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TISSUE-SPECIFIC STEM CELLS

STEM CELLS

Alternative splicing in mesenchymal stem cell differentiation

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Abstract

The differentiation and maturation of mesenchymal stem cells (MSCs) to mesodermal and other lineages are known to be controlled by various extrinsic and intrinsic signals. The dysregulation of the MSC differentiation balance has been linked to several pathophysiological conditions, including obesity and osteoporosis. Previous research of the molecular mechanisms governing MSC differentiation has mostly focused on transcriptional regulation. However, recent findings are revealing the underrated role of alternative splicing (AS) in MSC differentiation and functions. In this review, we discuss recent progress in elucidating the regulatory roles of AS in MSC differentiation. We catalogue and highlight the key AS events that modulate MSC differentiation to major osteocytes, chondrocytes, and adipocytes, and discuss the regulatory mechanisms by which AS is regulated.

KEYWORDS

adipogenic, alternative splicing, chondrogenic, ESC differentiation, mesenchymal stem cells, MSC differentiation, neural differentiation, osteogenic, RNA-binding proteins

1 | INTRODUCTION

Mesenchymal stem or stromal cells (MSCs) are multipotent and multifunctional stem cells that have the potential to differentiate into mesodermal cell types, including osteoblasts, chondrocytes, and adipocytes, and they also play important roles as immune regulators and a critical niche in hematopoiesis.[1,2] Due to the current debate surrounding their embryonic origin and in vivo functions, the term "MSC" has been suggested to be inaccurate as MSCs from different tissue depots clearly have different phenotypes. For example, not all MSCs support hematopoiesis. Thus, alternative terms such as "multipotent mesenchymal stromal cells" and "tissue specific stem cells" have been proposed. Given the broad therapeutic effects identified thus far for MSCs derived from various adult tissues and embryonic stem cells, MSCs are even suggested to refer to "medicinal signaling cells."[3-5] In this review, however, we use "MSC" throughout, for the simplicity with alluding to classically defined differentiation potential toward mesodermal derivatives in vivo and in vitro.[6,7]

Dissecting the governing molecular mechanisms of MSC differentiation into mesenchymal cell types is critical for understanding MSC function in vivo and evaluating and predicting the clinical outcome of MSC-based cell therapy. The balance of extrinsic signaling pathways through combinatorial or distinct sets of cytokines, growth factors, and hormones coordinates the activation or suppression of transcription factors that determine the fate of MSCs and the maturation of progenitors into terminally differentiated cell types.[8-10] One of the best known examples is Wnt signaling, which simultaneously activates and represses the expression of Runt-related transcription factor 2 (*RUNX2*) and peroxisome proliferator-activated receptor gamma (*PPARγ*), the master genes for osteogenesis and adipogenesis, respectively, and plays a critical role in lineage commitment during early MSC differentiation.[11]

The importance of alternative splicing (AS), which will be discussed in more detail below, in controlling MSC differentiation has gained less attention than the mechanisms of transcriptional control. A recent study by Aprile et al identified a novel alternative isoform of PPARγ, which modulates adipogenesis by blocking the function of wild type PPARγ in two ways.[12] Interestingly, the expression level of the PPARγΔ5 isoform in subcutaneous adipose tissues was shown to be directly proportional to body mass index (BMI) in two independent

cohorts of obese or type II diabetic patients, highlighting a potential link between AS and health risk of obesity.

Van de Peppel et al analyzed the gene expression dynamics for the early steps of human MSC (hMSC) differentiation into osteoblasts and adipocytes with a high-density temporal resolution.[13] Upstream regulator analysis revealed 34 transcriptions factors (TFs) with a role in the early differentiation of MSC. Unexpectedly, the expression change of the differentially expressed genes (DEGs) that is thought to be regulated by the 34 TFs did not match with the expression change of the TFs themselves during the early stage of MSC differentiation.[13] This suggests that additional gene expression regulatory mechanisms, including post-transcriptional regulation, may be needed to explain the aforementioned discrepancy in evidence for the control the expression of the MSC differentiation-associated genes.

Moreover, recent studies have demonstrated that pre-mRNAs encoding master transcription factors, the prerequisite for the early lineage specification of MSC differentiation, undergoes AS and encode multiple protein isoforms with functionally distinct activities. The pre-mRNAs of the osteogenic and adipogenic master regulators, *RUNX2* and *PPARγ*, generate multiple mRNA isoforms by AS that are subsequently translated into the protein isoforms with the regulatory activities.[12,14]

Girardot et al recently demonstrated that a master regulator for chondrogenesis, Sox9, regulates the splicing of hundreds of genes without affecting their transcription during chondrogenic differentiation.[15] The study indicates that the regulation occurs through the direct association of Sox9 with pre-mRNAs and other RNA-binding proteins (RBPs), rather than by altering the target RBP expression levels and indirectly modulating AS. Consistent with the notion, more than one-third of the regulators that control ES- and neural-specific AS were transcription factors, implying that some transcription factors have dual functions, regulating transcription and AS during ES and neural differentiation.[16] One of the surprising aspects of the study was that the regulatory transcription factors control AS and shape the cell fate-specificity by directly binding to target pre-mRNA.[16] Not surprisingly, the coupling between pre-mRNA splicing and transcription has been well-documented as transcription elongation rate controls AS, and AS controls the transcriptional rate.[17]

Yet, there is a lack of systematic review on the regulatory roles of AS in MSC differentiation despite the current research progress in the field. In this review, we will briefly discuss the regulatory mechanisms of AS, focusing on the dynamic interactions between cis-acting sequence elements within the pre-mRNA and trans-acting RBPs, and discuss the major AS of pre-mRNAs encoding the key transcription factors and critical factors that are involved in the cell fate determination, maturation, and metabolic status for MSC differentiation into osteocytes, chondrocytes, adipocytes, and neurons.

2 | AS AND ITS REGULATORY MECHANISMS

AS is one of the powerful regulatory mechanisms of gene expression by which a pre-mRNA generates multiple mRNA and protein isoforms to increase the functional capacity of cells. As high as 95% of all human genes undergo AS and the pattern of AS is highly variable among different human tissues, suggesting a correlation between the unique signature of the transcriptome, regulated by AS, and tissue-specific structure and function.[18] Thanks to the recent development of high-throughput RNA sequencing (RNA-Seq), more widespread, evolutionarily conserved, overall biological roles of AS in the gene expression landscape have been recently recognized.[19-21]

Significance statement

Mesenchymal stem cells (MSCs) are important cell sources for bone, fat tissues, cartilage, and other connective tissues inside body and in test tube. One needs a better understanding of molecular mechanisms how intrinsic regulatory network governs the differentiation of MSCs. In this review, the underrated role of alternative splicing is discussed, a process to produce multiple similar but functionally distinct proteins from one gene, in MSC differentiation.

In metazoans, introns are removed from pre-messenger RNAs (pre-mRNAs) to produce mature RNA. However, this seemingly simple chemical reaction is catalyzed by the largest known macromolecular complex inside the cell, called the spliceosome. This mega-sized machinery is made of more than 300 RNA and protein components.[22,23] The regulation of AS is controlled by the interaction between cis-acting elements and trans-acting factors, primarily RBPs. Trans-acting factors interact with different components of the spliceosome during the assembly of the spliceosome on the nascent pre-mRNA. Here, we summarize the general mechanism of regulation by which several groups of RBPs (heteronuclear ribonucleoproteins [hnRNPs], serine-arginine rich proteins [SRs], and KH domain-containing protein such as NOVA) function by promoting or inhibiting the binding of components of the splicing machinery to the target splice sites (Figure 1). These auxiliary splicing factors recognize a variety of cis-acting sequence elements within the pre-mRNA and are known as exonic splicing enhancers, exonic splicing silencers, intronic splicing enhancers, and intronic splicing silencers (ISSs), by either recruiting or blocking the assembly of small nuclear ribonucleoprotein (snRNP) complexes (Figure 1). These RBPs are expressed in a tissue- and developmental-specific manner and the relative expression level of critical RBPs in a specific cell determines a ratio of the isoforms by regulating whether or not a given exon will be included in the mRNA.

3 | REGULATION OF STEM CELL FATE DETERMINATION BY AS

One of the earliest evidence that AS plays a critical role in the pluripotency-differentiation axis in ESCs came from a study in which a shorter FGF4 isoform, FGF4si, attenuated activity of FGF4 by a novel fibroblast growth factor 4 (FGF4), thereby inducing ESC

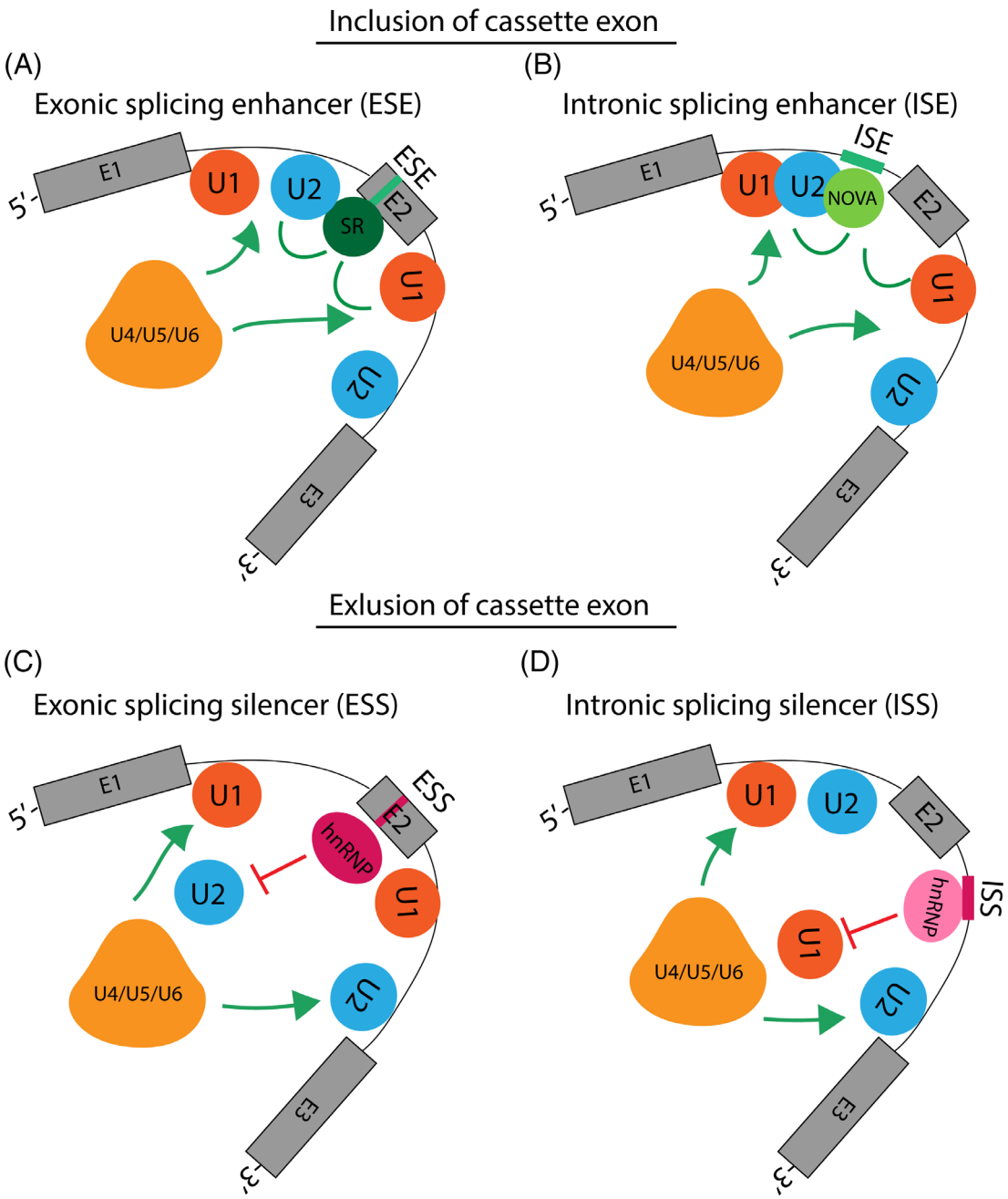

FIGURE 1 A schematic of the regulatory mechanisms by which interaction between cis-acting elements and trans-acting factors control the inclusion or exclusion of the cassette exon in three exons model. A, Serine-arginine (SR) proteins (green) bind to exonic splicing enhancer (ESE) and recruit U1 and U2 snRNP for the inclusion of exon 2. B, RNA binding protein, NOVA, binds to intronic splicing enhancer (ISE) and recruit U1 and U2 snRNP for the inclusion of exon 2. C, RNA binding protein, hnRNP, binds to exonic splicing silencer (ESS) to block the binding of U1 and U2 snRNP for the exclusion of exon 2. D, RNA binding protein, hnRNP, binds to intronic splicing silencer (ISS) to block the binding of U1 and U2 snRNP for the exclusion of exon 2

differentiation.[24] Later, Blencowe's group demonstrated that the muscleblind-like RNA binding proteins-mediated alternative splicing of a key transcription factor, FOXP1, makes a significant contribution to the switch between maintaining pluripotency and triggering differentiation of human ESCs.[25,26] Moreover, Paired box 6 (PAX6), RNA binding motif protein 24 (RBM24)-mediated, Tcf3, and Sal4 AS have been associated with ectodermal, mesodermal, and endodermal lineage determination of human and mouse embryonic stem cells.[27-31]

Su et al recently reported that RNA binding domain protein 4 (RBM4) regulates AS of PKM pre-mRNA, thereby promoting neural differentiation of hMSCs.[32] RBM4 was originally was shown to promote the differentiation of neuronal progenitor cells and the neurite outgrowth of cultured neurons via its role in splicing regulation. During neuronal differentiation, energy production shifts from glycolysis to oxidative phosphorylation. AS of Pyruvate kinase M (PKM) has been implicated as a fuel switch in determining the source of energy

production, glycolysis or oxidative phosphorylation. Inclusion of mutually exclusive exons, 9 and 10, results in two PKM isoforms, PKM1 and PKM2, differing in the formation of the tetramer, which is a limiting factor for the production of pyruvate. Tetramer formation is less efficient for PKM2, which results in the reduced production of pyruvate, substrates for oxidative phosphorylation, and in turn, promotes the production of energy through glycolysis. Indeed, a higher PKM2/PKM1 ratio is a hallmark of undifferentiated stem cells and cancer cells.[33] PKM pre-mRNA alternative splicing appears to be regulated by multiple RBPs including hnRNP A1 and A2, polypyrimidine-binding protein (PTB), RBM4, and SRSF3 by binding to an ISS to block the assembly or entry of U1 snRNP to the 5′ splice site, thus preferentially selecting exon 10 for the production of the *PKM2* isoform.[34-36]

Genome-wide RNA-seq analyses have revealed that a specific AS pattern is observed with ESCs and a global AS change in differentiated ESCs. Conversely, the global AS profile in differentiated cells reverts back to that of a pluripotent state during stem cell reprogramming. Although this finding does not provide the conclusion that AS program is the driving force of differentiation, it supports the notion that a signature pattern of AS for a particular cell type may contribute to the cell type-specific global gene expression landscape. More recently, it has been shown that the downregulation of U2AF, a core component of spliceosome, induces a switch in the AS of TFs involved in the differentiation of pluripotent stem cells and controlled cell-fate specific AS.[37] Besides, it has been shown that differential AS events were detected in MSCs derived from young and old donors, illustrating that AS is a widespread mechanism to shape the transcriptome landscape in various cell and tissue types, as well as during differentiation.[38,39]

The regulation of MSC differentiation by AS has recently been recognized as an additional mechanism that controls the precise cell fate determination and maturation to a terminally differentiated cell type. One mechanism of AS in cell fate determination is the production of two functionally distinct or opposite isoforms by AS, forming a molecular switch during cell fate commitment.[40,41] As mentioned above, pre-mRNAs of key transcription factors, which specify cell fate by controlling the expression of downstream target genes, undergo AS, resulting in alternative isoforms. These alternative isoforms play overlapping, distinct, and even opposite roles during MSC differentiation through various molecular mechanisms. Here, we describe the AS of key molecules that specify cell fate during early MSC differentiation and maturation steps (summarized in Table 1) and the major AS events associated with MSC differentiation (illustrated in Figure 2).

4 | REGULATION OF OSTEOGENIC DIFFERENTIATION BY AS

The activation of transcription factor RUNX2 is a prerequisite for the commitment to an early osteogenic lineage and is switched off later during osteocyte maturation.[65] The induction of RUNX2 in MSCs coincides with the commitment to osteogenic progenitors, and it also suppresses the cell fate specification toward an adipogenic lineage.[14] The first hint that AS may be involved in regulating osteogenic differentiation came from several studies that showed that a shorter RUNX2 protein isoform, produced by alternative splicing, exhibited distinct transcriptional activity.[14,66] Makita et al demonstrated the detection of four *RUNX2* mRNA isoforms; WT *RUNX2*, *RUNX2Δ5*, *Δ7*, and *Δ5Δ7*, through skipping one or both cassette exons (exon 5 and 7) in hMSCs.[14] Two of these isoforms generated from the skipping of exon 5 encoded RUNX2 isoforms that were devoid of nuclear localization, thereby eliminating their transcriptional activities. On the other hand, a third mRNA isoform, *RUNX2Δ7*, encodes an isoform with a deletion in the region near the activation domain at the carboxy terminus of RUNX2. The RUNX2Δ7 protein isoform localizes in the nucleus and exhibits a similar binding affinity and transcriptional activity for the osteocalcin (OC) gene as WT RUNX2. However, RUNX2Δ7 is less dependent on coactivators and corepressors (CBP, p300, and HDAC3) in transactivating the OC gene. This raises the reasonable possibility that RUNX2Δ7 may transactivate both the same common target genes as the WT and distinct target genes in a CBP/p53- and HDAC3-independent manner.

Nonetheless, the expression pattern of two isoforms, WT and RUNX2Δ7, is distinct between chondrocytes and osteocytes. Roughly equal expression levels of the isoforms are present in chondrocyte differentiated from hMSCs, while exclusively the wild-type isoform is present in osteocyte differentiated from hMSCs, indicating that the WT/ RUNX2Δ7 ratio may be a key determinant for cell fate of hMSCs. Mechanistically, RUNX2 WT and Δ7 possess distinct transcriptional activities for the target genes, including OC, and potentially provide the fine-tuning for osteogenic vs chondrogenic fate determination. It is also worth noting that the WT/ RUNX2Δ7 ratio varies among various osteoblast-like cells, including osteosarcomas, primary osteoblasts isolated from human donors, and differentiated osteocytes from hMSC, potentially the ratio of the two isoforms as a potential biomarker for cancers of osteocytic origin.

Although the regulation of *RUNX2* alternative splicing has not been clearly elucidated, skipping *RUNX2* alternative exons has been stimulated by knockdown (KD) of the components of U2 snRNP, namely, U2AF1, SF3A1, and SF3A3.[67] Interestingly, U2 snRNPs are components of the spliceosome and are considered essential for the catalysis of pre-mRNA splicing, rather than modulating AS. This finding is consistent with the previous studies that have shown that mutations in general splicing factors can indeed alter the global AS pattern in vitro and in vivo, rather than affecting global splicing defects in a cell- or tissue-specific manner.[68-70] Furthermore, osteogenesis-specific RUNX2 target genes, including OSC, COL1A1, OPN, BSP, and OSX, undergo alternative splicing and produce multiple mRNA isoforms, although the exact biological implication is not known at present.[67]

Of particular interest, OSX (Sp7) is an immediate downstream target gene of RUNX2, which drives the differentiation of pre-osteoblasts into mature osteoblasts and promotes bone formation, in part, by modulating the expression of many osteogenic genes including COL1A, SPARC, BSP-1, and BGLAP.[71] Two mRNA isoforms, encoding 413 (Sp7S) and 431 (Sp7L) amino acids long peptides, respectively, through 3′ alternative splicing of exon 3, are differentially expressed

TABLE 1 Summary of alternative splicing events for genes involved in the differentiation of embryonic stem cells (ESCs) and mesenchymal stem cells (MSCs)

Gene	Isoform	AS mode	Isoform function	Remarks	Regulation	Reference
ES cell differentiation						
FGF4	*FGF4 WT*	Full length	Promotes self-renew and pluripotency	Positive regulator of Erk1/2 phosphorylation	Unknown	24
	FGF4si	Exon skipping	Antagonizes self-renew and pluripotency	Negative regulator of Erk1/2 phosphorylation		
FOXP1	*FOXP1*	Mutually exclusive exon	Promotes hESC differentiation	Activates differentiation and suppresses pluripotency genes	MBNL1, RBFOX2	25
	FOXP1-ES	Mutually exclusive exon	Activates pluripotency genes	Activates pluripotency and suppresses differentiation genes		
PAX6	*PAX6a*	Exclusion of Cassette Exon	Neuroectoderm differentiation	Induces neuroectoderm	PNN, ESRP1	31
	PAX6b	Inclusion of Cassette exon	Cell proliferation	No effect on neuroectoderm differentiation		
Tcf3 (TCF7L1)	*Tcf3-E18a*	Mutually exclusive exon	High in undifferentiated mES cells	Maintains pluripotency of ES cells	hnRNP H, hnRNP F	29
	Tcf3-E18b	Mutually exclusive exon	High in differentiated mES cells	Promotes differentiation		
Sall4	*Sall4a*	Alternative 5′ splice site	Promotes pluripotency		Unknown	30
	Sall4b	Alternative 5′ splice site	Sufficient for pluripotency	Missing ZF domains, preferentially binds to ES-specific promoters		
Mesenchymal stem cell differentiation						
RUNX2	*RUNX2 WT*	Full length	Promotes osteogenesis	Promotes early osteogenesis, activates osteocalcin	U2 snRNP, U2AF, SF3A1, SF3A3	14,44
	RUNX2Δ5	Cassette exon	Not known	Missing NLS		
	RUNX2Δ7	Cassette exon	Chondrocytes specific	RUNX2Δ7 antagonizes RUNX2, no activation of osteocalcin		
	RUNX2Δ5Δ7	Cassette exon	Not known	Missing NLS		
FosB	*FosB WT*	Full length	Osteogenesis		Unknown	45,46
	ΔFosB	Cassette exon	High in osteoblasts, promotes osteogenesis and inhibits adipogenesis	Truncation of transactivation domain		
	Δ2ΔFosB	Alternative promoter, cassette exon	Promotes osteogenesis independent of AP-1 activity	Truncation of Fos-homology domain and transactivation domain		
OPN	*OPN-a*	Cassette exon	Promotes angiogenesis and osteogenesis		Unknown	47,48
	OPN-b (ΔE5)	Cassette exon	Not known	Exon 5 containing serine phosphorylation site		
	OPN-c (ΔE4)	Cassette exon	Promotes angiogenesis and osteogenesis	Exon 4 containing tyrosine phosphorylation site		
OSX (SP7)	*OSX-I*	Alternative 5′ splice site	Lower expression in osteoblasts		Unknown	49

(Continues)

TABLE 1 (Continued)

Gene	Isoform	AS mode	Isoform function	Remarks	Regulation	Reference
	OSX-s	Alternative 5′ splice site	Higher expression in osteoblasts and chondrocytes formation	Truncated N-terminal with distinct t/s activity		
Vegf-A	*Vegf-A WT*	Full length	High expression in high-frequency mechanical loading	Matrix bound	Unknown	50
	Vegf-A206	Cassette exon	High expression in high-frequency mechanical loading	Matrix bound		
	Vegf-A189	Cassette exon	High expression in high-frequency mechanical loading	Matrix bound		
	Vegf-A165	Cassette exon	High expression in high-frequency mechanical loading	Partially matrix/partially secreted		
	Vegf-A145	Cassette exon	High expression in low-frequency mechanical loading	Matrix bound		
	Vegf-A121	Cassette exon	High expression in low-frequency mechanical loading	Secreted		
Periostin (Postn)	*Postn-001*	Full length	Osteoblast differentiation	Maximum number of RD domains	Unknown	51
	Postn-001 - 008	Cassette exons	Increased attachment of osteoblasts in periosteum	Various number of RD domains		
NELL-1	*NELL-1-810*	Full length	Promotes osteogenesis		Unknown	52
	NELL-1-570	Cassette exon	Enhanced MSC proliferation and osteogenesis	Truncation of LamG domain, required for heterodimer formation		
PPARγ	*PPARγ WT*	Full length	Promotes adipogenesis		SRSF1	12
	PPARγΔ5	Cassette exon	Disrupts adipogenesis	Dominant negative against the WT protein		
PTHrP	*PTHrP-L*	Alternative 3′ splice site			Unknown	53,54
	PTHrP-S	Alternative 3′ splice site	Promotes chondrocytes, suppresses MSC hypertrophy	EGF-dependent 3'-UTR enables RNA stability		
mTOR	*mTOR-int5*	Removal of intron	Promotes adipocyte differentiation	Increased mTOR signaling	Sam68	55
	mTOR + Int5	Retained intron	Suppresses adipocyte differentiation	Introduction of PTC and reduced mTOR signaling		
LPIN1	*LPIN1 WT*	Full length	Promotes adipocyte differentiation	Nuclear	SFRS10	56-58
	LPIN1Δ7	Cassette exon	Suppresses adipocyte maturation	Cytoplasmic		
AMBN	*AMBN WT*	Full length	Promotes MSC proliferation and enamel formation	Exon 5 peptide promotes MSC proliferation and osteogenesis	Unknown	59
	$AMBN\Delta 6_{1\text{-}15}$	Alternative 3′ splice site	Promotes MSC proliferation and enamel formation	Exon 6 15 AA peptide suppresses MSC proliferation and osteogenesis		

TABLE 1 (Continued)

Gene	Isoform	AS mode	Isoform function	Remarks	Regulation	Reference
S6K1	*S6K1 WT*	Cassette exon	Promotes adipogenesis		Sam68	60,61
	S6K1Δ6a	Cassette exon	Suppresses adipogenesis	Activates mTOR signaling		
RUNX1T1	*RUNX1T1-L*	Cassette exon	Suppresses adipogenesis	Increased m6A leads to SRSF2 binding and inclusion of exon 6	FTO, SRSF2	62
	RUNX1T1-S	Cassette exon	Promotes adipogenesis	Reduced m6A by FTO leads to lower SRSF2 binding to exon 6		
Col2a1	*Col2a1 IIA*	Alternative 5′ splice site	High expression in chondroprogenitors		Tia1, Sox9	15,63,64
	Col2a1 IIB	Cassette exon	High expression in mature chondrocytes			
	Col2a1 IIC	Alternative 5′ splice site	Unknown	Alters IIA/IIB ratio		
	Col2a1 IID	Alternative 5′ splice site	Unknown	Alters IIA/IIB ratio		
TAF4	*TAF4*	Cassette exon			Unknown	65
	TAF4ΔTAFH	Cassette exon	Promotes MSC proliferation and chondrogenic differentiation	Cell cycle exit via p53 activation and regulates Wnt signaling		
PKM	*PKM1*	Mutually exclusive exon	Promotes differentiation, including neural precursor cells	Tetramer formation, prefers oxidative phosphorylation	RBM4, SRSF3	32,66
	PKM2	Mutually exclusive exon	Promotes dedifferentiation	Dimer formation, prefers glycolysis		

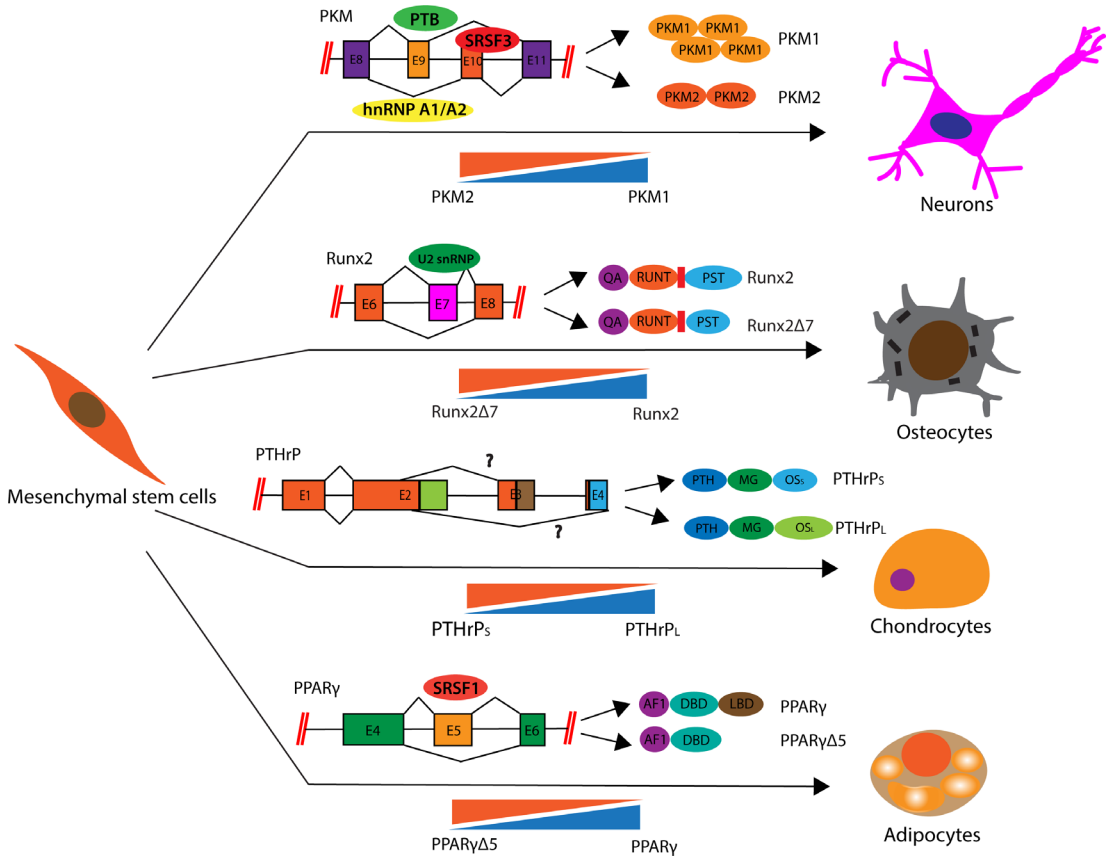

FIGURE 2 A schematic showing the important AS events involved in MSC differentiation to osteocytes, chondrocytes, adipocytes, and neurons. A schematic above each arrow represents the abbreviated splicing pattern with the resulting protein isoforms structure with indicated structural domains. A schematic below each arrow represents the relationship between expression level of each splicing isoform and functional consequence

between various cell and tissue types, especially fetal vs adult osteoblasts, and chondrocytes, suggesting a biological role of AS in cell or differentiation stage-specific AS.[47] Besides, multiple osteogenesis imperfecta patients harbor a splicing-sensitive mutation in the intron of COL1A1, implicating the mis-regulated intron retention as a causative mechanism for osteogenesis imperfecta.[72]

Vascular endothelial growth factor A (VEGF-A), encoded by *VEGFA*, is a multifunctional growth factor that was shown to promote osteogenic differentiation of MSC by promoting mineralization in autocrine manner and angiogenesis in a paracrine manner.[73] Faure et al demonstrated a correlation between different VEGF-A splicing isoforms and the mechanical stress in osteoblastic cells, that is, the upregulation of the membrane bound VEGF-A isoforms under high mechanical stress and that of the secreted VEGF-A isoforms under low mechanical stress.[48] Generation of membrane-bound and secreted isoforms is carried out by a differential inclusion of three cassette exons (6A, 6B, and 7), within VEGF-A pre-mRNA. Nonetheless, regulated AS of VEGF-A by mechanical stress may provide an additional regulatory mechanism toward osteogenic differentiation, especially during osteogenesis upon a bone fracture.

Mechanical stress plays an important role during the formation of osteoblast's extracellular matrix (ECM). Physiologically, the lack of mechanical loading reduces the integrity of the ECM structure of osteocytes in the bone and eventually leads to bone loss. Roosa et al recently analyzed the global AS pattern of post-mechanical loading and subsequent bone formation using rats as a model system.[74] Their study identified numerous AS changes after the mechanical loading, potentially linking its AS pattern to the response of osteocytes to mechanical stress. A deeper understanding of the biological functions of AS isoforms during mechanical loading can further shed light on the regulation of bone maintenance and bone formation in general by AS in response to mechanical loading.

One of the known osteogenic growth factors, called neural epidermal growth-like (NEL)-like factor 1 (NELL-1), promotes osteogenesis in vitro and in vivo.[75] This gene generates two alternative isoforms, the full-length isoform (NELL-1810) and a shorter isoform (NELL-1570), by a combination of an alternative promoter and a 5′ splice site for the inclusion of exon 2. Although both isoforms demonstrate an osteogenic differentiating effect, the shorter isoform had a stronger promoting effect on the proliferation of a murine primary MSC line and pericytes compared with the wild-type NELL-1.[50] However, the molecular mechanism by which NELL-1 splicing is regulated remains unknown.

Ameloblastin (*AMBN*) encodes a regulator of osteogenic differentiation, which is originally expressed in ameloblast for enamel formation and mineralization. AMBN pre-mRNA produces two isoforms, AMBN-

WT and -$\Delta 6_{1\text{-}15,}$ by choosing alternative 3′ splice sites of exon 6. It was recently shown that a synthetic peptide derived from the sequence of *AMBN* exon 5 promotes hMSC proliferation and activates expression of RUNX2 and OSX.[57] Intriguingly, 15 amino acid synthetic peptide corresponding to *AMBN* exon 6 abrogated the effect by exon 5 peptide. Although the regulatory mechanism by which ABMN exon 5/6 AS is regulated and the exact molecular function of the smaller AMBN isoform remain to be elucidated, AS of AMBN appears to be important for encoding the extracellular domain, the self-assembly of AMBN, and proliferation/differentiation of hMSC, particularly in osteogenesis.

5 | REGULATION OF CHONDROGENIC DIFFERENTIATION BY AS

SRY-related high-mobility group-box gene 9 (Sox9) is a master regulator of chondrogenesis.[76-79] Hata et al demonstrated that Sox9 exerts its molecular function as an activator of chondrogenic genes, at least in part, through interacting with an RBP called p54nrb in a specialized region of the nucleus called the paraspeckle, as originally described in a mouse study.[80] The paraspeckle is a ribonucleoprotein body located in the interchromatin region and has been hypothesized to function as the retention center for a specific set of mRNAs as a controlling mechanism for gene expression.[81] A recent report on a potential function of Sox9 in directly regulating alternative splicing raises an intriguing possibility that Sox9 may be a protein with dual functions—transcriptional and splicing regulation. Sox9 KD in colon tumor cancer cells induced a global change in AS without affecting their expression levels.[15,82] Girardot et al identified several mutants in the HMG domain, for example, W143R, Del400, and Del440, that lost transcriptional activity but not splicing.[15] This indicates that Sox9 may play a dual role in transcription and splicing, and that Sox9 can regulate AS independently of its transcriptional activity. This idea that Sox9 is a splicing regulator, as well as a transcriptional factor, was validated by the fact that Sox9 affects the splicing of *Col2A1*, a key chondrogenic gene, via physical interaction with p54nrb in paraspeckles.[80]

Another regulatory mechanism of chondrogenic differentiation via AS was described where a splicing variant of TATA binding protein-associate factor 4 (TAF4) repressed the proliferation of MSCs and promoted the chondrocyte differentiation of hMSCs.[63] *TAF4* pre-mRNA was shown to produce four *TAF4* mRNA isoforms, one of which produced an isoform lacking a TAFH domain (TAF4ΔTAFH), as a result of skipping two of the cassette exons (6 and 7). Kazantseva et al reported that the TAF4ΔTAFH expression level is directly correlated with the hMSC differentiation potential. Overexpression of the TAF4ΔTAFH isoform in hMSCs represses the proliferation of MSCs and preferentially promotes chondrogenic differentiation.[63] TAF4 is a subunit of the transcription factor IID (TFIID) complex of the transcriptional machinery, which typically controls a set of genes through the coordinated interaction of temporal- and spatial-specific transcriptional cofactors in response to external stimulation. Interestingly, Pijnappel et al recently demonstrated a higher expression level of several components of the TFIID complex in mouse ES cells compared with its fibroblast counterpart and that KD of these TFIID components resulted in the differentiation of mouse ES cells.[83] Thus, this raises the interesting hypothesis where the increased expression of splicing variant TAF4ΔTAFH could perturb the stoichiometry and activity of TFIID, thereby modulating MSC differentiation and, in particular, the chondrogenic pathway.

Parathyroid hormone-related peptide (PTHrP) is a paracrine peptide hormone that is essential for osteogenesis and chondrogenesis. PTHrP has also previously been implicated in chondrogenesis, as it is secreted by immature chondrocytes, and chondrogenesis was shown to be negatively affected in PTHrP knockout mice.[84] The biological activity of PTHrP is exerted by the proteolytic cleavage of the translated peptide to produce three shorter peptides: PTH-like (amino acids 1-34), middle region (amino acids 38-94), and osteostatin (amino acids 107-139). PTHrP protein isoforms are generated from three different mRNA isoforms via distinct 3′ terminal exons and a 3′-UTR through alternative 3′ splice site selection.[52] This results in three protein isoforms of 139, 141, and 173 amino acids with varying in the osteostatin domain, which have been implicated in differentially regulating chondrogenesis and hypertrophy in BM-MSCs.[51] Since PTHrP pre-mRNA AS encodes three distinct osteostatin domain peptides, one can speculate that PTHrP exerts an important regulatory role in chondrogenesis and hypertrophy by AS.

The alternative splicing of *PTHrP* pre-mRNA can modulate MSC differentiation in two ways. First, the three mRNA isoforms with three distinct 3′-UTRs are subjected to the variable half-life, as the 3′-UTR serves as the binding site for RBPs and miRNA. By modulating alternative 3′-splice site selection, one can speculate that mRNA with a differing 3′-UTR can determine the subcellular localization and/or stability of the mature mRNA, leading to a control mechanism for the PTHrP level. Second, it has been suggested that the three PTHrP isoforms with variable C-terminal ends are implicated in the differential paracrine activity by producing three structurally distinct osteostatin peptides.[52]

6 | REGULATION OF ADIPOGENIC DIFFERENTIATION BY AS

One genome-wide study examined the diversity of 3′-UTR structures of nascent transcripts bound to polysomes during adipogenesis.[85] The study not only found massive changes in the lengthening or shortening of the 3′-UTR after initiation of adipogenesis but also found a significant correlation between 3′-UTR length and the expression levels of the DEGs during adipogenesis.[85] The notion that there is a direct association between changes in gene expression and an increase in 3′-UTR diversity and length via alternative polyadenylation and splicing has been previously demonstrated in mouse embryogenesis.[86] The differentially expressed components of the polyadenylation machinery and pre-mRNA splicing are mainly responsible for generating different 3′-UTRs, as described above, leading to control of the expression levels of key regulators of adipogenesis through differential subcellular localization of mRNAs and translational repression.

The process of adipogenesis is activated by two key transcription factors: peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα).[87] PPARγ is a nuclear hormone receptor protein that binds to retinoic acid receptor alpha (RXRα) and activates downstream target genes associated with regulating fatty acid uptake and synthesis, thereby promoting adipogenesis.[87] Recently, a naturally occurring dominant negative isoform of PPARγ has been discovered to regulate PPARγ activity. Skipping cassette exon 5 generates a shorter isoform, PPARγΔ5, which is devoid of the entire ligand-binding domain, which may compete with WT PPARγ by interacting with an unidentified coreceptor, thereby reducing the adipogenic differentiation of hMSCs.[12] Interestingly, the PPARγΔ5/PPARγ ratio in human fat tissues was determined to be positively correlated to the BMI index, suggesting that adipose tissue dysfunction may be associated with the level of PPARγΔ5 isoform. The inclusion of exon 5 appears to be regulated by SRSF1 (SR domain-containing splicing regulator), also known as ASF/SF, which directly binds to *PPARγ* pre-mRNA and promotes the exon's inclusion.[12] This represents a typical negative feedback loop in which the activation of adipogenic differentiation by *PPARγ* expression, in response to external signaling cues, may be controlled by a counteractive balancing of the PPARγΔ5 expression via AS. Subsequently, an increased level of PPARγ activation results in the reduction of SRSF1-mediated inclusion of exon 5, thereby producing more of PPARγΔ5 and negatively regulating the activity of WT PPARγ.

LPIN1 was initially discovered as a protein associated with diseases of adipose tissue degeneration and was shown to be expressed predominantly in adipose tissues.[54] Peterfy et al demonstrated that *LPIN1* pre-mRNA undergoes alternative splicing to produce two isoforms, LPIN1α and LPIN1β, and observed that the isoform switch is accomplished during adipocyte differentiation from pre-adipocytes to mature adipocytes. Although the exact mechanism by which LPIN1 isoforms control adipocyte differentiation has not been completely elucidated, the differential subcellular localization patterns of the two isoforms may be involved in regulating adipocyte differentiation.[54]

Src-associated substrate during mitosis of 68 kD (Sam68) is an RBP that modulates alternative splicing by binding to its consensus binding site, the U(U/A)AA motif. Sam68 has been shown to regulate the alternative splicing of various pre-mRNA substrates, such as *CD44*, *Bcl-x*, *Survival of Motor Neuron* (*SMN*), *Srsf1*, and *Neurexin 1* (*Nrxn1*).[88-92] Sam68-dependent *mTOR* pre-mRNA splicing was shown to be correlated with adipogenesis and metabolism. Sam68 binds to the intron flanking exon 5 and 6 of *mTOR* pre-mRNA and recruits spliceosome to promote the splicing of the intron. Upon depletion of Sam68, the intron is retained and introduces a premature termination codon, resulting in the degradation of the *mTOR* mRNA and, subsequently, lower protein levels. Lower mTOR signaling, as a result, reduces the adipogenic differentiation. Song et al demonstrated that Sam68 expression in pre-adipocytes (3T3-L1 cells) suppresses the skipping of three cassette exons, exon 6a, b, and c, within *ribosomal S6 kinase* (*S6K1*) pre-mRNA, thereby inhibiting the expression of the dominant negative form, known as p31S6K1. The authors further demonstrated that the ectopic expression of p31S6K1 causes the abrogation of adipogenesis by disrupting the mTOR pathway.[59]

7 | CONCLUSION AND PERSPECTIVES

The biological functions of MSCs have many clinical implications, and it is imperative to understand their true cellular and molecular characteristics. In this review, we have summarized and emphasized the critical role of AS of key transcription factors and growth factors in MSC differentiation. The alternative splicing of key transcriptional regulators such as RUNX2, PPARγ, and PTHrP during MSC differentiation into osteocytes, adipocytes, and chondrocytes plays an underappreciated role in modulating the proliferation and cell fate determination of MSCs. An expression of a specific isoform generated by AS contributes to determining the cell type specification by activating isoform-specific transcriptional targets or antagonizing the function of the WT protein. Furthermore, AS of many pre-mRNAs, which encode the critical determinants of MSC differentiation, ensures the MSC differentiation by fine-tuning the balance of isoform expressions, controlling the subcellular localization, and other intermolecular interactions such as RNA-protein and protein-protein interactions. AS of key transcription factors and growth factors is regulated by the differential expression of various RBPs (regulated both temporally and spatially) and modulates the binding of the spliceosome to the target splice site. One interesting regulatory mechanism that has been revealed in several studies suggests that key transcription factors regulate AS by directly binding to RNA and further recruiting splicing regulators to cis-acting elements. One potential of most work reported thus far in the role of AS in MSC differentiation are artifacts in the in vitro cultures, especially associated with origin, development, and endogenous functions of MSCs. Future studies with a focus on elucidating more detailed mechanisms and exact molecular functions in vivo will help to enhance the understanding of MSC biology and strengthen the therapeutic benefits of MSCs in the future.

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CONFLICT OF INTEREST

Ren-He Xu declared Intellectual property rights and consultancy role with ImStem Biotechnology, Inc. All of the other authors declared no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Jung Woo Park, Siyi Fu, Borong Huang, Ren-He Xu: wrote the manuscript; Jung Woo Park, Ren-He Xu: approved the manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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