new bone formation and regulation of osteoblast differentiation. Furthermore, Dlx3 is expressed in ex vivo osteoblasts, whilst overexpression and RNAi knock down result in increased and decreased osteogenesis respectively [29]. In contrast to the Dlx transcription factors, Msx2 is expressed in the proliferating osteogenic precursors, and not the differentiated cells [29]. Overexpression of Msx2 prevented osteogenic differentiation and mineralisation, while overexpression of the antisense mRNA resulted in decreased proliferation and enhanced osteogenesis [30].

12.2.2 Adipogenesis

Peroxisome proliferator activated receptor-γ (PPARγ) is a nuclear hormone receptor, thought to be the master regulator of adipogenesis. There are two isoforms of PPARγ, generated by alternate splice sites. PPARγ1 is ubiquitously expressed whilst PPARγ2 is restricted to adipose tissues and appears to be a more potent stimulator of adipogenesis [31]. PPARγ was discovered as key player in adipogenesis through its interaction with the 5′-flanking region of the adipocyte P2 gene, a gene capable of inducing adipocyte

specific gene expression. It was subsequently shown to be expressed very early in the differentiation of adipocytes, with forced overexpression of PPARγ inducing adipogenesis in cultured fibroblasts [32]. Interestingly, this induction was not limited to fibroblastic cells; myoblastic cell lines can also be transdifferentiated to adipocytes [33]. Once again complementary experiments have been performed, in which PPARγ was deleted in fibroblasts, resulting in reduced adipogenesis (<2 % efficiency) even with the addition of C/EBPα, another regulator of adipogenesis [34]. These results and others suggest PPARγ is both sufficient and indispensable for adipogenic differentiation. While PPARγ is widely considered the master regulator of adipogenesis, it has also been implicated in the reciprocal regulation of adipogenesis and osteogenesis. Akune et al. [35] showed that embryonic stem cells from homozygous PPARγ-deficient mice would spontaneously differentiate into osteoblasts, while PPARγ haploinsufficiency resulted in enhanced bone formation with increased osteogenesis from bone marrow progenitors both in vivo and ex vivo [35].

CAAT/enhancer binding proteins (**C/EBPs**) are members of the basic-leucine zipper class of transcription factors, which function as homo- or heterodimers with other C/EBP family members. There are three C/EBPs which play a role in adipogenesis, C/EBPα, β and δ, of which C/EBPα has the most prominent role. A dramatic demonstration of this effect was shown by the overexpression of C/EBPα in fibroblastic cells, resulting in the induction of adipogenesis in up to 50 % of the cells [36]; conversely antisense mRNA knockdown resulted in reduced adipose phenotype in differentiated 3T3-L1 cells [37]. Similar results were obtained in mouse models, in which C/EBPα expression was restricted to the liver, showed reduced adipose tissue [38]. When studying the levels of endogenous C/EBPs, during adipogenesis of cultured cells, it was noted that C/EBPα is expressed late in the differentiation process immediately prior to the activation of the many adipo-specific genes, while C/EBPβ and δ are only transiently expressed, accumulating during the early stages of differentiation, before diminishing prior to terminal differentiation [39]. C/EBPβ and δ act early in the differentiation process to relay the hormonal signals, leading to the activation of C/EBPα [39]. This signal transduction is likely to function through the activation of PPARγ, via C/EBP binding sites in the PPAR promoter. This PPARγ expression is then thought to activate C/EBPα, which then enters a positive feedback loop, increasing the expression of PPARγ (Fig. 12.2). This process is apparent through the generation of PPARγ and C/EBPα null cell lines [40, 41], where PPARγ null cells fail to express C/EBPα despite normal early differentiation [40]. Additionally, C/EBPα null fibroblasts have reduced levels of PPARγ expression, which can be rescued by retroviral transfection and expression of C/EBPα [41]. It is thought that this positive feedback loop maintains the expression of these two important transcription factors through to terminal differentiation of the adipocytes.

Another transcription factor of note for its pro-adipogenic effects is **Sterol regulatory binding element protein-1** (**SREBP1**)/Adipocyte differentiation and determination factor-1 (Add1). Dominant negative expression of SREBP1 in 3T3-L1 (pre-adipocyte line) cells sharply repressed adipogenic differentiation, while overexpression of SREBP1 in the fibroblastic line, NIH-3T3, increased adipogenesis in a synergistic manner with PPARγ overexpression, suggesting its involvement in this pathway [42] (Fig. 12.2). SREBP1 exerts it pro- adipogenic effects through the interaction with E-box domains in the PPARγ promoter regions, allowing further regulation of PPARγ gene expression [43].

As with osteogenesis, there are also transcription factors involved in the inhibition of adipogenesis. **C/EBP homologous proteins** (**CHOPs**) negatively regulate adipogenesis through interactions with C/EBPs. CHOP10 for example binds to C/EBPβ early during differentiation, preventing it from binding PPARγ, thereby delaying the terminal differentiation of adipocytes, allowing for the initial clonal expansion step [44]. The activity of SREBP1 is also negatively regulated during adipogenesis, by the binding of **Inhibitor of DNA binding** (**Id**) proteins which prevent SREBP1

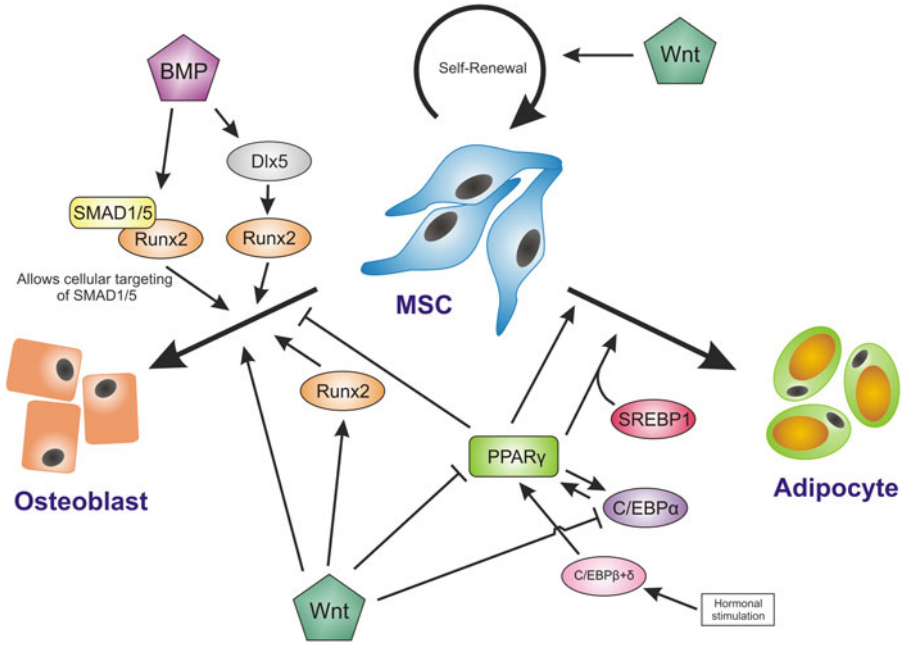

Fig. 12.2 Role of Wnt and BMPs in Osteoblast/Adipocyte lineage commitment. Wnt signalling is vital for the commitment decision of MSCs between osteoblasts and adipocytes, acting through the inhibition of PPARγ to prevent adipogenesis and activate osteogenesis via Runx2. BMPs induce the osteogenic differentiation of MSCs via Dlx5 and Runx2. The BMP signalling components SMAD1/5 can also be directed to transcriptional foci by Runx2

from binding to the E-box DNA regulatory sequences [45]. Another transcription factor important in the negative regulation of adipogenesis is **GATA binding transcription factor** (**GATAs**) family. GATA2 and 3 have been shown to be expressed in pre-adipocytes, and their down regulation leads to enhanced adipogenesis. Forced expression of GATA2 and 3 prevents the switch from pre-adipocytes to mature adipocytes, in part through binding directly to PPARγ [46], but also through the formation of protein complexes with C/EBPα or β [47].

12.2.3 Chondrogenesis

As with both adipogenesis and osteogenesis, there is an apparent master regulator of chondrogenesis, **Sox9**. Sox9 is a member of a family of transcription factors that contain a HMG-type DNA binding domain, and is expressed throughout chondrogenic differentiation until the cells become hypertrophic, where it is rapidly shut off [48]. The requirement for Sox9 is clearly demonstrated in the work by Akiyama et al. [49], where deletion of Sox9 expression in the mesenchymal cells of limb buds lead to the complete absence of chondrogenic mesenchymal condensations in the developing limbs, while deletion of the Sox9 gene in mesenchymal condensations lead to the arrest of chondrogenesis at this stage [49]. These results clearly demonstrated that Sox9 was vital for chondrogenesis, and plays important roles in both mesenchymal condensation and for chondrogenic progression.

Furthermore, Sox9 was identified as part of a triad of Sox genes which are sufficient for the induction of chondrogenesis in embryonic stem cells [50]. Two other members of the Sox family of transcription factors also play a role in chondrogenesis **L-Sox5 and Sox6**. L-Sox5 and Sox6 differ from Sox9 in that they do not possess a

transactivation domain and therefore do not affect gene expression directly, but are thought to alter gene expression through the recruitment of other transcriptional activators [51]. L-Sox5 and Sox6 are coexpressed with Sox9 during chondrogenesis and therefore share expression patterns with the chondrogenic marker Col2A1, prompting further studies into the role of these transcription factors in chondrogenesis. L-Sox5- and Sox6-deficient mice present chondrogenic defects, with the dual knockout generating a more severe phenotype, suggesting some redundancy. However, in contrast to Sox9-deficient mice, Sox5/6-deficient mice do develop chondrogenic mesenchymal condensations [52], implicating their role as being later in the differentiation process. It is thought that these three Sox transcription factors work in collaboration to activate chondrocyte-specific markers, with enhanced Col2A1 reporter expression when all three Sox genes are coexpressed in non-chondrogenic cells [53]. Similarly the three Sox proteins have been shown to cooperatively activate the chondrocyte marker Col11A2 [54]. As discussed above, Sox transcription factors are required for the progression of chondrogenesis, but over expression of the Sox triad also causes chondrogenesis arrest in the pre-hypertrophic cells preventing terminal differentiation [50]. It is thought that this terminal differentiation inhibition is at least in part due to the action of two genes, S110A1 and S100B, members of the S100 protein family which carry the Ca^{2+}-binding EF-hand motif. These proteins are expressed during the late proliferative and pre-hypertrophic stages of chondrogenesis, and when overexpressed in chondrogenesis inhibited the terminal differentiation step. Furthermore, S100B protein expression is responsive to the Sox triad through enhancer elements in the 5′ flanking region [55].

As described above, **Runx2** is a master regulator of osteogenesis, but it also has important roles in regulating chondrogenesis. The initial evidence for this was presented in the Runx2 null mice used to identify its function in osteogenesis. It was noted that these mice also had cartilage defects as well as the more obvious bone defects [56]. Runx2 null mice had a lack of hypertrophic chondrocytes, implying an important role for Runx2 in this step. The expression levels of Runx2 are at their highest in chondrocytes during the hypertrophic stage [56], and overexpression of Runx2 during hypertrophy caused enhanced maturation and increased endochondral ossification [57].

12.3 Signalling Pathways Controlling MSC Differentiation

Multiple signalling pathways have also been found to be involved in lineage commitment and MSC behaviour. For example, studies have identified the involvement of bone morphogenetic proteins (BMPs), Hedgehog (Hh) and Wnt signalling (Fig. 12.3) in the regulation of MSC differentiation [58–60].

12.3.1 Wnt Signalling Pathway

Wnt signalling has been implicated by multiple studies to play an important role in the regulation of skeletal function, and in particular osteoblast differentiation and activity. Wnt molecules are a family of cysteine-rich secreted glyco-lipoproteins that regulate many processes including development, cell proliferation and cell fate [61]. Wnt signalling acts through two known pathways, the canonical pathway involving β-catenin, and the β-catenin-independent pathway termed the non-canonical pathway. Canonical Wnt ligands mediate their effects by binding to their receptors frizzled (Fzd) and co-receptors, low-density lipoprotein receptor related protein (LRP) 5 and 6. This causes activation of intracellular Dishevelled which in turn inhibits a protein destruction complex. This results in the stabilisation and nuclear translocation of β-catenin, inducing gene transcription via the LEF/TCF family of transcription factors. In the absence of Wnt signalling, the destruction complex is not inhibited and can therefore perform its function to phosphorylate β-catenin, through glycogen synthase kinase 3β (GSK3β) leading to degradation by ubiquitination (Fig. 12.3a).

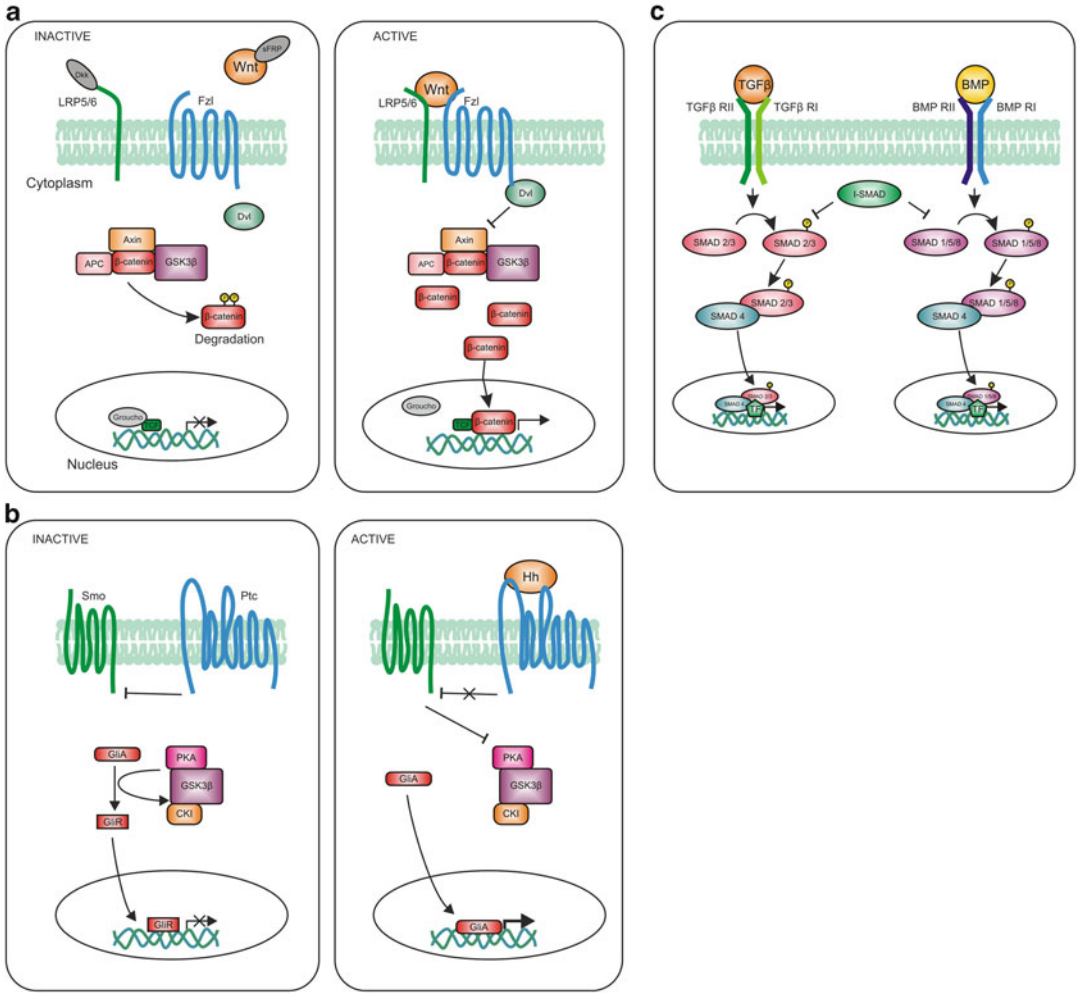

Fig. 12.3 Signalling pathways. (**a**) The Canonical Wnt signalling cascade. Canonical Wnt signalling mediates its effect by binding to their receptors frizzled (*Fzd*) and co-receptors, LRP 5/6. This causes activation of intracellular Dishevelled (*Dvl*) which, in turn, inhibits glycogen synthase kinase-3 β (*GSK3β*). This results in the stabilisation and nuclear translocation of β-catenin, inducing gene transcription via the LEF/TCF family of transcription factors. In the absence of Wnt signalling, a complex containing GSK3β phophorylates β-catenin, leading to degradation by ubiquitination. (**b**) The Hedgehog signalling cascade. In the absence of any Hedgehog ligand the Hedgehog signalling complex phosphorylates the Gli family of transcription factors, leading to degradation or proteolytic cleavage to transcriptional repressors. In the presence of Hedgehog ligand, signalling is mediated through the binding of Hedgehog to their receptor Patched (*Ptc*). This causes the inhibition of a second transmembrane protein, Smoothened (*Smo*), to be relieved. Smo is then able to inhibit the Hedgehog signalling complex preventing the phosphorylation of the Gli proteins, priming them for transcriptional activation. (**c**) TGFβ/BMP signalling cascade. TGFβ/BMPs signal through their receptors on the cell surface which phosphorylate and activate their respective R-SMADs, which in turn can then bind to the Co-SMAD (SMAD 4). This R-SMAD/Co-SMAD complex can then enter the nucleus where it interacts with transcription factors to induce gene expression

12.3.2 Wnt Signalling in Lineage Commitment

The role of Wnt signalling in bone regulation was first identified in osteoporosis pseudoglioma syndrome patients (characterised by low bone mineral density) with loss of function mutations in the co-receptor LRP5 [62]. Conversely, mutations in the N-terminus of LRP5 that reduce the affinity with the Wnt signalling inhibitor Dkk1 are associated with high bone mass [58]. These observations have been reinforced by using mouse models

in which LRP5 overexpression [63] and reduced inhibition of Wnt signalling by sFRP1 knock down [64] resulted in similar results with increase bone mass and density.

In an attempt to elucidate the molecular basis for this response to Wnt, many studies have been carried out using various activators and inhibitors of the Wnt signalling pathway both in vivo and vitro. One process by which Wnt signalling may act to increase bone formation is through the stimulation of osteoblast development. Inhibition of GSK3β enzyme activity using LiCl or small molecules, caused increased β-catenin nuclear translocation, stimulated mouse mesenchymal precursors to differentiate into osteoblasts [6, 7]. GSK3β is involved in other pathways and may therefore cause these effects through means other than the Wnt pathway, however Wnt3a, Wnt1, Wnt10 [65] and constitutively active β-catenin also stimulate osteoblastogenesis, while Dkk1 reduces osteoblast differentiation [66]. Further to this, in vivo work has shown that administration of LiCl, a GSK3β inhibitor, to C57BL/6 mice for 4 weeks dramatically increased bone formation rate [67]. One route by which Wnt is thought to promote osteogenesis is through the direct stimulation of Runx2 expression [68] (Fig. 12.2). Gaur et al. [68] identified a TCF binding site in the promoter of Runx2 and demonstrated an increase in Runx2 expression in response to co-expression of TCF and canonical Wnt proteins.

However, while there is a good deal of evidence in mouse in vivo and vitro for the role of Wnt in inducing osteogenic differentiation, the research in human MSCs is much less conclusive and straightforward. This difference is clearly demonstrated by the work carried out by Boland et al. [69], which demonstrated that Wnt3a conditioned media, leading to canonical Wnt signalling, caused inhibition of osteogenic differentiation demonstrated by reduced ALP mRNA and activity and decreased mineralisation [69]. Induced Wnt signalling did however appear to increase the proliferation rate of human MSCs, whilst at the same time reducing apoptosis (Fig. 12.2). Similar results have been shown in human MSCs by inducing Wnt signalling at different stages of the canonical pathway, including LRP5 and TCF1 [70]. Interestingly these studies also identify the non-canonical Wnt signalling pathway, induced by Wnt5a, as an activator of osteogenesis in human MSCs, capable of inhibiting the effect of Wnt3a activity.

More recently, research has focused on deciphering these apparent variations in response to canonical Wnt signalling. Eijken et al. [71] used a human foetal osteoblastic cell line, with which they generated a non-differentiating and differentiating population, through the addition of the synthetic glucocorticoid dexamethasone [71], while Quarto et al. [72] used a range of human and mouse multipotent and pre-osteoblastic cells to study the effect of Wnt manipulation on osteogenic differentiation [72]. These studies, amongst others [73] have demonstrated that the response to Wnt signalling is dependent on the level of activation and the differentiation state of the target cells. Collectively it seems that canonical Wnt stimulates differentiation of cells committed to the osteogenic lineage, but can inhibit the differentiation of multipotent cells, and prevent the terminal differentiation of mature osteoblasts.

Canonical Wnt signalling is also well studied with relation to adipogenic differentiation, with multiple studies showing reduced adipogenesis with Wnt signalling [74], both in vivo and in vitro. Upon canonical Wnt stimulation, adipogenesis of 3T3-L1 cells is completely inhibited. Canonical Wnt activation does not affect the expression of the early adipocyte transcription factors, C/EBPβ and δ, but blocks C/EBPα and PPARγ and the downstream gene aP2 [75] (Fig. 12.2). The inhibition of PPARγ is thought to be via the activation of chicken ovalbumin upstream promoter transcription factor II, leading to the recruitment of the silencing mediator of retinoid and thyroid hormone receptors co-repressor complex. This binds to the PPARγ gene, maintaining the chromatin in a hypoacetylated state repressing its expression [76]. Conversely, the expression of Wnt inhibitors, reducing endogenous Wnt, causes the spontaneous adipogenic differentiation of pre-adipocytes [75]. This work, along with related findings, identifies canonical Wnt as an important switch in the lineage decisions of MSCs, with canonical Wnt maintaining the cells in a multipotent state until its coordinated removal results in adipogenesis. Recently, work has been

carried out studying the relationship between adipogenesis and osteogenesis in response to canonical Wnt signalling [77]. Liu et al. [77] were able to show that human MSCs under dual osteogenic and adipogenic conditions, preferentially formed osteoblasts in response to Wnt3a administration. This response was shown to be due to differential inhibition of the two differentiation processes, where adipogenesis is totally inhibited at low Wnt stimulation, and osteogenesis is only partially inhibited. This suggests that under dual lineage differentiation conditions, differences in sensitivity to Wnt inhibition may alter the equilibrium and shift the commitment from adipocytes toward osteoblasts. This work correlates with that discussed earlier, in which PPARγ-deficient embryonic stem cells would spontaneously differentiate into osteoblasts [35], again implicating Wnt signalling as important regulatory element of lineage decision and commitment.

Canonical Wnt signalling is also influential in the differentiation of MSCs into chondrocytes. This was demonstrated by Day et al. [78] who generated mice with ectopic induction of canonical Wnt signalling in the developing limb bud. These mice showed enhanced ossification and reduced chondrocyte formation. Furthermore, inactivation of β-catenin, therefore preventing canonical Wnt, created the opposite phenotype, with ectopic chondrocyte differentiation, and reduced osteogenesis [78]. In vitro work also confirms a role for Wnt signalling in chondrogenesis, with the overexpression of Wnt8c, 9a or β-catenin causing enhanced chondrocyte hypertrophy in chick upper sternal chondrocytes. Canonical Wnt activation led to decreased Sox9 and Col2A1 expression, whist increasing the hypertrophic markers Col10A1 and Runx2. Canonical Wnt exerts these effects, at least in part, through the LEF/TCF activation of Runx2 and in turn induces the expression of Col10A1 [79]. These findings correlate with those in human MSC culture, where inhibition of canonical Wnt signalling by secreted frizzled-related proteins and Dickkopf overexpression causes enhanced chondrogenesis, with up regulation of Col2A1, Sox9 and glycosaminoglycan expression, and a decrease in Col1A1. However, Wnt inhibition does not induce the expression of Col10A1 [80], suggesting Wnt inhibition can induce early chondrocyte differentiation, but has no effect of final maturation and hypertrophy (Fig. 12.4).

12.3.3 Hedgehog Signalling Pathway

Another well studied signalling pathway shown to be involved in bone development is the hedgehog (Hh) pathway [81–83]. Hedgehog was first discovered in *Drosophila* as a single gene that regulates many diverse aspects of embryonic and adult patterning. Hh signalling has since been found to be present in mammalian cells, but differs initially in that there are three Hh proteins; Sonic hedgehog (Shh), Desert hedgehog (Dhh), and Indian hedgehog (Ihh). Some functional redundancy can be seen between these types, however they do express distinct expression profiles with little overlap [84].

All Hh proteins signal through the same receptors and signalling pathway. The Hh pathway is triggered by the binding of Hedgehog to its receptor, Patched (Ptc). In the absence of any Hh interaction, Ptc acts to inhibit the activity of a 7-transmembrane protein, Smoothened (Smo). In contrast, in the presence of Hh binding to Ptc, Smo repression is alleviated leading to signal transduction and the conversion of the Gli family of transcription factors to an activating state. There are three Gli proteins in mammals, Gli1, 2 and 3, compared to the single transcription factor, Ci, in *Drosophila* [85].

Smo exerts its effect on signal transduction by inhibiting the hedgehog signalling complex, primarily consisting of glycogen synthase kinase, protein kinase A and casein kinase (GSK3β, PKA and CSK, respectively). Under inactive conditions, when Smo activity is inhibited by Ptc, this complex acts to phosphorylate the Gli transcription factors, priming the Gli proteins for degradation or proteolytic cleavage. This has the overall effect of increasing the transcriptional repressor forms of the Gli proteins, preventing target gene transcription. Conversely, the release of inhibition of Smo, by Hh binding to Ptc, results

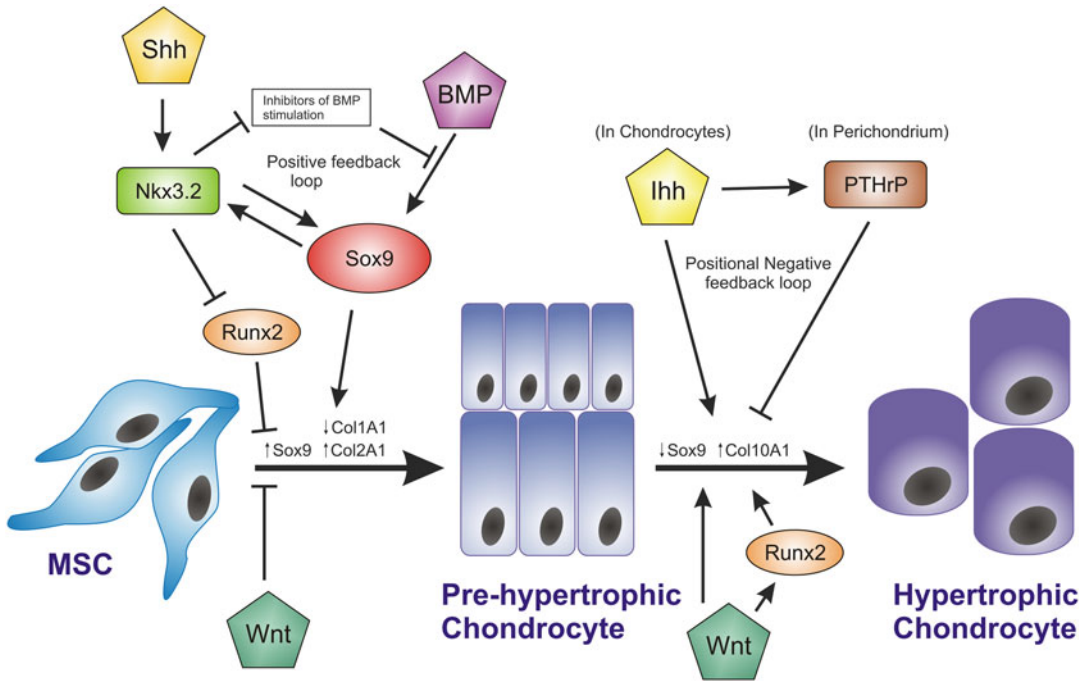

Fig. 12.4 Chondrogenic differentiation of MSCs. Shh and BMPs act together to generate positive feedback loop with Sox9 and Nkx3.2, stimulating the differentiation of MSCs into prehypertrophic chondrocytes, while Wnt signalling inhibits the initiation of chondrogenic differentiation. Wnt signalling is however required for the switch between prehypertrophic and hypertrophic chondrocytes leading to reduced Sox9 expression and increased Col10A1. Ihh expression in prehypertrophic chondrocytes stimulates the switch to hypertrophic chondrocytes, yet also causes PTHrP expression in the surrounding perichondrium, which in turn inhibits hypertrophy in the leading edge of the developing limb, generating a positional negative feedback loop

in inhibition of the Hedgehog signalling complex, and therefore prevents phosphorylation of the Gli proteins. The predominant Gli state is therefore converted to activatory, leading to the transcription of target genes (Fig. 12.3b).

12.3.4 Hh Signalling in Lineage Commitment

Indian hedgehog (Ihh) signalling is indispensable for osteoblast development during endochondral ossification. This was strikingly shown in $Ihh^{-/-}$ mice, which demonstrated a complete failure of osteoblast development in endochondral bones [82]. Further to this, genetic manipulation of Smo, resulting in removal of Smo from the perichondral cells using a Cre-LoxP system, resulted in the failure of osteoblast differentiation [81]. In addition to these in vivo experiments, the role of Hh signalling in mesenchymal commitment has been studied in vitro. The induction of the hedgehog pathway, by addition of recombinant Hh protein, in C3H10T1/2 cells, induced osteogenesis, with ALP activity detectable after just 2 days of treatment [83].

There is now also evidence for interactions between the Hh and Wnt pathways in relation to osteogenesis. $Ihh^{-/-}$ mice showed a disrupted Wnt signalling phenotype at E14.5 and E16.5, with an absence of nuclear β-catenin staining in the perichondral cells, as compared to the positive staining of the wild type mice [83]. To investigate the functional relationship between Hh and Wnt signalling as inferred by the $Ihh^{-/-}$ mice, the same group used an in vitro C3H10T1/2 differentiation model, in which Ihh overexpression led to ALP expression in the Ihh-expressing cells. This osteogenic differentiation was however reduced by ~50 % when the cells were co-transfected with

either Dkk or double negative Tcf4 constructs. In addition to this, *Wnt5a*, *Wnt7b* and *Wnt 9a* mRNA levels were significantly induced over controls in response to 24–48 h of Hh treatment. This body of work suggests that Hh signalling acts upstream of Wnt signalling and that Wnt signalling is required, at least in part, for the osteogenic inducing potential of Hh.

Ihh is also implicated in the switch between pre- and hypertrophic differentiation, where it is thought to act with parathyroid hormone-related protein (PTHrP) to generate a negative feedback loop regulating the onset of hypertrophy. Ihh signalling by the developing chondrocytes, targets the surrounding perichondrium, where it leads to PTHrP expression. PTHrP then signals to the pre-hypertrophic chondrocytes preventing the initiation of hypertrophic differentiation [86]. It is postulated that the level of Ihh/PTHrP signalling regulated the distance between the joint region and the onset of hypertrophy (Fig. 12.4).

12.3.5 TGFβ-Superfamily Signalling Pathways

The TGFβ-superfamily contains many transcription factors and morphogens, including two families involved in MSC differentiation, TGFβ and BMPs. As members of the TGFβ-superfamily, both TGFβ and BMPs are dimeric secreted ligands, which generally exist as homodimers. Binding of these ligands to their corresponding receptors, leads to the phosphorylation of the receptor SMADs (R-SMADs). This in turn leads to the interaction of the R-SMADs with SMAD4 (Co-SMAD), and translocation to the nucleus. Here the SMADs interact with transcription factors to activate gene expression. The R-SMADs are specific to either TGFβ signalling or BMP signalling, with SMADs 2/3 for TGFβ and SMADs 1/5/8 for BMPs, generating specificity between the two pathways. Inhibitor SMADS (I-SMADs) also play a role in this pathway by generating a feedback control loop (Fig. 12.3c).

12.3.6 TGFβ- Superfamily Signalling in Lineage Commitment

BMPs were first identified as proteins that were capable of inducing endochondral bone formation and increasing osteoblast differentiation in vitro [87]. However it is now known that BMPs play vital roles in a wide variety of embryonic processes, including gastrulation, neural development and endothelial cell function [88, 89]. This review will concentrate on the roles of BMPs on the differentiation of MSCs. As stated above, the application of recombinant BMPs to in vitro pre-osteoblast cultures results in increased osteoblastogenesis, demonstrated by increased ALP, osteocalcin expression and matrix mineralisation [87], while the blocking of BMP signalling both arrests osteogenesis and prevents the programmed cell death of mature osteoblasts. BMPs are thought to induce osteoblast differentiation through the activation of Runx2 [90]. Recent work in multiple mouse cell lines has demonstrated that this increase in Runx2 activity in response to BMPs is indirect and acts through Dlx5 [91]. Runx2 is also thought to interact with SMAD1 and 5. These SMAD-Runx2 complexes are directed by the Runx2 targeting signals to sub-nuclear foci where gene targets for both transcription factors are present. This suggests that SMAD transcriptional activation is at least in part dependent on the targeting factors of Runx2 [92]. It is interesting to note that BMP2 stimulation of Dlx5 stimulates the expression of osterix independently of Runx2, implicating Dlx2 as an important regulator of early and late BMP-induced osteogenesis [93].

BMPs not only induce osteoblast differentiation, but also have pro-chondrogenic characteristics, and have been shown to increase the expression of type II and X collagen in growth plate cultures [94]. BMPs exert their effect on chondrogenesis through the chondrogenic master regulator Sox9. Beads soaked in BMP4 implanted into mouse mandibular explants induced ectopic cartilage formation in the proximal position of the explants. These same areas also had upregulation

of the Sox9 transcription factor, implicating its role in BMP induced chondrogenesis. Interestingly, BMP4-soaked beads did not induce chondrogenesis in the rostral position, despite similar up regulation of Sox9. However, upregulation of the homeodomain transcription factor Msx2 was also seen in the areas surrounding the beads, and to a much greater degree in rostral region of the explants. Furthermore, ectopic expression of Msx2 prevented the BMP4 induced chondrogenesis, and reduced endogenous chondrogenesis [95]. This body of work demonstrates that BMP induction of chondrogenesis via Sox9, is also dependent on the expression of Msx2, generating a threshold for chondrogenesis, thereby providing a means for positional regulation of chondrogenesis in vivo.

Chondrogenesis is also reliant on the complex cross-talk between BMPs and the Shh/Ihh signalling pathways. One transcription factor of interest in the regulation of chondrogenesis is the homeobox protein, Nkx3.2. Shh signalling initiates the expression of Nkx3.2, while BMP signals act to maintain its expression [96], allowing the transcription repressor activity of Nkx3.2 to block the activity of inhibitors of BMP-induced chondrogenesis. Interestingly, Nkx3.2 also acts to repress Runx2 activity which prevents the onset of differentiation [97]. Furthermore, Nkx3.2 can induce the expression of Sox9 [98], which in turn can increase expression of Nkx3.2, generating a positive feedback loop maintaining the expression of pro-chondrogenic factors. In summary, BMP acts to induce Runx2, stimulating osteogenic differentiation, yet acting alongside Shh signalling during chondrogenesis, generates and maintains high levels of Nkx3.2 leading to the down regulation of Runx2 and increased Sox9, allowing the onset of chondrogenesis (Fig. 12.4).

TGFβ signalling also plays a role in the regulation of MSC differentiation. Unlike BMP-specific SMADs, TGFβ SMADs do not induce osteogenesis, but in fact act to repress the pro-osteogenic effects of BMPs. This inhibition is mediated through SMAD3, which interacts with Runx2 repressing its transcriptional activity [99]. In contrast to this inhibitory effect on osteogenesis, TGFβ is required for the in vitro chondrogenic differentiation of multipotent mesenchymal cells, acting through the p38, ERK-1, and JNK MAP Kinases [100].

12.4 Additional Regulators of MSC Differentiation

12.4.1 miRNAs in MSC Differentiation

The discovery of microRNAs (miRNAs) as a mechanism for regulating gene expression in the early 2000s [101] opened up a new avenue of investigation in the hunt for regulators of MSC differentiation. miRNAs are small non-coding RNAs that regulate the translation of protein coding genes by binding to the 3′ untranslated region and in most cases causing degradation of the mRNA. Li et al. [102] found that following BMP-induced osteoblast differentiation, 22 miRNAs could be detected as downregulated [102]. They then showed that two of these downregulated miRNAs acted directly on genes important in osteoblast differentiation. MiR-133 directly targets Runx2, the master regulator of osteogenesis, while miR-135 acts upon SMAD5, an important transducer of the BMP signal. Similarly, Hassan et al. [103] showed that Runx2 negatively regulates a cluster of miRNAs, 23a~27a~24-2, and that these miRNAs act to suppress osteogenesis by suppression of SATB2, a protein that acts synergistically with Runx2 during osteogenesis [103]. It is thought that this generates a feed forward loop, whereby Runx2 expression can de-repress SATB2, enhancing osteogenic progression.

12.4.2 Mechanical Stimulation in MSC Differentiation

The effect of mechanical stimuli on the differentiation of MSCs is another growing area of research. McMahon et al. [104] demonstrated that MSCs grown in a 3D collagen type I-glycosaminoglycan (GAG) scaffold could

differentiate into chondrocytes when supplemented with inductive medium. This differentiation could then be enhanced by the application of 10 % cyclic tensile loading for 7 days, measured by increased GAG synthesis [104]. Similarly, Sim et al. [105] created a novel micro cell chip system to apply compressive pressures to MSCs. MSCs grown in this system could differentiate into osteoblasts when treated with an osteogenic cocktail, measured by ALP expression, and this was further enhanced by intermittent cyclic compression for 1 week [105].

12.5 Summary

The regulation of MSC differentiation is complex and multilayered, comprising an interwoven combination of genetic, bio-chemical and mechanical influences. Since the early work of Friedenstein and colleagues [1, 2], great advances have been made in the molecular description of MSC control, but challenges do remain. There is a lack of consensus in the field on appropriate MSC isolation techniques, caused largely by the absence of a truly selective and universally adopted MSC marker. Much work therefore relies on the use of heterogeneous MSC-like populations of cells, sorted, somewhat crudely perhaps, by their adherence to a plastic surface. Transgenic models have given a penetrating insight into the genetic determinants of skeletal tissue development and organisation, but inter-species variations in MSCs persist in mouse and man. However, there is a common belief, supported by an admirable resolve and talented research, that these obstacles and others will be overcome, and that the information gathered on MSC biology will continue to add to the scientific and clinical appeal of these precious cells.

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