Regulation of Alternative Splicing during Epithelial-Mesenchymal Transition

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Abstract

Alternative splicing is an essential mechanism of gene regulation, giving rise to remarkable protein diversity in higher eukaryotes. Epithelial-mesenchymal transition (EMT) is a developmental process that plays an essential role in metazoan embryogenesis. Recent studies have revealed that alternative splicing serves as a fundamental layer of regulation that governs cells to undergo EMT. In this review, we summarize recent findings on the functional impact of alternative splicing in EMT and EMT-associated activities. We then discuss the regulatory mechanisms that control alternative splicing changes during EMT.

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Introduction

Epithelial-mesenchymal transition (EMT) is a conserved developmental process critical for metazoan embryogenesis [Thiery, 2003; Thiery et al., 2009]. First described in a chick primitive streak formation model, EMT

is a process where epithelial cells acquire mesenchymal characteristics such as increased motility and invasive capacity [Nieto, 2013]. During embryogenesis, EMT is an essential step for epithelial cell migration to various future organ locations [Kalluri and Weinberg, 2009]. At the secondary sites, these cells undergo mesenchymal-epithelial transition (MET), the reverse process of EMT, to differentiate and establish future organs [Acloque et al., 2009]. EMT and MET are two fundamental processes that provide cells with the flexibility to switch cell states according to developmental needs, and they are tightly regulated to ensure proper tissue and organ formation.

EMT is activated primarily by extensive crosstalk between extracellular stimuli, EMT-inducing signaling pathways, and transcription factors [Polyak and Weinberg, 2009; Nieto et al., 2016]. During EMT, epithelial cells lose their characteristic apical-basal polarity and intercellular junctions, detaching cells from the basement membrane. Their cobblestone-like morphology transitions towards a spindle-like, elongated morphology while acquiring increased migration and invasion capacity [Pérez-Pomares and Muñoz-Chápuli, 2002; Thiery and Sleeman, 2006; Acloque et al., 2009]. Mesenchymal cells can disintegrate the extracellular matrix and invade surrounding tissues, and acquire elevated resistance against apoptosis [Radisky et al., 2005; Morel et al., 2012; Nieto,

2013; Lamouille et al., 2014; Brabletz et al., 2018]. During development, cycles of EMT and MET are especially important for gastrulation, neural crest cell emigration, and proper formation of the embryonic heart [Nakajima et al., 2000; Acloque et al., 2009; Kim et al., 2017]. In adult tissues, normal EMT is mainly associated with wound healing and tissue regeneration, whereas pathological dysregulation of EMT can lead to organ fibrosis, carcinogenesis, and metastasis [Kalluri and Weinberg, 2009; Dongre and Weinberg, 2019]. Classification of EMT into three categories is widely used: Type I EMT refers to EMT during embryonic development, Type II EMT is involved in myofibroblast production and tissue fibrosis, and Type III EMT is related to cancer [Kalluri and Weinberg, 2009]. In this review, we will focus on Type III cancer-related EMT and one of its regulators, alternative splicing, which is especially important for cellular plasticity.

EMT plays a critical role in various key aspects of tumorigenesis [Tsai and Yang, 2013; Brabletz et al., 2018; Pastushenko and Blanpain, 2019]. In tumors, the EMT process elevates chemoresistance and promotes resistance to apoptosis [Chaffer et al., 2016], increasing likelihood of recurrence, which poses significant obstacles to treatment. EMT also increases the migration and invasion capacity of carcinogenic epithelial cells to form the invasive front, placing EMT as one of the major contributors to metastasis. It affects various aspects of the metastatic cascade, including invasion, migration, intravasation/extravasation, dissemination, and angiogenesis [Chaffer and Weinberg, 2011; Yeung and Yang, 2017]. Although the importance of EMT during metastasis is widely acknowledged, its direct contribution has been a topic of intense debate. Recent studies have proposed a theory that EMT is not required for metastasis in breast and pancreatic cancer models [Fischer et al., 2015; Zheng et al., 2015]. However, limitations in experimental design still cannot rule out the determining role of EMT during the metastatic cascade [Aiello et al., 2017; Nieto, 2017; Ye et al., 2017].

As tumors are not homogeneous, the ability of cells to shift towards a more mesenchymal state, where it can resist radiation and apoptosis, but also transition back towards a rapidly dividing epithelial state is critical to establishing a metastatic tumor [Chaffer et al., 2007]. Stable mesenchymal cells have been shown to be ineffective metastatic progenitors [Chao et al., 2010; Lecharpentier et al., 2011]. Circulating tumor cells (CTCs) displaying both epithelial and mesenchymal characteristics are found in the bloodstream, and more mesenchymal CTCs are found in patients who responded poorly to chemo-

therapy [Aktas et al., 2009; Kasimir-Bauer et al., 2012; Nieto, 2013; Yu et al., 2013; Aceto et al., 2014]. These observations suggest that the ability to undergo EMT and MET in cancer cells provides maximum plasticity for effective dissemination, metastatic colonization, and therapeutic resistance.

EMT is a complex biological process orchestrated by multiple layers of regulation. Extensive research in the EMT field has focused on transcription factors such as TWIST1, SNAI1/2, and ZEB1/2, signaling pathways such as TGF- β , Wnt/ β -catenin, and Notch, as well as cytokines and miRNA regulation [Peinado et al., 2007; Lamouille et al., 2014]. In addition to these well-studied master regulators of EMT, recent research has also highlighted other mechanisms of gene regulation, such as epigenetics, post-transcriptional RNA processing, and post-translational modifications, which are essential for fine-tuning EMT [Lee and Kong, 2016; Pradella et al., 2017]. Alternative splicing is one of the key post-transcriptional RNA processes that regulates crucial activities involving EMT such as signaling, invasion, and migration [Biamonti et al., 2012; Baralle and Giudice, 2017; Pradella et al., 2017; Aiello and Kang, 2019]. This review will focus on the essential role of alternative splicing and how it is regulated during EMT.

Alternative Splicing and EMT

Alternative splicing is a critical component of gene regulation and represents a major contributor to mRNA complexity and proteome diversity, emerging as one of the key regulators of tissue identity and cell state. Pre-mRNA is processed into mature mRNA by splicing out introns and properly joining together exons. This essential step is carried out by the spliceosome, which is a protein complex composed of small nuclear ribonucleoproteins (snRNPs) along with other protein and RNA components [Wahl et al., 2009; Shi, 2017]. It is estimated that up to 95% of human genes undergo alternative splicing, where specific exons are skipped or included to produce different mRNA products [Pan et al., 2008; Wang et al., 2008]. Common modes of alternative splicing include cassette exon (also called exon skipping), mutually exclusive exons, using alternative 3' splice sites or 5' splice sites, and intron retention (Fig. 1a) [Wang et al., 2008; Liu and Cheng, 2013]. Less common modes of alternative splicing such as circRNA splicing also exist and can produce unique transcripts with functional significance (Fig. 1a) [Jeck and Sharpless, 2014]. Exon skipping is the most

Fig. 1. Alternative splicing is a fundamental regulator of EMT. **a** Schematic depiction of the common modes of alternative splicing. Exons are depicted as rectangles and introns as lines. **b** Alternative splicing changes during EMT give rise to various EMT-associated phenotypes. The blue to yellow gradient represents the transition from the epithelial state to mesenchymal state. Alternatively spliced isoforms during EMT are depicted and they contribute to many aspects of EMT-associated phenotypes such as EMT-associated signaling, cytoskeletal remodeling, loss of cell-cell junctions, invasion, CSCs, and metastasis.

prevalent mode of alternative splicing, accounting for about 40% of alternative splicing events [Keren et al., 2010].

Alternative splicing produces functionally diverse transcripts. By including or excluding exons encoding functional domains, alternative splicing can alter enzymatic activity, change binding affinity for other proteins and form diverse protein complexes [Romero et al., 2006; Baralle and Giudice, 2017]. Similarly, alternative splicing can change subcellular localization and protein solubility [Kelemen et al., 2013]. Many alternative exons are divisible by three, maintaining the open reading frame with insertion or deletion of a segment of peptides. However, alternative exons that are not divisible by three can alter the open reading frame to produce completely different proteins from the same gene locus [Magen and Ast, 2005; Xing and Lee, 2005]. They can also trigger nonsense-mediated decay through the generation of a premature stop

codon, resulting in reduction of mRNA expression. Alternative splicing of terminal exons may produce mRNA transcripts with distinct 3'UTRs, thus affecting gene expression by including different miRNA binding sites [Sandberg et al., 2008]. Some RNA transcripts generated through alternative splicing are nonprotein coding but have regulatory roles, such as long noncoding RNAs (lncRNA) and circular RNAs (circRNA) [Kung et al., 2013; Barrett and Salzman, 2016; Marchese et al., 2017; Kristensen et al., 2019].

Well-coordinated regulation of alternative splicing ensures normal cellular function, while aberrant regulation of alternative splicing is increasingly recognized as a driver of disease. Misregulation of alternative splicing is prominent in many aspects of the hallmarks of cancer [Liu and Cheng, 2013], one of which is EMT. As a matter of fact, alternative splicing of FGFR2 during EMT was considered as one of the first generation of EMT markers

[Orr-Urtreger et al., 1993; Savagner et al., 1994]. In recent years, various splicing events have been shown to be critical during EMT. Here we will summarize the functional importance of alternative splicing that impact the EMT process. We will then discuss the mechanisms of alternative splicing regulation during EMT.

Functional Importance of Alternative Splicing Changes during EMT

The EMT process is tightly regulated by orchestrating signaling cascades, resulting in drastic changes in cellular morphology and how cells interact with the surrounding extracellular matrix. Alternative splicing events during EMT play an indispensable role, as splicing changes are found in genes involved in EMT-associated signaling, cytoskeletal remodeling, invasiveness, tumor-initiating capacity, and metastasis (Fig. 1b). Most of these alternatively spliced genes do not change significantly at gene expression levels, indicating that these genes are more functionally relevant to EMT at the post-transcriptional regulation level. These results demonstrate that alternative splicing regulation is an important process of gene regulation distinct from gene expression.

EMT-Associated Signaling

Several genes encoding surface receptors and kinases undergo alternative splicing during EMT. The signaling function of EMT-associated splice isoforms is altered through protein structure changes, accelerating EMT. Early research found the fibroblast growth factor receptor 2 (FGFR2) to be alternatively spliced during EMT. FGFR2 has 2 mutually exclusive exons IIIb and IIIc. In epithelial cells, FGFR2-IIIb is the predominant isoform, and in mesenchymal cells, FGFR2-IIIc is majorly expressed, changing the structure of an immunoglobulin-like domain to alter binding affinity to FGF (Fig. 2a) [Orr-Urtreger et al., 1993; Savagner et al., 1994; Yeh et al., 2003; Zhang et al., 2006]. This splicing event, along with the splicing of another FGF receptor, FGFR1, are further observed in breast cancer and clear cell renal carcinoma [Warzecha et al., 2009; Zhao et al., 2013; Hopkins et al., 2017]. Functionally, FGFR splice isoforms differ in ligand binding preferences. The mesenchymal isoforms have stronger affinity for FGF, enhancing FGFR downstream signaling to increase cancer cell invasiveness [Zhang et al., 2006; Shirakihara et al., 2011; Hopkins et al., 2017].

CD44 is a transmembrane glycoprotein that takes part in multiple signaling cascades [Ponta et al., 2003]. The

CD44 gene undergoes extensive alternative splicing, changing the length of the extracellular stem region. Human CD44 encodes nine variable exons flanked by a total of nine constitutive exons. Alternative splicing generates two major families of CD44 splice isoforms. The CD44 variant isoforms (CD44v) are a collection of isoforms that contain at least one of the variable exons, whereas the CD44 standard isoform (CD44s) is devoid of all variable exons. CD44v and CD44s both contain the same ligand-binding domain for hyaluronan, but CD44s shows higher affinity [Aruffo et al., 1990; Culty et al., 1990; Miyake, 1990]. Aside from binding canonical ligands, CD44v contains additional binding sites. For example, CD44v3 contains heparin-binding domains that bind to HB-EGF [Bennett et al., 1995]. Different isoforms of CD44 are known to activate distinct pathways important for respective cell states. CD44v6, one of the epithelial dominant isoforms of CD44, helps sustain a Ras/MAPK feedback loop that promotes cell proliferation [Matter et al., 2002; Cheng, 2006]. The mesenchymal dominant isoform, CD44s, sustains PI3K/Akt signaling and activates PDGFR β /Stat3 pathway to enhance cancer stem cell (CSC) characteristics (Fig. 2b) [Liu and Cheng, 2017; Zhang et al., 2019a].

Ron is a receptor tyrosine kinase that contains important docking sites for many downstream signaling adaptors. When phosphorylated, Ron promotes cell growth, controls cell motility, and supports the invasion of extracellular matrices [Camp et al., 2005]. The variable exon 11 of Ron encodes 49 amino acids in the extracellular domain of the β -subunit (Fig. 2c). Skipping of exon 11 produces the Δ Ron isoform that is constitutively active even in the absence of its ligand MSP [Ghigna et al., 2005]. Δ Ron is upregulated in tumors and during EMT. Overexpression of Δ Ron promotes cell motility and induces a mesenchymal morphology [Zhou et al., 2003; Ghigna et al., 2005].

Rac1 is a member of the Rho GTPase family important for regulation of actin cytoskeleton organization. Rac1 contains a variable exon 3b, inclusion of which leads to a 19-amino acid insertion in the GDP/GTP binding region [Schnelzer et al., 2000; Singh et al., 2004]. This mesenchymal isoform, Rac1b, exists predominantly in the GTP-bound active conformation and stimulates the NF- κ B pathway, promoting cell cycle progression and enhancing anti-apoptotic response. Aberrant splicing of Rac1b stimulates mitochondrial production of ROS, which promotes EMT through inducing SNAIL1 expression (Fig. 2d) [Matos and Jordan, 2005; Radisky et al., 2005; Gonçalves et al., 2009]. These examples demonstrate that alternative

Fig. 2. Critical alternative splicing events during EMT. **a** Schematic of FGFR2 alternative splicing during EMT. Isoform switching from the epithelial FGFR2-IIIb to the mesenchymal FGFR2-IIIc changes the structure of an immunoglobulin-like domain to enhance the binding affinity to FGF. **b** Schematic of CD44 alternative splicing during EMT. Epithelial isoform CD44v has a longer extracellular stem region than mesenchymal isoform CD44s. The two isoforms activate the Ras/MAPK pathway and PI3K/Akt, PDGFR β /Stat3 pathways, respectively. **c** Schematic of Ron alternative splicing during EMT. The mesenchymal isoform Δ Ron lacks a 49-amino acid region in the extracellular domain of the β -subunit and is constitutively active. **d** Schematic of Rac1 alternative splicing during EMT. The mesenchymal Rac1b isoform is in the GTP-bound active conformation, generating ROS and induces SNAIL expression. **e** Schematic of p120-catenin (CTNND1) splicing during EMT. Mesenchymal isoform 1 is the longest isoform that contains a coiled-coil domain and a regulatory domain, inhibiting RhoA activity and promoting Rac1 activity. The epithelial isoform 4 lacks both domains and promotes RhoA activity.

splicing during EMT produces isoforms that sustain EMT signaling and subsequently causes morphological and functional changes associated with mesenchymal cells.

Cytoskeleton Remodeling, Cell Junctions, and Invasiveness

During EMT, cells undergo extensive cytoskeletal remodeling to disrupt cell-cell junctions, change cellular morphology, and enhance invadopodia formation. Multiple splicing events have been shown to directly affect cell-cell junctions and the invasive front.

p120-catenin (CTNND1), a crucial regulator of adherens junctions, is alternatively spliced during EMT. The full-length isoform 1, containing a coiled-coil domain and a regulatory domain, is overexpressed in mesenchymal cells and tumors, promoting invasiveness through the inhibition of RhoA GTPase activity. In contrast, p120 isoforms 3 and 4 which lack the coiled-coil and regulatory domains, are dominantly expressed in the epithelial cell state, blocking invasiveness by increasing binding to and stabilization of E-cadherin at adherens junctions (Fig. 2e) [Yanagisawa et al., 2008; Ishiyama et al., 2010; Slorach et al., 2011; Faux et al., 2021]. TJP1 (ZO-1), a junction protein located at the cytoplasmic membrane surface of tight junctions, is also regulated by alternative splicing in a similar manner. By excluding its variable exon 20, TJP1 loses its activity at tight junctions but enhances actin stress fiber assembly, increasing migration [Kim et al., 2019].

Alternative splicing also regulates genes involved in actin regulation and invadopodia formation. MENA (ENAH) is an actin regulatory protein that antagonizes actin capping proteins [Gertler et al., 1996; Barzik et al., 2005; Applewhite et al., 2007]. hMENA Δ v6 is expressed in invasive cancer cells while the epithelial isoform, hMENA^{11a}, is expressed in premalignant cells and facilitates F-actin organization. The two isoforms have antagonistic functions. The hMENA Δ v6 mesenchymal isoform shows increased invasive ability while the hMENA^{11a} epithelial isoform reduces migration and invasion by decreasing the amount of filopodia [Modugno et al., 2012]. Other examples of alternative splicing involved in cytoskeletal rearrangement include Exo70-M that regulates actin branching, CD44s isoform that is required for the formation of invadopodia, and Rac1b isoform that serves as an important regulator of the cytoskeleton [Lu et al., 2013; Ishii et al., 2014; Zhao et al., 2016]. In summary, alternative splicing of both cytoskeletal modulators and junction proteins are crucial to the regulation of cell-cell and cell-matrix interactions during EMT.

Tumor-Initiating Capacity

CSCs, also called tumor-initiating cells, are recognized as cells that are highly resistant to treatment and have strong tumor-initiating capacity. CD44 is a widely used CSC marker in various types of cancers. Previous work has illustrated the causal role of CD44 isoform switching in EMT and metastasis [Brown et al., 2011; Mima et al., 2012; Roca et al., 2013; Preca et al., 2015; Park et al., 2016; Tripathi et al., 2016]. Both CD44s and CD44v are shown to promote tumor progression in a tissue-specific manner, playing distinct roles. Mesenchymal-dominant CD44s promotes triple-negative breast cancer cell survival and metastasis [Ouhtit et al., 2007; Brown et al., 2011; Mima et al., 2012; Hiraga et al., 2013; Xu et al., 2014; Zhao et al., 2016; Zhang et al., 2019a], whereas epithelial-dominant CD44v stimulates tumor cell proliferation and promotes gastric cancer progression [Cheng, 2006; Yoshikawa et al., 2013; Lau et al., 2014; Todaro et al., 2014; Zeilstra et al., 2014]. Switching isoform expression between CD44s and CD44v determines breast CSC and non-CSC cell states [Zhang et al., 2019a]. CD44s can activate the PDGFR β /Stat3 pathway and ZEB1 transcription factor to promote mammosphere formation ability [Wellner et al., 2009; Preca et al., 2015; Zhang et al., 2019a]. This is also found in ovarian cancer models where CD44s expression increases aldehyde dehydrogenase activity, tumor spheroid formation, and chemoresistance [Bhattacharya et al., 2018]. CD44s is more potent at initiating tumor formation and promoting breast tumor recurrence than CD44v in mice, and CD44s is positively correlated with a mammary stem cell signature in TCGA [Brown et al., 2011; Zhang et al. 2019a].

Other alternative splicing events involved in CSC characteristics include the splicing of the surface molecule α 6 integrin. Within a CD44^{high}/CD24^{low} CSC population, the epithelial-like cells express α 6A, while the mesenchymal-like cells express α 6B, and the two isoforms differ in their cytoplasmic domains. The α 6B splice isoform promotes CSC characteristics and enhances tumor initiation potential through sustaining VEGF signaling [Goel et al., 2014]. It will be interesting to investigate whether there are additional alternative splicing events that are crucial to maintaining the CD44^{high}/CD24^{low} CSC population and its tumor initiation capacity.

Noncoding RNAs Produced from Alternative Splicing

Apart from producing protein-coding RNA variants, alternative splicing also regulates noncoding RNAs. This includes lncRNAs and circRNAs that are functionally important for EMT regulation, such as MALAT1, PNUTS,

Fig. 3. Regulation of alternative splicing by splicing enhancers and silencers and RBPs. Exonic splicing enhancers and silencers (ESE, ESS) and intronic splicing enhancers and silencers (ISE, ISS) are shown in the alternative exon and downstream intron flanking the alternative exon. These short sequences are bound by RBPs which either function as a splicing repressor or activator. Binding of these RBPs directly act upon the U1 and U2 snRNP, two core components of the spliceosome, to dictate the splicing outcome.

and CiRS-7 [Conn et al., 2015; Dhamija and Diederichs, 2016; Grelet et al., 2017; Gugnoni and Ciarrocchi, 2019]. As an example, alternative splicing of the PNUTS gene generates a PNUTS mRNA and an lncRNA-PNUTS by using different splice acceptor sites of exon 12. The lncRNA-PNUTS is upregulated during EMT and correlates with ZEB1/ZEB2 mesenchymal marker expression. Functionally, the lncRNA-PNUTS antagonizes the epithelial regulator miR-205, serving as a miRNA sponge to promote EMT. When overexpressed, lncRNA-PNUTS promotes tumor initiation and metastasis [Grelet et al., 2017].

circRNAs are produced from a particular type of alternative splicing, where the splice donor of one exon is ligated to the splice acceptor site of an upstream exon (Fig. 1a) [Jeck and Sharpless, 2014; Conn et al., 2015]. RNA sequencing analysis has identified hundreds of circRNAs with an increased expression during EMT. Many of them are produced from genes involved in EMT, including SMARCA5, POLE2, SHPRH, and DOCK1. These results suggest that circRNA may play important roles in regulating EMT, an area warranting further investigation [Conn et al., 2015; Pillman et al., 2018].

In recent years, RNA-seq has been extensively involved in discovering global alternative splicing changes in processes including EMT. Numerous splicing events have been identified, and their encoding genes are commonly involved in actin cytoskeleton regulation, cell-cell junctions, migration, and wound healing [Shapiro et al., 2011; Vanharanta et al., 2014; Li et al., 2018; Pillman et al., 2018; Ahuja et al., 2020]. While at this stage only a handful of these splicing changes have been connected to functional properties, it will be important to investigate these many other alternative splicing events on their biological roles in EMT in the future.

Cell State-Specific Regulation of Alternative Splicing during EMT

In order to generate cell state-specific isoforms during EMT, alternative splicing needs to be tightly controlled by cellular machinery. The 5' and 3' splice sites located at intron-exon boundaries are essential for recognition by the spliceosome. Variations in the splice site sequences determine their strength. Weaker splice sites are less often recognized by the spliceosome, resulting in exon skipping, whereas stronger splice sites are more likely to be recognized by the spliceosome, leading to exon inclusion in the mature mRNA product. Regulation of the recognition of splice sites, especially the weaker ones, are modulated by *cis*-acting elements contained within the intron and exon sequences. These *cis*-acting elements include exonic splicing enhancers (ESEs), intronic splicing enhancers (ISE), exonic splicing silencers (ESSs), and intronic splicing silencers (ISSs) (Fig. 3) [Fu and Ares, 2014]. Other *cis*-acting elements, including secondary and tertiary RNA structures, are also determinants of alternative splicing [Huang et al., 2017]. These sequence and structural elements can be recognized and bound by *trans*-acting elements, which are mainly RNA-binding proteins (RBPs). Through binding to the *cis*-acting enhancer or silencer sequences, these RBPs either enhance or reduce recruitment of the spliceosome and thus promote exon inclusion or skipping, respectively (Fig. 3). Importantly, RBPs are dynamically regulated during EMT to control splicing events for enhanced oncogenic signaling, cytoskeletal remodeling, and increased cell death resistance [Valacca et al., 2010; Shirakihara et al., 2011; Horiguchi et al., 2012; Reinke et al., 2012; Venables et al., 2013; Hopkins et al., 2017; Yang and Carstens, 2017; Pillman et al., 2018; Gordon et al., 2019].

Regulation of Epithelial Splicing Patterns

The epithelial splicing regulatory proteins 1/2 (ESRP1/2) are the first RBPs identified to specifically regulate epithelial alternative splicing [Warzecha et al., 2009]. The ESRPs are by far the most well-characterized epithelial-type specific master regulators of alternative splicing and epithelial cell state. Loss of the ESRPs is an essential event during EMT while re-expression of the ESRPs in the mesenchymal state can partially restore an epithelial splicing program along with epithelial marker expression. As EMT occurs during early stages of embryogenesis, ESRP1 knockout mice were neonatally lethal due to cleft lip and cleft palate [Beebe et al., 2015; Lee et al., 2020]. Similar palate development defects were also observed in ESRP1/2 knockout zebrafish, demonstrating the conserved function of ESRPs in vertebrate development [Carroll et al., 2020]. ESRP1 and ESRP2 double knockout embryos fail to form lung and salivary glands, indicating that the ESRPs are essential for branching morphogenesis. Moreover, these double knockout mice exhibit epidermal hypoplasia and hair follicle developmental defects [Beebe et al., 2015]. These phenotypes are consistent with the phenotype of FGFR2-IIIb knockout mouse, which causes developmental defects in the lung, thymus, salivary gland and kidney [Moerlooze et al., 2000]. ESRP1 knockout also demonstrates tissue-specific defects in mice. ESRP1 knockout disrupts cochlear development, resulting in hearing loss, while germline knockout of ESRP1 in female mice caused infertility. Both phenotypes are caused by aberrant splicing of FGFR2 after ESRP1 loss [Rohacek et al., 2017; Yu et al., 2021]. These results indicate that ESRPs play an indispensable role in embryogenesis through regulating downstream alternative splicing events such as FGFR2 during developmental EMT.

ESRP1 binds a consensus GU-rich motif generally located in the proximal introns of a variable exon [Warzecha et al., 2010]. It regulates a large cohort of splicing events found to be critical during EMT, such as FGFR2, CTNND1, CD44, ENAH, Exo70, α 6-integrin, and ARHGEF11 [Warzecha et al., 2009; Brown et al., 2011; Modugno et al., 2012; Lu et al., 2013; Goel et al., 2014; Lee et al., 2018; Faux et al., 2021]. When ESRP1 is depleted, epithelial cells undergo accelerated transition to the mesenchymal state in response to EMT stimuli [Brown et al., 2011]. The ESRP1 promoter contains E-boxes, which recruits the SNAI1 transcription factor, resulting in transcriptional suppression of ESRP1 during EMT, further affecting a large cohort of downstream splicing events [Reinke et al., 2012].

Other RBPs regulating the epithelial cell state have been identified. Loss of RBM47 during EMT enhances breast cancer metastasis [Vanharanta et al., 2014]. This effect is mediated through both regulation of alternative splicing and mRNA stability [Vanharanta et al., 2014; Yang et al., 2016]. RBM47 depletion during EMT increases TJP1- α production to increase invasion [Kim et al., 2019]. AKAP8 is another recently discovered epithelial RBP that suppresses breast cancer metastasis by antagonizing mesenchymal splicing of CD44 and CLSTN1 [Hu et al., 2020]. Specific RNA secondary structures can also act as important regulators of alternative splicing. One example is the RNA G-quadruplex, which contains two or more planar structures formed by Hoogsteen hydrogen bonding of four guanines. Recent work has shown that the CD44 intron contains RNA G-quadruplexes, which facilitate the recruitment of hnRNPF. hnRNPF promotes exon inclusion in G-quadruplex-containing introns, therefore inhibiting EMT by regulating inclusion of important EMT-related alternative splicing events such as CD44 [Huang et al., 2017]. hnRNPF regulation of splicing can be perturbed by disrupting the G-quadruplex structure [Zhang et al., 2019b]. While most of these epithelial RBPs are studied in the context of cancer, it will be interesting to investigate whether they play an equally important role in developmental EMT.

Regulation of Mesenchymal Splicing Patterns

hnRNPM, RBFOX1/2 and QKI are examples of RBPs that play an indispensable role in regulating mesenchymal splicing patterns. hnRNPM, a member of the hnRNP family, is often associated with splicing suppression [Han et al., 2010]. It was found to be a key regulator of mesenchymal splicing [Xu et al., 2014]. hnRNPM inhibits CD44 variable exon inclusion and promotes the production of CD44s mesenchymal isoform. By acting on CD44 alternative splicing, hnRNPM potentiates TGF- β signaling, accelerates EMT, and promotes breast cancer lung metastasis [Xu et al., 2014]. In addition to CD44, hnRNPM also promotes splice isoform switching of a large set of genes, including previously identified EMT-related splicing events EXOC7 and TCF7L2 [Weise et al., 2010; Lu et al., 2013; Harvey et al., 2018].

RBFOX factors alter the splicing direction by binding up or downstream of the alternative exon. Binding upstream tends to repress splicing and vice versa [Underwood et al., 2005; Zhang et al., 2008; Yeo et al., 2009]. RBFOX2 expression is induced by TGF- β in breast cancer cell lines, which promotes the mesenchymal splicing of TAK1 exon 12 to sustain multiple signaling pathways, in-

Fig. 4. Combinatorial regulation of EMT-associated alternative splicing by RBPs. **a** CD44 splice isoform switching during EMT is coregulated by several RBPs. ESRP1, hnRNPF, and AKAP8 promote the epithelial CD44v splicing pattern, while hnRNPM promotes the mesenchymal CD44s splicing pattern. **b** Epithelial and mesenchymal RBPs share overlapping alternative splicing targets. Cooperative or competitive regulation of RBPs on EMT-associated splicing events determine the splicing outcome, which further affects EMT cell states.

cluding JNK, p38/MAPK, and NF- κ B [Tripathi et al., 2019]. On a global scale, depleting RBFOX2 in mesenchymal cells can induce phenotypes similar to an epithelial state [Shapiro et al., 2011], whereas its overexpression promotes several mesenchymal-specific splicing patterns [Braeutigam et al., 2014]. The STAR family RBP Quaking (QKI), along with RBFOX1, a homolog of RBFOX2, are identified as critical regulators that induce a mesenchymal cell state in breast cancer cells [Li et al., 2018; Pillman et al., 2018]. QKI promotes a broad spectrum of EMT-associated alternative splicing events. Similar to the binding position dependency of RBFOX factors, QKI binding upstream of an alternative exon often promotes skipping, while binding downstream promotes exon inclusion [Pillman et al., 2018]. It will be interesting to investigate the function of these splicing events in EMT in more detail.

Combinatorial Regulation of Alternative Splicing

Multiple studies regarding alternative splicing regulation have created a blueprint for constructing networks consisting of alternative splicing events co-regulated by a

set of RBPs. It is increasingly recognized that a single splicing event is co-regulated by multiple RBPs in different cell states. Furthermore, a single RBP can regulate a cohort of downstream events that are interconnected with other RBP regulation networks. For instance, although the ESRPs are strong epithelial regulators, other RBPs have shown the ability to regulate splicing events in both cell states. RBM47 regulates mostly epithelial splicing programs, but can antagonize some ESRP-regulated events [Yang et al., 2016]. RBFOX2 regulates a majority of mesenchymal splicing events, but can enhance some epithelial splicing patterns [Braeutigam et al., 2014; Venables et al., 2013; Lapuk et al., 2010; Dittmar et al., 2012]. Thus, it is important to understand how RBPs are tightly regulated in a tissue and cell state-specific manner, and how the expression of epithelial-specific and mesenchymal-specific RBPs act antagonistically on the same splicing event to determine splicing outcome.

An early example of coregulation of the same event by multiple RBPs during EMT was demonstrated by the event of Ron splicing. SRSF1 promotes the production of mesenchymal Δ Ron, while hnRNP A1 inhibits its pro-

duction [Bonomi et al., 2013]. The ratio of ESRP1 and RBFOX2 expression dictates the outcome of hMENAX exon 11a splicing in breast cancer [Ahuja et al., 2020]. In CD44 alternative splicing, inclusion of variable exons is promoted by ESRP1, hnRNPF, and AKAP8 in the epithelial state, while skipping of variable exons is enhanced by hnRNPM (Fig. 4a) [Brown et al., 2011; Xu et al., 2014; Huang et al., 2017; Hu et al., 2020]. In fact, ESRP1 and hnRNPM compete for binding at the same GU-rich motifs, coregulating a large cohort of splicing events antagonistically during EMT [Harvey et al., 2018].

Recently, through analysis of breast cancer specimens in BRCA-TCGA datasets, a combinatorial network of splicing regulation by RBPs was identified as a common mechanism in controlling EMT-associated alternative splicing events [Qiu et al., 2020]. In support of previous experimental findings, this study demonstrates that RBPs have strongly overlapping regulation networks, and that cooperative or competitive modes of RBP regulation on alternative splicing are essential for the splicing outcome during EMT (Fig. 4b).

Conclusions and Perspectives

EMT is a critical step during normal embryogenesis and wound healing, but dysregulation of EMT is common in many types of cancer and affects invasion, migration, survival, and metastasis. EMT is also critical for phenotypic heterogeneity in tumors as cells can transition between epithelial and mesenchymal states to obtain a balance between proliferation and survival. Alternative splicing, as one of the major contributors to proteome diversity, provides a mode of versatile gene regulation during EMT. Dysregulation of alternative splicing leads to disruption of the epithelial cell state, increasing metastasis and enhancing survival. EMT-associated alternative splicing are mostly found in genes involved in signaling, cytoskeletal remodeling, invasiveness, CSCs, and metastasis. RBPs are critical *trans*-acting regulators of alternative splicing that specifically regulates epithelial or mesenchymal splicing patterns. Balanced regulation of RBPs determines the outcome of global alternative splicing through an intricate interaction network between RBPs and alternative splicing events. Thus, the regulation of RBPs serves as an essential mechanism to control EMT and metastasis in cancer.

As EMT-related alternative splicing is an intricately regulated process that contributes to the heterogeneous nature of solid tumors, its regulators and resulting iso-

forms have become targets of interest for precision medicine. Although research in this area is still in its early stages, therapeutic agents against pathogenic isoforms in diseases such as spinal muscular atrophy have been tested and approved for patient care. An antisense oligo against SMN2 exon 7 became the first FDA-approved drug against a pathogenic isoform in spinal muscular atrophy [Hua et al., 2010]. Targeting the regulators and products of alternative splicing holds enormous promise for developing novel therapeutics.

Although hundreds of splicing changes have been identified during EMT, much is still unknown concerning the functional role underlying the switched expression of splice isoforms. A key future direction is to understand the function and regulation of EMT-associated alternative splicing isoforms. It would also be interesting to understand how alternative splicing cooperates with other post-transcriptional regulatory methods, such as RNA modifications and epigenetic regulation to ensure rapid and flexible gene regulation during EMT.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

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Author Contributions

J.L. and C.C. wrote the manuscript.

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