Transcriptional Control of Mesenchymal Stem Cell Differentiation

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Key Words

MSCs · Cbfa-1 · PPAR- γ · Sox9 · Transcription factors

Summary

In recent years, transcriptomics and proteomics have provided us with a great deal of information about the expression profiles of various cell types and how these change under different conditions. Stem cell research is one area where this has had a major impact by providing an insight into events at the molecular level that control stem cell growth and differentiation. This includes mesenchymal stem cell (MSC) biology where knowledge about the mechanisms governing differentiation is vital for the development of future therapeutic strategies. Although there is still much to learn, we are starting to build up a picture of the main events in these differentiation processes. This review will discuss control of MSC differentiation at the transcriptional level. Not all the factors which have been shown to play a role in lineage-specific mesenchymal differentiation can be covered here. Instead, we will focus specifically on the key factors that contribute to the regulation of osteogenesis, adipogenesis, and chondrogenesis.

Schlüsselwörter

MSCs · Cbfa-1 · PPAR- γ · Sox9 · Transkriptionsfaktoren

Zusammenfassung

Transkriptionskontrolle der mesenchymalen Stammzelldifferenzierung

In den vergangenen Jahren bescherte uns die Transkriptomik und Proteomik eine Menge an Informationen über die Expressionsprofile von verschiedenen Zelltypen und darüber, wie sich diese unter verschiedenen Bedingungen ändern. Stammzellforschung ist ein Bereich, in dem dies einen großen Einfluss hatte, weil dort Einblicke erzielt wurden, wie Stammzellwachstum und -differenzierung auf molekularer Ebene kontrolliert werden. Das schließt auch die Biologie der mesenchymalen Stammzellen (MSCs) mit ein, wo das Wissen über die Mechanismen, die die Differenzierung steuern, entscheidend ist für die Entwicklung zukünftiger therapeutischer Strategien. Obwohl immer noch vieles nicht bekannt ist, beginnt man die Hauptereignisse innerhalb der Differenzierungsprozess zu verstehen. Die Kontrolle der MSC-Differenzierung auf Transkriptionsebene bildet den Schwerpunkt der vorliegenden Übersicht, wenn auch nicht alle Faktoren, die bei der abstammungsspezifischen mesenchymalen Differenzierung eine Rolle spielen, hier dargestellt werden können. Stattdessen wollen wir uns besonders auf die Schlüsselfaktoren, die zur Regulation der Osteogenese, der Adipogenese und der Chondrogenese beitragen, beschränken.

Osteogenesis

The differentiation of mesenchymal stem cells (MSCs) into mature osteoblasts involves several phases, starting with cellu-

lar proliferation before progressing on to extracellular matrix (ECM) maturation and finally matrix mineralisation. Throughout this process, the cells mature through a series of intermediates, from committed osteoprogenitors to pre-

Fig. 1. Expression levels of transcription factors and osteoblast-related genes during osteogenesis. The onset of differentiation is marked by a decrease in Twist and an increase in Cbfa-1 and Osterix expression. Committed pre-osteoblasts show increased expression of Msx and Dlx proteins which interact and, together with Cbfa-1 and osterix, coordinate the expression of osteoblast-specific genes. Alkaline phosphatase is expressed during the early stages of osteogenesis, and osteonectin, osteocalcin, and osteopontin are expressed by more mature osteoblasts during the phases of matrix maturation and mineralisation.

osteoblasts and terminally differentiated osteoblasts. Osteogenesis is regulated by many of the major developmental signalling pathways including bone morphogenetic protein (BMP), Wnt and Hedgehog signalling. These converge on, and exert their effects through, transcription factors that regulate gene expression profiles and consequently cell behaviour (fig. 1). BMP-2 is a potent inducer of osteogenesis and can be used to promote differentiation in vitro [1]. It is also common to use an osteogenic cocktail of dexamethasone, ascorbate, and beta-glycerolphosphate. Although both of these methods are sufficient to induce osteogenic differentiation, it is likely that they act through different signalling mechanisms and induce different transcriptional activities.

Cbfa-1

Cbfa-1 (core binding factor $\alpha 1$, also known as Runx2/Pebp2 α A/AML3) is a runt-domain transcription factor expressed at sites of bone formation in the developing embryo [2] and more specifically in early osteoblast progenitors [1, 3]. Cbfa-1 was cloned as the factor that bound to an osteoblast-specific cis element in the osteocalcin promoter [4]. Overexpressing Cbfa-1 in MC3T3-E1 preosteoblastic cells demonstrated increased expression of the bone-associated markers type-I collagen, osteopontin, and bone sialoprotein [4], and binding sites for Cbfa-1 have been identified in the promoters of type-I collagen, bone sialoprotein, osteopontin, and osteocalcin [4, 5], indicating a role for Cbfa-1 as an osteoblast-specific transcription factor. Cbfa-1-null mice showed a complete absence of bone, with a purely cartilaginous skeleton. Together with an absence of mature osteoblasts, these data suggested

that a lack of Cbfa-1 prevents osteogenic differentiation [6, 7]. Later studies went on to show that Cbfa-1 can induce osteogenesis in vitro and in vivo. MSCs overexpressing Cbfa-1 differentiated into osteoblasts in the absence of osteogenic supplements, with increased expression of several osteoblastic markers and matrix mineralisation, and accelerated the healing of critical size defects in the skulls of mice [2].

There are conflicting reports on the potency of Cbfa-1, with some showing that ectopic expression of Cbfa-1 is sufficient to promote osteogenesis [2], whilst others conclude that overexpression of Cbfa-1 actually reduces the amount of bone formation. Transgenic mice, with osteoblasts expressing Cbfa-1 under the control of the type-I collagen promoter, had osteopenia, multiple fractures, and fewer mature osteoblasts than wild-type controls [8]. The discrepancy in these results may reflect the fact that Cbfa-1 could have differential effects on early and late osteogenesis, promoting differentiation at early stages but having an inhibitory effect on terminal differentiation [8]. Cbfa-1 is a downstream target of BMP and transforming growth factor β (TGF β) signalling, both of which are known to be potent inducers of osteogenesis [9–11]. Treatment with BMPs upregulates Cbfa-1 expression in osteogenic precursors [4, 9]. It is also known that Cbfa-1 interacts with other transcription factors and that these interactions can alter the transcriptional profile, providing additional levels of control to the signalling mechanisms governing osteogenesis.

Osterix

Nakashima et al. [9] identified Osterix (Osx) as a zinc finger-containing transcription factor specifically expressed in all de-

veloping bones. *Osx*-null mice lacked bone formation and were born with a cartilaginous skeleton. This phenotype was similar to the *Cbfa-1* knockout mice, although these also showed some cartilage defects whilst the effects of *Osx* deficiency were restricted solely to osteoblasts. Investigation of the cartilage elements of *Osx*-null mice revealed that they had been invaded by mesenchymal cells but these could not deposit bone matrix, suggesting that *Osx* was required for the differentiation of MSCs into osteoblasts. Importantly, these mesenchymal cells expressed *Cbfa-1*, indicating that *Osx* acts downstream of *Cbfa-1* in osteogenesis. It has been suggested that *Cbfa-1* commits MSCs to osteochondral lineages, and that later expression of *Osx* restricts differentiation to osteogenesis [12].

Studies on the effects of *Osx* overexpression on osteogenesis have variable results. Some indicate that *Osx* alone is sufficient to induce osteogenesis [9, 13, 14] whilst others indicate a positive-regulatory role that is also dependent on other factors [15]. These studies cover the expression of a variety of osteoblast-specific genes expressed at different stages of differentiation and more recently it has become accepted that *Osx* promotes the earlier stages of osteogenesis but is not sufficient to orchestrate events through to terminal differentiation [16]. It has also been demonstrated that *Osx* overexpression increases the proliferation of mesenchymal cells as well as providing commitment to the osteogenic lineage [15, 14].

Msx/Dlx Proteins

Msx and *Dlx* homeodomain transcription factors are homologues of the *Drosophila* Distal-less and muscle-specific homeobox genes, respectively. The *Dlx* and *Msx* proteins have diverse roles in regulating development and patterning whilst *Dlx3*, *Dlx5*, *Dlx6*, and *Msx2* have been specifically implicated in osteogenic differentiation. The involvement of *Dlx* proteins in osteogenesis was suggested by their expression both at sites of bone formation during embryogenesis and in osteoblasts themselves [17–20]. It was later shown that the expression levels of the various *Dlx* proteins changes during the course of osteogenic differentiation. *Dlx3* is expressed early in osteogenesis with *Dlx5* and *Dlx6* expressed at later stages. *Dlx5* and *Dlx6* levels then remain elevated through to matrix mineralisation [21–23]. *Dlx5* and 6 are the most widely studied in connection with osteogenesis, and have some functional redundancy as well as individual roles. *Dlx5*-deficient mice have craniofacial abnormalities and a mild delay in ossification of the long bones whilst *Dlx5/Dlx6* double mutants have more severe defects [24–26]. In addition to this, *Dlx5* is upregulated during osteogenesis and has a positive effect on differentiation inducing osteogenic markers and promoting mineralisation of preosteoblasts in culture [19, 23, 27, 28]. *Dlx3* also promotes osteogenesis although its specific function is less clear as *Dlx3* knockout mice die early in development before sufficient skeletal development [29]. Overexpression of *Dlx3* in osteoprogenitors induces expression of osteogenic markers, and

inhibition of *Dlx3* by RNAi reduces their expression, indicating that a positive-regulatory role for *Dlx3* is similar to that described for *Dlx5* and *Dlx6* [22].

It has recently been suggested that *Dlx5* can act in a *Cbfa-1*-dependent and independent manner [30]. *Dlx5* is necessary for BMP2-induced osteogenesis [31, 32]. It is expressed simultaneously with BMP2 and BMP4 during development and is induced by BMP signalling in vitro [32–36]. BMP2, through *Dlx5*, increased both *Osx* and alkaline phosphatase levels in *Cbfa-1*-deficient cells, challenging the requirement for *Cbfa1* in all aspects of osteogenic development [6, 37]. In addition, *Dlx5* induction was not affected by cyclohexamide inhibition of de novo protein synthesis while the response of *Cbfa1* to BMPs was [20, 38]. These findings suggest that BMP signalling activates *Dlx5* which induces *Cbfa1* to regulate osteogenesis. *Cbfa-1* can also upregulate *Dlx5* and may form part of a positive feedback loop [16]. It remains to be seen how relevant this signalling mechanism is in vivo. Liu et al. [37] transplanted diffusion chambers, containing BMP2- and *Cbfa-1*-deficient cells, into the peritoneal cavity of mice. As predicted from in vitro observations, these cells expressed osteoblastic markers but failed to form bone. Therefore, it appears that whilst a *Cbfa-1*-independent pathway exists for *Dlx5* in vitro it is unlikely to play a prominent role in vivo.

Msx2 is one of 3 members of the *Msx* gene family and is thought to promote osteoblast proliferation and inhibit maturation. It was identified under conditions where the expected positive-regulatory actions of *Cbfa-1* were not observed, pointing to the existence of an osteogenic inhibitor [21]. Later studies confirmed that *Msx2* overexpression inhibited osteogenesis whilst a reduction in endogenous *Msx2* promoted matrix mineralisation [39, 40]. In addition, *Msx2* is found associated with the osteocalcin promoter only when the gene is inactive [22]. However, *Msx2* mutant mice have skull defects, resulting from reduced osteoblast numbers, and *Msx1/Msx2* double-knockout mice also show severe limb defects. These observations would suggest that *Msx2* was involved in promoting osteogenesis in vivo but could also reflect the positive influence of *Msx2* on osteoprogenitor proliferation [41–43]. Recent work has begun to identify the cooperative actions of the *Dlx* and *Msx* proteins in osteogenesis, showing that their effects are brought about by interactions with other key transcription factors, including *Cbfa1* and *Osx*. Several possible mechanisms have been proposed, all of which may have some influence. Despite the in vivo evidence to support a positive role for *Msx2* in osteogenesis, it is more widely accepted that *Msx2* and *Dlx5* act antagonistically, with *Msx2* inhibiting and *Dlx5* promoting osteogenesis. The phenotype observed in the *Msx2* knockout mouse may also not strictly represent the specific influence of *Msx2* alone, but the overall effect when multiple signalling interactions are interrupted. One proposed mechanism suggests that *Msx2* binds to elements in osteoblast-specific genes, and *Cbfa-1*, competing with and blocking *Dlx5* binding [30]. Bound *Msx2* would then repress gene

Fig. 2. Expression levels of transcription factors and adipocyte-related genes during adipogenesis. C/EBP β and C/EBP δ initiate adipogenic differentiation and are expressed during commitment and mitotic clonal expansion (MCE) of pre-adipocytes. Expression levels of the inhibitor CHOP-10 decrease at the end of MCE, and allow expression of C/EBP α . C/EBP α and PPAR γ as well as SREBP1 are expressed during adipocyte maturation, activating the expression of many adipocyte-specific genes including LPL, aP2, FAS, and adiponectin.

transcription and promote proliferation. In the later stages of osteogenesis, increased levels of *Dlx5* would displace *Msx2* and activate transcription through *Cbfa-1* promoter interactions [44]. The detailed repertoire of signalling interactions is likely to be more complex than this as the function of other factors is identified. *Dlx3* can also bind *Cbfa-1* but in this case the association was inhibitory and *Dlx3* binding directly to the osteocalcin promoter activated transcription [22].

Twist

Twist-1 and *Twist-2* are basic helix-loop-helix (bHLH) transcription factors which have an inhibitory effect on osteogenesis [45, 46]. Homozygous *Twist-1* knockout mice die before birth from a failure of the neural tube to close, but heterozygotes have craniosynostosis, increased bone formation in the skull caused by premature osteoblast differentiation [47, 48]. The inhibitory effect of *Twist* on osteogenesis therefore seems to be important in ensuring the correct timing of osteogenic differentiation and create correctly patterned skeletal structures. In vitro experiments support this hypothesis, showing that overexpression of *Twist-1* suppresses BMP-induced differentiation [49]. *Twist*-mediated repression of osteogenesis acts through 2 pathways involving either *Cbfa-1* or histone deacetylases. Repression through *Cbfa-1* is mediated via direct binding of *Twist* to *Cbfa-1* through interactions between the *Twist*-box domain of *Twist* and the DNA-binding domain of *Cbfa-1*. This interferes with the binding and activation of osteogenic promoters by *Cbfa-1* [50, 51]. Both *Cbfa-1* and *Twist-1* are expressed by preosteoblasts. *Twist-1* levels then decrease during the onset of differentiation. This helps to explain the observation that *Cbfa-1* expression occurs 4 days before

osteoblasts become apparent in the developing embryo [51]. More recently, it was shown that *Twist-1* suppresses osteogenic differentiation following BMP treatment. Co-immunoprecipitations revealed complexes containing *Twist-1*, *Smad4*, and histone deacetylase 1 (HDAC1) in MC3T3-E1 cells. Addition of an HDAC1 inhibitor increased BMP-signalling and osteogenesis, indicating that *Twist* may also repress osteogenesis by recruiting HDAC1 to *Smad* complexes thereby inhibiting transcription of osteogenic target genes [49]. Whilst we have discussed the major transcriptional regulators here, there are also a whole host of other factors that are involved in the regulation of osteogenesis acting independently or as co-regulators. These include activating transcription factor 4 ATF4, and p68 Ddx5 which are positive regulatory factors, and NKX3.2 and Pitx2 which negatively regulate transcription.

Adipogenesis

In a manner similar to osteogenesis, adipogenic differentiation of MSCs is initiated with the commitment to pre-adipocytes which express adipocyte-specific genes and mature into terminally differentiated, functional adipocytes. MC3T3-L1 cells are a pre-adipocyte cell line that can faithfully recapitulate the progression of adipogenesis, when treated with pro-adipogenic factors in vitro, and have been widely used, alongside primary MSCs, in studies to elucidate events controlling the adipogenic differentiation programme. In vitro, a cocktail of dexamethasone, isobutylmethylxanthine (IBMX), indomethacin, and insulin is used to promote differentiation. The addition of adipogenic supplements causes MC3T3-L1 cells to move from a

growth-arrested state through rounds of proliferation (mitotic clonal expansion, MCE) to develop into mature adipocytes. The induction of adipogenesis is finely regulated by a balance between pro-adipogenic and anti-adipogenic signalling mechanisms. The pro-adipogenic factors include fibroblast growth factors (FGFs), insulin and insulin-like growth factor (IGF-1), prostaglandins, and glucocorticoids whilst Wnt3a, Wnt7a, TGF β , and Sonic hedgehog (Shh) have been shown to inhibit adipogenesis [52]. These upstream signalling pathways coordinate the actions of a number of signalling factors, most notably peroxisome proliferator activated receptor- γ (PPAR γ) and the CAAT/enhancer binding protein (C/EBP) family. The regulatory actions of these transcription factors then directs expression of fatty acid bind protein (aP2, otherwise known as FABP4), fatty acid synthase (FAS), lipoprotein lipase (LPL), and glucose transporter 4 (GLUT4) and other adipocyte-specific genes, all of which produce the changes in ECM production, metabolism, and lipid accumulation associated with adipocyte maturation (fig. 2).

PPAR γ

Peroxisome proliferator activated receptor- γ (PPAR γ) is a nuclear hormone receptor that is widely acknowledged to be the master regulator of adipogenesis. Two different isoforms of PPAR γ are produced by alternative splicing of the PPAR γ gene. PPAR γ 1 is ubiquitously expressed whilst PPAR γ 2, with an additional 28 amino acids at the N-terminus, has a more restricted expression pattern and is found mainly in adipose tissue [53, 54]. PPAR γ 2 seems to be a more potent inducer of adipogenic differentiation [55]. PPAR α and PPAR β are related proteins that have reduced adipogenic activity compared to PPAR γ [56].

PPAR γ is both necessary and sufficient to induce adipogenic differentiation. Importantly, many other factors regulating adipogenesis mediate their effects through PPAR γ . Overexpression of PPAR γ 2 in cultured fibroblasts promoted adipogenic differentiation [57] and caused the transdifferentiation of myoblasts into adipocytes [58], but PPAR γ -deficient cells could not form adipocytes, even with the addition of other regulators of adipogenesis [59]. This key role of PPAR γ has been confirmed in vivo with PPAR γ knockout mice having decreased amounts of adipose tissue compared to wild-type littermates [60]. The mechanism of PPAR γ -induced transcription is initiated by the binding of ligand to the PPAR γ receptor. In vivo, PPAR γ ligands include fatty acids and eicosanoids. Thiazolidinediones (TZDs) are synthetic PPAR γ ligands which increase sensitivity to insulin and are used as a treatment for diabetes [61, 62]. Ligand-binding induces a conformational change and allows the PPAR γ receptor to complex with retinoid $\times$ receptor α (RXR α) forming a heterodimer. This, in turn, binds to peroxisome proliferator response elements (PPREs) and activates transcription of adipogenic target genes [63]. PPREs contain 2 repeats of AGGTCA separated by a single base pair [64].

Additional levels of PPAR γ control include phosphorylation events and the activities of co-activator and repressor complexes. There are several potential phosphorylation sites in PPAR γ , and when one or more of these sites are phosphorylated, transcriptional activity is decreased [65]. Co-factors interact with the PPAR γ -RXR α heterodimer to influence transcriptional activity and there may be as many as 200 proteins that are capable of doing this. In the absence of ligand, co-repressors bind to PPAR γ -RXR. Many of these either have intrinsic histone deacetylase activity, or recruit histone deacetylases, which keeps the chromatin tightly bound and represses transcription. The conformational change induced by ligand binding results in disassociation of repressors from the PPAR γ -RXR α complex and allows association of co-activators [66]. Co-factors of PPAR γ include members of the steroid receptor co-activator (SRC) family. These, again, act through modification of the chromatin, but also allow recruitment of additional co-factors such as p300/CBP which are general transcriptional activators and interact with the basal transcriptional machinery [67–71].

C/EBP α , β and δ

The CAAT/enhancer binding proteins (C/EBPs) are transcription factors with a C-terminal basic DNA-binding domain and a leucine zipper domain that facilitates homo- or heterodimerisation with other C/EBP family members. Of the 3 family members, C/EBP α plays the most prominent role in coordinating adipogenesis by interacting with PPAR γ and up-regulating adipogenic gene transcription. C/EBP binding motifs are found in the promoters of genes such as PPAR γ and aP2, and expression of C/EBP α increases immediately prior to the expression of these markers [54, 72]. C/EBP α -null mice die shortly after birth due to the lost energy metabolism function of C/EBP α . However, mice in which C/EBP α expression is restricted to the liver survive and show reduced adipose tissue formation caused by reduced numbers of adipocytes from arrested differentiation [73]. Loftus and Lane [56] confirmed this finding in vitro by antisense-mediated knockdown of endogenous C/EBP α in 3T3-L1 pre-adipocytes. This reduced the expression of aP2 and GLUT4, and prevented lipid accumulation. In contrast, 3T3-L1 cells overexpressing C/EBP α were directed towards the adipogenic lineage, demonstrating that C/EBP α is sufficient to promote differentiation.

C/EBP β and C/EBP δ are involved earlier on in adipogenesis than C/EBP α and seem to be initiators of differentiation rather than contributing to the maturation process, which appears to be the primary function of C/EBP α . Both C/EBP β and C/EBP δ are induced by adipogenic hormones, such as insulin and glucocorticoids. The major role of these factors is to promote the expression of C/EBP α and PPAR γ which then drive forward terminal differentiation [56]. Mouse embryonic fibroblasts (MEFs) deficient in C/EBP β do not progress through even the earliest stages of adipogenic differentiation, an effect that could be rescued by expression of functional,

but not a dominant-negative C/EBP β [74]. Further work showed that C/EBP β activity impacts upon the MCE, which helps to explain why it is expressed immediately upon adipogenic induction, but does not bind to nor activate the promoters of C/EBP α and PPAR γ until several hours later [72]. The delay in the transcriptional action of C/EBP β is caused by a requirement for C/EBP β to be phosphorylated in order to release it from the inhibitory activity of various cofactors, including C/EBP homologous proteins (CHOPs) [74, 75]. However, C/EBP β also plays a direct role in MCE, and C/EBP β -deficient cells are unable to undergo this round of expansion [60, 72]. Exit from MCE is controlled by C/EBP α and C/EBP β . C/EBP α inhibits mitosis and is downregulated in the early stages of adipogenesis. When C/EBP α is induced by C/EBP β , it exerts its anti-mitotic effect thereby ending MCE and driving the cells onto the next stage of differentiation [75]. After their activation, PPAR γ and C/EBP α act in a coordinated manner to propagate adipogenesis. Although PPAR γ can stimulate adipogenic differentiation in the absence of C/EBP α [59], C/EBP α cannot in the absence of PPAR γ . However, the adipogenic effect of PPAR γ on fibroblasts lacking C/EBP α is greatly reduced [76]. These C/EBP α -deficient cells express reduced levels of PPAR γ , and cells deficient in PPAR γ have reduced C/EBP α levels. This shows the co-stimulatory effect of both factors together, with PPAR γ increasing C/EBP α expression and C/EBP α increasing PPAR γ expression. This positive feedback loop maintains the high expression levels of both factors through to terminal differentiation, and it has been suggested that they are required for maintenance of the adipogenic phenotype as well as the initial differentiation process [59, 77, 78]. The expression of both PPAR γ and C/EBP α together is sufficient to promote transdifferentiation of myoblasts into adipocytes [58].

SREBP1/Add1

Sterol regulatory binding element binding protein-1 (SREBP1), also known as adipocyte differentiation and determination factor-1 (Add1), is another pro-adipogenic transcription factor. It is synthesised as a precursor, tethered to the endoplasmic reticulum and cleaved, producing an N-terminal fragment that translocates to the nucleus to activate target gene transcription by binding both E-boxes and Sterol regulatory elements (SREs) [53, 79, 80]. Ectopic expression of SREBP1 in non-adipogenic NIH3T3 cells caused upregulated FAS and LPL expression, but did not result in overt adipocytic differentiation. Dominant-negative SREBP1 diminished the level of differentiation in 3T3-L1 adipocyte cultures [81]. SREBP1 augments PPAR γ -induced adipogenesis by directly stimulating PPAR γ expression [82] and enhancing levels of PPAR γ ligands [83].

Negative Regulators of Adipogenesis

In addition to the positive regulation of adipogenesis by PPAR γ , C/EBP α , β , δ , and SREBP1, there are other factors

that negatively regulate the transcription of adipocyte genes. These include the GATA-binding transcription factors (GATAs), CHOP, and inhibitor of DNA binding (Id) proteins. GATA factors were first connected to adipogenesis through their influence on fat body formation in *Drosophila* [84]. Tong et al. [85] showed that this adipogenic role was conserved in mammals with GATA factors acting as inhibitors of adipogenesis. Ectopic expression of GATA2 and GATA3 held cells as pre-adipocytes whilst embryonic stem cells deficient in GATA2 and GATA3 showed an increased propensity to form adipocytes. In this initial work, it was demonstrated that GATA factors could bind directly to PPAR γ , inhibiting its transcriptional activity. Later it was shown that GATA2 and GATA3 formed complexes with both C/EBP α and C/EBP β and that this interaction was necessary for GATA-mediated repression [86].

The pro-adipogenic factor SREBP1 is negatively regulated by Id proteins. The family consists of 3 members of which Id2 and Id3 have been shown to interact with SREBP1 and control adipocytic gene expression. The expression of Ids declines during adipogenesis [87] allowing SREBP1 to exert its positive effect. Under non-adipogenic conditions, Id proteins physically interact with SREBP1 and prevent it from binding to DNA-regulatory sequences [88].

CHOPs negatively regulate adipogenesis through interactions with C/EBPs. In early adipogenesis, CHOP-10 binds to and sequesters C/EBP β preventing it from binding to DNA regulatory sequences to activate transcription. This is a key regulatory stage because inhibition of C/EBP β prevents it from activating C/EBP α which inhibits the necessary clonal expansion of pre-adipocytes. During S-phase, CHOP-10 is downregulated, releasing C/EBP β which in turn activates C/EBP α and ends MCE. The continued effects of C/EBP α and PPAR γ then allow adipogenesis to progress [75, 89]. CHOP function is also influenced by physiological conditions. For example, CHOP expression is elevated under conditions of stress, including low glucose availability [90]. In this case, it may function to prevent the formation of adipocytes under conditions where the body does not have the resources to accumulate fat. It is interesting to note that all of the anti-adipogenic regulators seem to function through either PPAR γ , C/EBP α , or both. This only serves to highlight their importance in the regulation of adipogenesis.

Integration of the Actions of Pro- and Anti-Adipogenic Factors to Coordinate Adipogenesis

The separate roles of each of the major adipogenic transcriptional regulators have been discussed above, but the actions of each of these intersect in a tightly regulated mechanism to drive adipogenesis (fig. 3). To summarise, pro-adipogenic hormones including insulin and glucocorticoid induce expression of C/EBP β and C/EBP δ in pre-adipocytes. There is a lag in the transcriptional activities of C/EBP β during which time it

Fig. 3. Network of transcriptional regulation during adipogenesis. Adipogenic hormones induce the expression of C/EBP β and C/EBP δ which activate C/EBP α and PPAR γ . Expression levels of C/EBP α and PPAR γ are maintained throughout differentiation in a positive-feedback loop. These promote the expression of genes involved in adipocyte maturation and function, including lipoprotein lipase (LPL) and fatty acid synthase (FAS). Negative regulators, such as CHOP-10, and Id2 and Id3 provide an additional level of control to regulate the process.

fulfils a role in promotion of the essential proliferative MCE. Phosphorylation of C/EBP β releases it from interactions with inhibitory CHOPs upon which it activates expression of C/EBP α and PPAR γ . C/EBP α has anti-mitotic activity which terminates MCE and moves cells into overt differentiation. C/EBP α promotes expression of both itself and PPAR γ , whilst PPAR γ also increases transcription of C/EBP α resulting in a positive feedback loop which maintains the expression of both of these factors through to terminal differentiation. C/EBP α and PPAR γ are the transcription factors that then initiate expression of the adipocyte-specific genes that change the phenotype of the cells. This includes expression of aP2, GLUT4, FAS, LPL, and other genes which give rise to fatty acid synthesis and lipid accumulation. Other factors are also involved in this mechanisms including SREBP1 which promotes adipogenesis through the actions of PPAR γ and GATA factors which are anti-adipogenic.

Chondrogenesis

The first stage of chondrogenic differentiation involves the condensation of mesenchymal cells, which is mimicked by the formation of micromass pellets when promoting chondrogenic differentiation in vitro. Concomitant with condensation are in-

creased cell-cell and cell-ECM interactions, mediated by the upregulation of N-cadherin (N-cad), neural cell adhesion molecule (NCAM), and fibronectin (Fn) [91, 92]. During this phase, the cells become committed to the chondrogenic lineage and are described as pre-chondrocytes. After condensation, the cells progress into a highly proliferative phase, during which they are termed chondroblasts. These cells change their expression of ECM components from a matrix rich in type-I collagen typical of MSCs, to a more cartilaginous matrix containing type-II, type-IX, and type-XI collagen, aggrecan, and cartilage oligomeric matrix protein-1 (COMP-1). The proliferating chondroblasts have a flattened morphology and form highly ordered columns of cells before becoming progressively larger as they develop into pre-hypertrophic and then hypertrophic chondrocytes. Chondrocyte hypertrophy is marked by the expression of type-X collagen which is not expressed at any other stage of chondrogenesis. During endochondral ossification, these hypertrophic chondrocytes terminally differentiate and then undergo apoptosis to allow replacement of the cartilaginous template with bone [93, 94].

The many stages of differentiation are regulated by BMP, FGF, TGF β , Wnt, and Indian hedgehog (Ihh) signalling, amongst others. Both BMP and TGF β can induce differentiation by promoting cellular condensation [92]. BMP also acts during later stages to control chondrocyte proliferation and

Fig. 4. Expression levels of transcription factors and chondrocyte-related genes during chondrogenesis. Sox9 is expressed from the onset of chondrogenesis when mesenchymal cells condense. Sox9 activates L-Sox5 and Sox6, and together they promote the early stages of differentiation when chondroblasts proliferate and form columnar chondrocytes. The transition into hypertrophy is controlled by Sox9 and Cbfa-1. During chondrogenesis, the expression of extracellular matrix proteins changes from a type-I collagen matrix to one rich in type-II collagen, type-IX collagen, and aggrecan. Hypertrophic chondrocytes are marked by expression of type-X collagen.

hypertrophy [95]. Signalling by Ihh with parathyroid hormone related peptide (PTHrP) is one of the best understood chondrogenic signalling pathways. Ihh is produced by proliferating chondrocytes and induces perichondrial cells, at the ends of the bone, to produce PTHrP. Together, these factors work in a negative feedback loop to control the progression of proliferative cells to chondrocyte hypertrophy [96]. FGF and Wnt signalling mechanisms also act during multiple stages of differentiation [97–100]. These signalling mechanisms ultimately exert their effects through a series of transcriptional regulators. Some of the most influential and widely studied of these include Sox5, Sox6, Sox9 and Cbfa-1 (fig. 4).

Sox 9

Sox9 is a sex-determining region Y (SRY)-related high motility group (HMG) box transcription factor. It has regulatory roles in both chondrogenic differentiation and sex determination as is demonstrated by the disease campomelic dysplasia (CD) which is caused by mutations in Sox9 and results in cartilage defects and XY sex reversal. Sox9 is the earliest known marker of chondrogenesis and has crucial roles in several stages of the differentiation process. Expression of Sox9 is evident in the condensing mesenchymal progenitors that will become chondrocytes, and its expression remains continuous until chondrocyte hypertrophy [101]. An integral role for Sox9 in cartilage development was shown by Bi et al. [102]. Their work used embryonic stem cells deficient in Sox9 to produce mouse chimeras. There was an absence of the Sox9-deficient cells in any of the cartilage elements. Teratomas produced

from the embryonic stem cells also showed an absence of any cartilage formation.

Sox9 functions during both early and late chondrogenesis. Mice in which Sox9 could be removed at different stages of development using Cre/LoxP recombination helped to define the temporal role of Sox9 during chondrogenesis. Removal of Sox9 before mesenchymal condensation resulted in mice with absent cartilage development. Loss of Sox9 after condensation caused generalised chondrogenic dysplasia, with the arrest of differentiation at the condensation stage, indicating that cells could not complete chondrocyte differentiation [103]. These data demonstrated that Sox9 was necessary at more than one stage of chondrogenic differentiation, and confirmed results found using heterozygous Sox9 mutant mice. These mice died perinatally displaying cleft palate defects and deformities in many of their skeletal structures that were attributed to defects in cartilage condensation. Analysis of the embryos at different stages of gestation revealed premature mineralisation of many bones and enlarged zones of hypertrophic chondrocytes indicating that Sox9 also controls the movement of pre-hypertrophic chondrocytes into hypertrophy [104]. Sox9 can delay hypertrophy whilst increasing progression through the cell cycle [105, 106].

The chondrogenic effects of Sox9 are mediated by its ability to activate genes encoding key components of the cartilage matrix. It has been demonstrated to bind to and activate elements in the promoters of Col2a1, Col9a1, Col11a2, and aggrecan [101, 107–110]. In many of these promoters, the Sox9 elements are arranged in pairs. Changing the number of nu-

cleotides separating these elements in the Col11a2 promoter, blocked activation by Sox9 suggesting that it functions as a homodimer, with each Sox9 monomer binding to one recognition site [110]. Subsequent work has also identified Sox9 sites arranged in 2 pairs in the Col9a1 promoter, and it has been suggested that two Sox9 dimers may bind these sites together in order to activate transcription [111]. There are multiple ubiquitination sites on Sox9 through which it is targeted for degradation. Akiyama et al. [112] showed that this was a mechanism used to control the transcriptional activity of Sox9 in chondrogenesis by regulating the amount of the protein present. They used inhibitors of the 26S proteasome and demonstrated both increased levels of Sox9 protein and increased levels of Col2a1 transcription.

L-Sox5 and Sox6

Sox5 and Sox6 are highly homologous with 67% identity as a whole and over 90% homology in the DNA-binding HMG box. In contrast, they share only 50% homology with the HMG-box of Sox9 and lack any significant homology outside of this region [113]. A major difference between the structures of Sox5 and 6 and that of Sox9 is that Sox9 has a transactivation domain whilst Sox5 and 6 do not. This means that Sox9 can directly activate transcription of target genes whilst Sox5 and 6 are thought to exert their effects through the recruitment and organisation of other transcriptional regulators. There are two isoforms of the Sox5 protein of which the long form L-Sox5 is involved in chondrogenesis. This contains an N-terminal coiled-coil domain through which it can dimerise either with other L-Sox5 monomers or with Sox6 to form homo and heterodimers [114].

Both Sox5 and the Sox6-null mice have a few mild skeletal defects, but Sox5/Sox6 double-knockout mice show evidence of a severe generalised chondrodysplasia [113]. This indicates redundant functions for Sox5 and Sox6 in chondrogenic differentiation. The chondrocytes in the double-knockout mice expressed diminished levels of cartilage matrix genes resulting in the poor cartilage formation. However, condensation of the progenitor cells had occurred properly, indicating that the overlapping roles of Sox5 and Sox6 are different to that of Sox9 which is necessary for mesenchymal condensation as well as chondrogenic differentiation. Sox9 induces transcription of Sox5 and Sox6, and their expression is fully dependent upon activation by Sox9. This has been demonstrated in vitro but also by the absence of Sox5 and Sox6 in Sox9-null mice. Sox9 is not dependent on Sox5 and Sox6 as its levels are unaffected in mice with loss of Sox5 and Sox6 [103, 113, 115]. The major role of Sox5 and Sox6 is to enhance the effects of Sox9. This advocates a mechanism whereby Sox9 induces Sox5 and Sox6 as well as other cartilage-specific genes in pre-chondrocytes. The combined effects of Sox5, Sox6, and Sox9, often referred to as the Sox trio, then act cooperatively to further up-regulate the expression of type-II, type-IX, and type-XI collagen and aggrecan in chondroblasts [116]. When expressed to-

gether, the Sox trio are sufficient to induce chondrogenesis in a variety of cell types, including non-chondrogenic cells [117].

Cbfa-1

In addition to its role in osteogenesis, Cbfa-1 also regulates chondrocyte development. Evidence for this function is present in the Cbfa-1-null mice which were used to define the pro-osteogenic role of Cbfa-1. Closer study confirmed that there were defects in the cartilage elements as well as the lack of bone formation caused by the absence of Cbfa-1-induced osteogenesis. These defects signified a failure of the chondrocytes to undergo terminal differentiation as demonstrated by the absence of cells expressing type-X collagen [118–120]. There were also defects in vascularisation of the chondrogenic elements [121].

Expression levels of Cbfa-1 peak during chondrocyte hypertrophy supporting a role for Cbfa-1 in regulating this process [122]. Overexpression of Cbfa-1 confirmed this observation by promoting hypertrophy in vitro and in vivo. Cbfa-1 overexpression in chick embryos caused joint fusion due to increased cartilage production, and in mice in whom Cbfa-1 expression was targeted to prehypertrophic chondrocytes, hypertrophy and endochondral ossification were increased [120]. Experiments have also been performed using dominant-negative Cbfa-1, and these demonstrated inhibition of hypertrophy [120, 123, 124]. Together, these data provide convincing evidence that Cbfa-1 induces chondrocyte hypertrophy. Rescue of Cbfa-1 expression in the mesenchymal condensations of Cbfa-1-null mice restores chondrocyte hypertrophy without affecting osteogenesis, indicating that this effect is direct and not just an artefact of the functions of Cbfa-1 in osteoblast differentiation [125].

Conclusions

MSCs are candidates for a range of therapeutic applications, through their self-renewal capacity and broad differentiation potential. Identifying the transcriptional regulators that control these activities will help realise this goal. Significant progress has been made on determining the factors that influence osteogenic, chondrogenic, and adipogenic differentiation. However, many findings have been drawn from the use of transgenic animals, non-human cell lines, or mixed populations of primary bone marrow-derived human MSCs which may not necessarily reflect the behaviour of true MSCs in vivo considering interspecies disparities and imprecise MSC identity. This is also true for those MSCs that have been identified in different sources that would be exposed to unique, tissue-specific environments and signalling cues. Much less is known about the differentiation of MSCs into non-mesenchymal lineages and how this may be regulated at the transcriptional level. As technology advances alongside our knowledge of MSC biology, these issues are increasingly likely to be resolved.

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