

CHAPTER 7

ALTERNATIVE SPLICING IN STEM CELL SELF-RENEWAL AND DIFFERENTIATION

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Abstract: This chapter provides a review of recent advances in understanding the importance of alternative pre-messenger RNA splicing in stem cell biology. The majority of transcribed pre-mRNAs undergo RNA splicing where introns are excised and exons are juxtaposed to form mature messenger RNA sequences. This regulated, selective removal of whole or portions of exons by alternative splicing provides avenues for control of RNA abundance and proteome diversity. We discuss several examples of key alternative splicing events in stem cell biology and provide an overview of recently developed microarray and sequencing technologies that enable systematic and genome-wide assessment of the extent of alternative splicing during stem cell differentiation.

INTRODUCTION

Stem cells are a unique resource for studying the bases of pluripotency, self-renewal and lineage specification. Embryonic stem cells remain undifferentiated in culture for long periods and are readily induced towards the three germ layers, differentiating in vitro into most if not all of the lineages that comprise a healthy organism. Thus, embryonic stem cells are a useful platform to study healthy and disease states in a multitude of lineages. Generation of cell populations enriched with a particular differentiated cell type and ongoing, detailed characterization of these cells before and after differentiation will continue to provide insight into the molecular basis of cell identity.

Gene expression studies have documented global transcriptional differences during the process of differentiation into several lineages,¹⁻⁴ but are limited in that most studies do not distinguish among alternatively spliced isoforms from the same gene locus. In

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this chapter, we will present several examples of alternatively spliced genes implicated in important stem cell processes and review recently available techniques that allow identification and quantitative measurement of alternative splicing events. Early work to identify splicing factors that influence alternative splicing will also be discussed.

INTRODUCTION TO ALTERNATIVE SPLICING

Transcription produces pre-messenger RNA (pre-mRNA) transcripts which are dominated by long, noncoding intronic sequences interspersed with short, 150 base exonic sequences. Intron removal, exon ligation and splice site selection is highly regulated as splicing errors can generate aberrant proteins or prevent translation of the mRNA. This process is mediated by a protein-RNA complex called the spliceosome whose stringent functional requirements are reflected in its complex makeup; it consists of five RNAs and hundreds of proteins.⁵⁻⁶ This machinery interacts with cis-regulatory elements encoded in pre-mRNAs and trans-acting regulators called splicing factors.

The interactions between cis-elements, splicing factors and the spliceosome define a set of rules called the “splicing code.” The components of this code determine which splice sites are chosen to generate different versions of mature mRNAs from the same pre-mRNA in the process called alternative splicing (Fig. 1). The splicing code is still largely a mystery, but its defining elements are rapidly being identified. Splicing factors include spliceosome particles, members of the serine-arginine (SR) protein family, heterogeneous nuclear ribonucleoproteins (hnRNPs) and auxiliary factors that are typically RNA binding proteins (RBPs).⁷⁻⁸ Recent studies indicate that the majority of human genes are alternatively spliced, greatly increasing the protein coding potential of the genome⁹⁻¹⁰ and genome-wide efforts to identify alternatively spliced genes in stem cells has begun.¹¹ The protein diversity generated by alternative splicing events is largely unexplored and provides an opportunity to better understand stem cell biology.

ALTERNATIVE SPLICING OF GENES IMPLICATED IN STEMNESS AND DIFFERENTIATION

Evidence of the influence of alternative splicing in stem cells is rapidly growing. Recent studies indicate that levels of some splice variants of stem cell-enriched genes (stemness genes) correlate with particular stages of differentiation. Some of the same studies have demonstrated disparate and reproducible phenotypes correlated with overexpression of splice variants of a stemness gene. These results hint at both the ability of splice variants from a single gene locus to differentially influence the phenotype and the importance of understanding posttranscriptional gene expression regulation afforded by alternative splicing. Here we present several examples of alternatively spliced genes important to stem cell biology.

The *POU5F1* gene is an example of a central stemness gene that is regulated by alternative splicing. This gene encodes a POU domain transcription factor, OCT4, which is a key transcriptional regulator of stem cell pluripotency. OCT4 is highly expressed in stem cells and expression of OCT4 appears to be essential for reprogramming differentiated cells to an induced pluripotent state.¹²⁻¹⁴ The utility of OCT4 as a stemness marker was questioned after it was detected in a few somatic cell types¹⁵⁻¹⁶ although finer discrimination

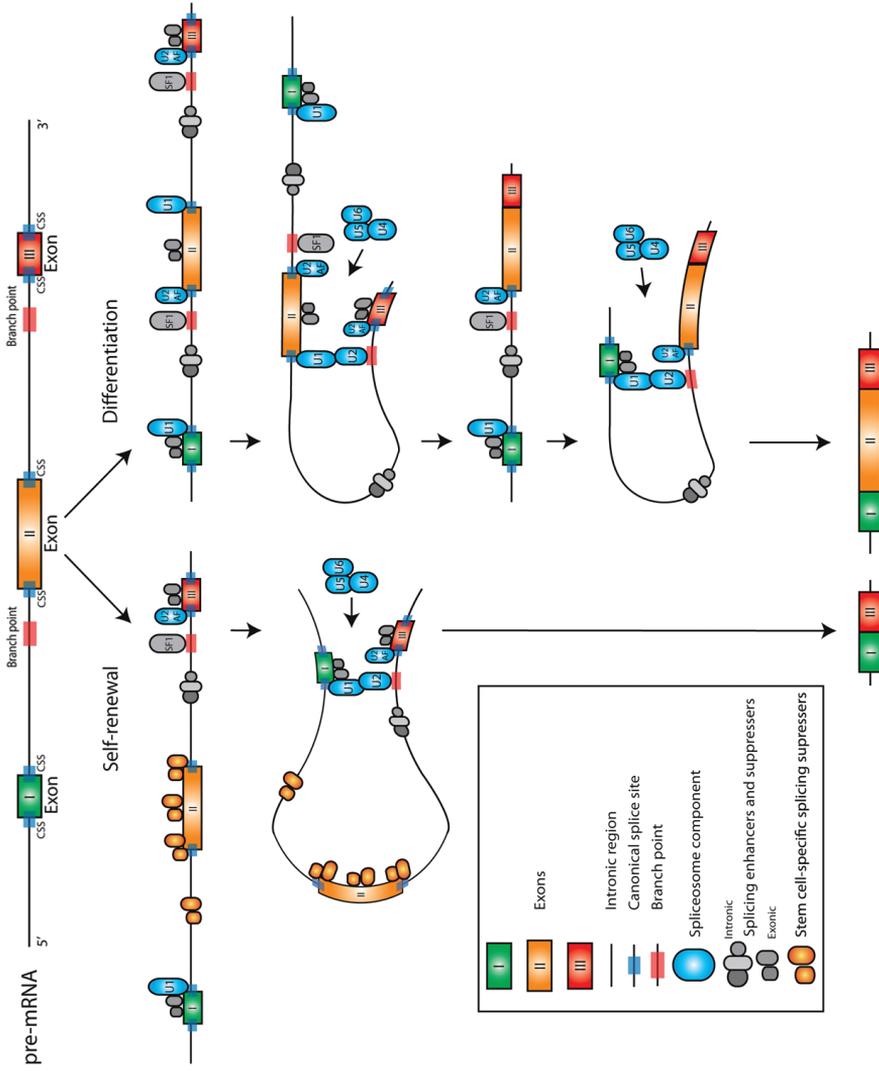


Figure 1. Legend viewed on following page.

Figure 1. Figure viewed on previous page. This diagram outlines the role of splicing factors during RNA splicing in pluripotent stem cells and differentiated cells. On the left, stem cell-specific splicing suppressors block assembly of the spliceosome near exon II and result in excision of exon II along with its flanking introns. On the right, differentiating stem cells that lack the splicing suppressor result in mRNA that includes all three exons. Splicing suppressors prevent splicing by inhibiting assembly of the spliceosome or by other mechanisms. In both cases, the core spliceosome small nuclear RNA proteins are pictured (U1, U2, U4, U5, U6) and their assembly results in catalyzed removal of a pre-mRNA region. The 5' exon end is marked by U1 while the 3' exon end is decorated with U2 auxiliary factors (U2AF). As the spliceosome assembles, the SF1-marked branch point recruits U2 and associates with U1 forming a loop. Next, U4, U5 and U6 are recruited and the intron bound to the 5' exon is cleaved, bound to the branch point and the exons are ligated. The resulting "lariat" (not pictured) and joined exons are released and the spliceosome disassembles.

among *POU5F1* gene products revealed differentially regulated splice variants. One variant called OCT4A is restricted to embryonic stem and embryonal carcinoma cells and can initiate expression at OCT4-initiated promoters.¹⁷ OCT4B, in contrast, does not initiate expression at OCT4 promoters.¹⁸⁻¹⁹ To complicate matters, a third isoform termed OCT4B1 is also highly expressed in embryonic stem and embryonal carcinoma cells, while the OCT4B isoform is expressed at low levels in many differentiated cell types.¹⁷ The roles of these isoforms are apparently distinct but largely unknown and hint at another level of regulation of OCT4's function by alternative splicing.

Although not as well characterized as its family member OCT4, OCT2 is highly expressed in the developing central nervous system and in the adult mouse brain.²⁰ Its splice variants also seem to influence stem cell differentiation: the OCT2.2 variant is sufficient to induce neural phenotypes when artificially overexpressed in mouse embryonic stem cells while OCT2.4 inhibits induction of neural phenotypes even in the presence of another known inducer of neuronal differentiation.²¹ Therefore, a detailed characterization of OCT2's splice variants could provide insight into neural lineage specification.

DNA methyltransferases comprise another group of genes with distinctly different alternative splicing patterns among stem cells and differentiated cells. By methylating DNA, methyltransferases epigenetically influence which genes are transcribed and provide a heritable form of expression regulation. Initial explorations revealed that DNA methyltransferase 3B (*DNMT3B*) is highly alternatively spliced; nearly 40 isoforms have been identified.²²⁻²³ Gopalakrishnan et al recently discovered a *DNMT3B* splice variant missing exon 5 in the NH₂-terminal regulatory domain called DNMT3B3Δ5 that, in contrast to DNMT3B3, is highly expressed in embryonic stem cells and brain tissue and is down-regulated during differentiation, up-regulated in reprogrammed fibroblasts and like DNMT3B3 lacks the catalytic segment involved in methylation.²⁴ Importantly, DNMT3B3Δ5's increased DNA affinity compared to DNMT3B3 could indicate its role as a blocker of active forms of *DNMT3B* to prevent hypermethylation of DNA. *DNMT3B* is known to interact with a variety of other DNA methyltransferases²⁵ and splice variants like DNMT3BA5 might add a new dimension to these interactions.

The *PKCδ* gene is also highly influenced by alternative splicing and an important regulator of gene expression. *PKCδ* has long been implicated in activation of apoptotic cascades and is known to positively regulate transcription of a host of apoptotic proteins.²⁶ *PKCδ* is also involved in homeostatic and antiapoptotic pathways.²⁷⁻²⁹ This dualism can be better appreciated in terms of *PKCδ*'s splice variants. PKCδI is cleavable by caspase 3, which yields a catalytic fragment known to induce apoptosis.³⁰ Sakurai et al discovered that another isoform, PKCδII, balances PKCδI's activity as it is insensitive to cleavage by caspase 3.³¹ Neural differentiation correlates with splicing towards the PKCδI isoform,

which supports apoptosis-mediated remodeling in the developing nervous system.³² Thus, the inclusion of intronic base pairs that distinguish PKC δ I from PKC δ II is sufficient to dramatically alter the regulation of apoptosis in teratocarcinoma cells before and after differentiation induction.

Alternative splicing also mediates production of splice variants with opposing functions in self-renewal. Fibroblast growth factors (FGFs) are known to positively regulate important self-renewal pathways in human embryonic stem cells.³³⁻³⁴ Mayshar et al discovered that a *FGF4* splice variant referred to as FGF4 is down-regulated after human embryonic stem cell (hESC) differentiation while another splice variant called FGF4si is expressed in both pluripotent and differentiated hESCs.³⁵ FGF4's self-renewal potential is based upon its ability to phosphorylate ERK1/2 and activate MEK/ERK signaling and introduction of soluble FGF4si seemed to dramatically reduce phosphorylation of ERK1/2. This counteraction among splice variants demonstrates strong modulation of a pluripotency-related pathway by alternative splicing and reveals a regulatory network among splice variants from the same gene.

Adult stem cells' multipotency and self-renewal are also heavily influenced by alternative splicing. Insulin-like growth factor 1 (*IGF-1*) generates splice variants that enhance proliferation and block differentiation of muscle stem cells (IGF-IEc) or induce muscle cell growth via anabolic pathways (IGF-IEa). These splice variants are expressed in a sequential fashion in response to mechanical stress which facilitates muscle growth and repair.³⁶ Low levels of IGF-IEc were associated with muscle wasting in diseased patient muscle tissue,³⁷ which hints at the therapeutic potential of this splice variant.

Vascular endothelial growth factor A (*VEGFA*) strongly influences mesenchymal stem cells (MSCs) and is also regulated by alternative splicing. The therapeutic utility of MSCs is hinged upon their unique paracrine signaling³⁸⁻³⁹ and splice variants of the mouse VEGF homolog affect paracrine signaling and other phenotypes in MSCs. In particular, Lin et al demonstrated that VEGF120 and VEGF188 induce expression of growth factors and immunosuppressant cytokines while VEGF164 affects expression of genes associated with remodeling and endothelial differentiation. VEGF188 also induces osteogenic phenotypes in MSCs.⁴⁰ Prospective tissue therapies rely upon VEGF to increase the regenerative potential of MSCs and only recently has the importance of choosing appropriate VEGF isoforms become apparent.

In addition to cis-acting alternative splicing, protein diversity is also amplified by trans-splicing events. Trans-splicing is the union of pre-mRNA segments from more than one gene to create a novel mRNA transcript. Few trans-spliced gene products associated with stemness have been identified, but trans-spliced mRNA from RNA binding motif protein 14 (*RBM14*) and *RBM4* indirectly affects splicing of important gene products. *RBM14* alone generates splice variants CoAA and CoAM that enhance and inhibit, respectively, cotranscriptional splicing of a variety of genes. During differentiation of embryonic stem cells via embryoid bodies, splicing switches to CoAM which inhibits the action of CoAA and induces expression of the differentiation marker SOX6.⁴¹ When *RBM4* and *RBM14* are trans-spliced, splice variants and splicing regulators CoAZ and ncCoAZ generate a complex network that affects cotranscriptional splicing of the tau pre-mRNA at exon 10.⁴² While the functions of these splice variants is not clear, their distinct expression profiles before and after differentiation hint at their importance to stem cell biology.

These initial studies reveal that alternative splicing regulates genes associated with almost every facet of the stem cell state (Table 1). DNA methylation is influenced by

Table 1. Summary of alternatively spliced gene products with distinct expression profiles before and after stem cell differentiation and/or gene products that mediate important stem cell processes

Gene	Isoforms	Activities	Reference
<i>IGF-1</i>	IGF-1Ec, IGF-1-Ea	IGF-1Ec promotes proliferation and inhibits differentiation of muscle progenitors, IGF-1Ea activates anabolic pathways	37
<i>POU5F1</i> (OCT4)	OCT4A, OCT4B, OCT4B1	OCT4A and OCT4B1 expressed in stem cells, OCT4B in differentiated cells	17
RNA binding motif protein 4 (<i>RBM4</i>) and RNA binding motif protein 14 (<i>RBM14</i> , CoAA)*	CoAZ, ncCoAZ	CoAZ and ncCoAZ influence cotranscriptional splicing	42
DNA Methyltransferase 3 Beta (<i>DNMT3B</i>)	DNMT3B3, DNMT3B3Δ5	DNMT3B3Δ5 expressed in ES cells and functionally distinct from DNMT3B3	24
<i>VEGFA</i>	VEGF120, VEGF164, VEGF188	All promote MSC proliferation; some amplify paracrine signaling, osteogenic, or endothelial differentiation	40
<i>FGF4</i>	FGF4, FGF4si	FGF4 is important to stem cell maintenance, while FGF4si antagonizes some of FGF4's activity	35
Protein Kinase C Delta (<i>PKCδ</i>)	PKCδI, PKCδII	PKCδI and PKCδII are caspase-cleavable and incleavable, respectively	32
<i>POU2F2</i> (<i>OCT2</i>)	OCT2.2, OCT2.4	OCT2.2 is sufficient to induce neural differentiation in mouse ES cells, OCT2.4 is sufficient to block neural differentiation	21
RNA binding motif protein 14 (<i>RBM14</i> , CoAA)	CoAA, CoAM	CoAA is down-regulated in favor of CoAM during early embryonic development	41

* pre-mRNAs from each are trans-spliced into a single mRNA.

highly alternatively spliced DNA methyltransferases. The activity of transcription factors both central and peripheral to stemness is also modulated by alternative splicing. Even the splicing machinery itself is regulated by trans-spliced products of *RBM4* and *RBM14*. Many of these genes' splicing patterns correlate with discrete stages of differentiation and sometimes depend on the terminal lineage of the stem cell. The majority of splice variants from stemness genes remain unidentified, but new genome-wide alternative splicing detection methods will dramatically increase the rate and reduce the cost of detecting splicing variants. Once functionally characterized, these splice variants

and the underlying splicing code will reveal a previously underappreciated layer of posttranscriptional gene regulation.

GENOME-WIDE METHODS TO IDENTIFY AND DETECT ALTERNATIVE SPLICING EVENTS

Until recently, detection of alternative splicing relied upon reverse transcriptase PCR of individual mRNA fragments. Throughput was greatly increased when systematic measurement of large segments of the transcriptome was enabled by whole-genome tiling and splicing-sensitive oligonucleotide microarrays.⁴³⁻⁴⁵ By designing nucleotide probes for exon sequences or exon-exon junctions for all known and predicted exons in the genome, genome-wide interrogation of alternative splicing become possible. Computational algorithms are being developed to identify differentially spliced exons from microarray data. Our group has utilized this platform to study neural differentiation of hESCs.⁴⁶ Our results revealed that alternative splicing is prevalent in groups of genes such as serine/threonine kinases and helicases. Comparative genome analysis within the intronic regions proximal to alternatively spliced exons identified putative cis-regulatory sequences that may regulate alternative splicing during neural differentiation. This approach provides a framework for comparison among other progenitor cells to identify alternative splicing-mediated pathways towards cell type specificity. For instance, a recent study comparing undifferentiated hESCs and hESC-derived progenitors revealed common and specific splicing events among cardiac and neural progenitors.⁴⁷ Further work is required to relate contrasting splicing patterns and splice variant functions to resulting phenotypes.

Unfortunately, microarray-based approaches have distinct shortcomings. Physical limitations on probe density, cross-hybridization caused by probe sequence bias and insensitivity to sparingly expressed transcripts makes detection of some alternative splicing events expensive or impossible. Additionally, arrays cannot detect un-annotated genes. Tag-based profiling methods such as massively parallel signature sequencing (MPSS), serial analysis of gene expression (SAGE), cap analysis of gene expression (CAGE) and polony multiplex analysis of gene expression (PMAGE) have much higher sensitivity and enable the discovery of novel transcripts but are cost-ineffective and time-consuming. These issues are sidestepped by next-generation sequencing technologies that produce hundreds of millions of RNA sequence reads which reveal the transcriptome's content in an inexpensive and quantitative way (Fig. 2). A comparison of mouse embryonic stem cells and embryoid bodies revealed novel alternative splicing events and demonstrated the power of this "shotgun" approach to transcriptome profiling.⁴⁸

REGULATION OF ALTERNATIVE SPLICING BY RNA BINDING PROTEINS

A number of RNA binding proteins (RBPs) are likely to be involved in regulation of alternative splicing in pluripotent stem and differentiated cells. While relatively little is known about the splicing factors important to stem cell maintenance, several have been implicated in neural differentiation. It is thought that the majority of splicing events are regulated by splicing factors that interact directly with regions in pre-mRNA. Identifying the RNA targets of splicing factors and understanding their mechanism of action is necessary to decipher the rules of alternative splicing during stem cell differentiation.

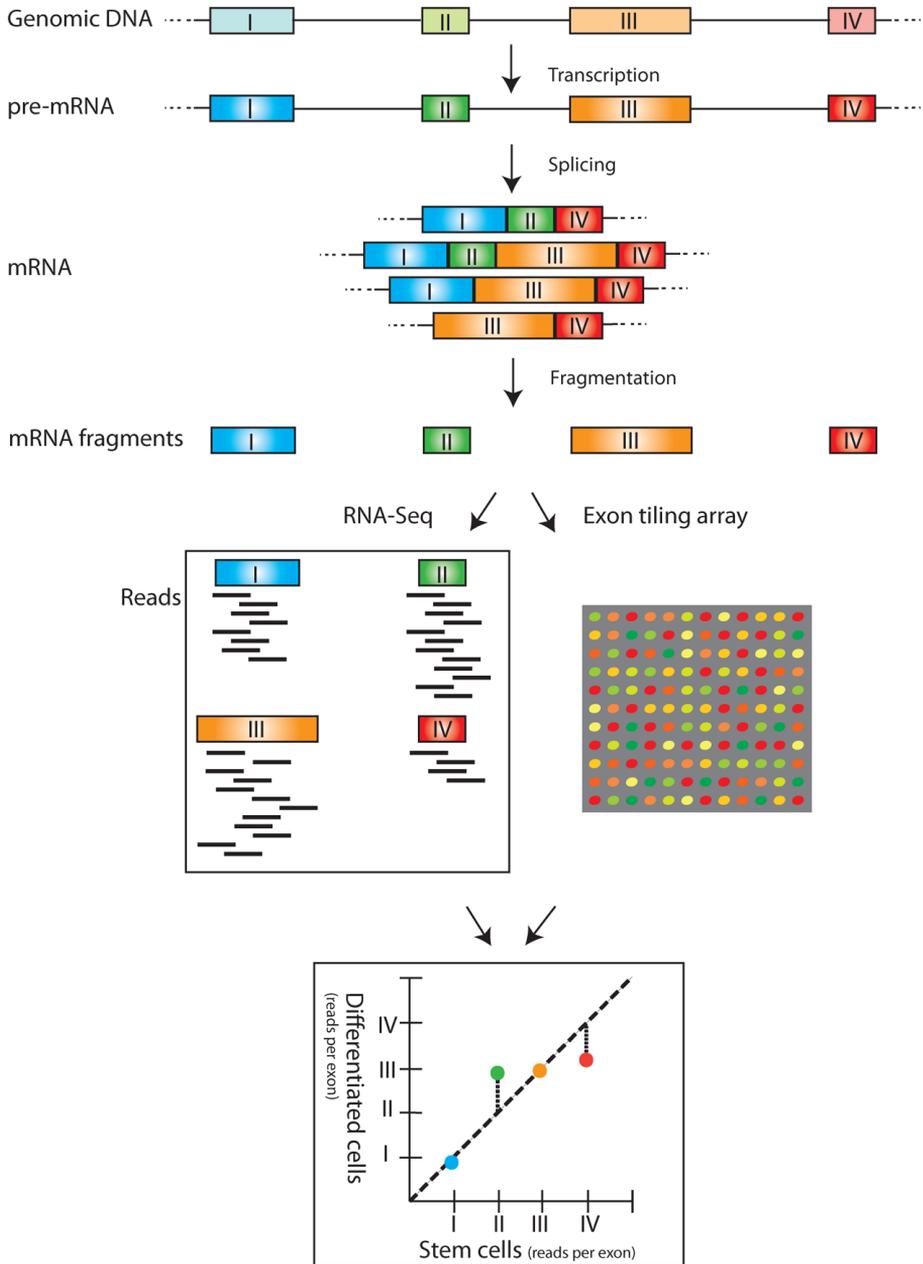


Figure 2. This schematic outlines two alternative splicing detection methods: RNA-seq and splicing-sensitive arrays. In the cell, genomic DNA is transcribed to RNA and processed into various splice variants. These splice variants are digested into short fragments and either sequenced or hybridized to a microarray. In the case of RNA-seq, short reads are aligned to the human genome and computational algorithms parse out which splice variants are expressed. Microarrays rely upon fluorophore-labeled fragments whose relative intensity on the chip reveal ratios of exon representation in mRNA. By comparing splice variant expression among differentiated and stem cells, variants enriched or depleted in stem cells can be identified.

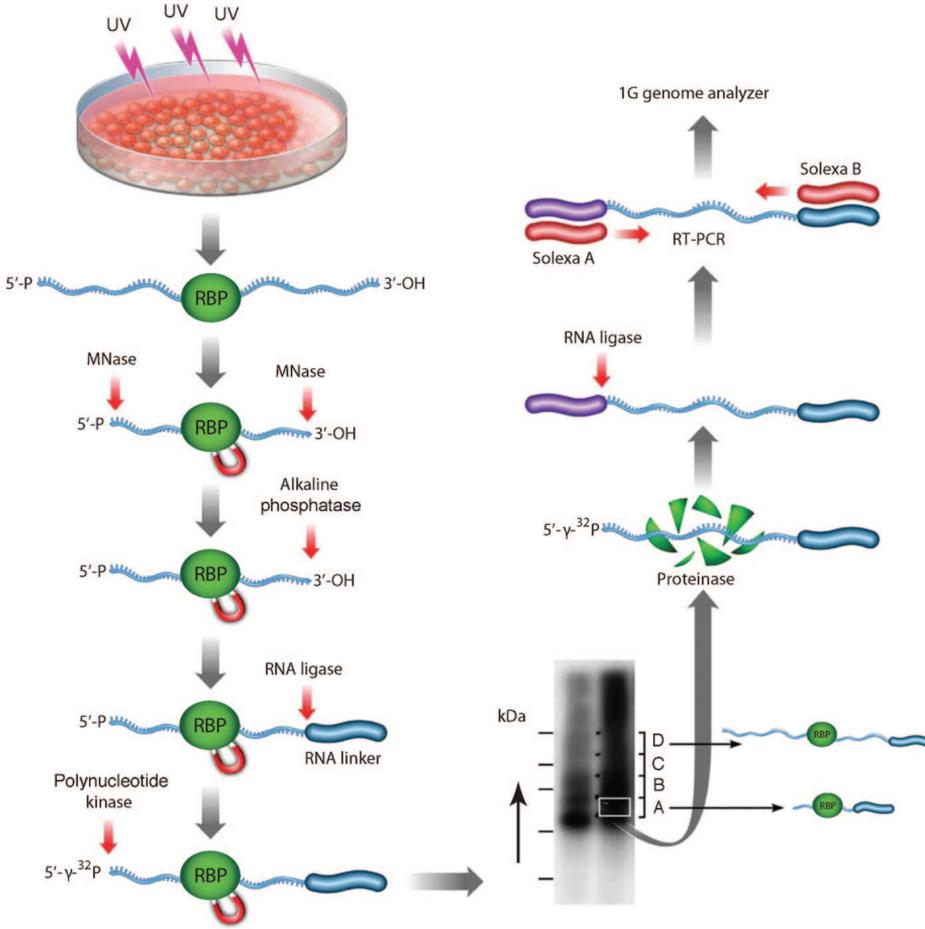


Figure 3. This schematic outlines CLIP-seq approach used with stem cells. RNA complexed with RNA binding proteins (RBPs) from UV-irradiated stem cells is enriched with an anti-RBP antibody. RNA in the complex is trimmed by MNase at two different concentrations, followed by autoradiography as illustrated. Protein-RNA covalent complexes corresponding to bands A and B are recovered following SDS-PAGE. Finally, bound RNA is amplified and then sequenced. Modified from reference 50.

In our study of neural differentiation of hESCs, we identified a conserved ‘GCAUG’ motif occurring near splice sites involved in differential alternative splicing.⁴⁶ This motif corresponds with the FOX1/2 splicing factor binding site, hinting at the important role of FOX splicing factors in splice site selection in hESCs.

To further investigate the role of FOX2 RBP-RNA interactions, we employed UV cross-linking and immunoprecipitation (CLIP). This technique facilitates stabilization of an RNA-protein complex *in vivo* via UV radiation.⁴⁹ We have utilized a modification of this technique to allow extraction of bound RNA for high-throughput sequencing in stem cells (CLIP-seq, Fig. 3). Application of CLIP-seq to isolate the RNA regions that interact with FOX2 in hESCs resulted in the identification of more than three thousand FOX2 bound regions in the human transcriptome. This RNA map of FOX2

binding sites identifies alternative splicing events regulated by FOX2.⁵⁰ FOX2 is evolutionarily conserved in mammals and is highly expressed in hESCs. Knockdown of FOX2 generated a rapid cell death phenotype in hESCs but not in neural stem cells or other cell lines. These results hint at FOX2 as an important alternative splice site selector in hESCs and further characterization may reveal its interactions with other components of the splicing machinery.

The polypyrimidine tract-binding protein (PTB) is another splicing factor that is widely expressed in the early embryo. PTB regulates alternative exon inclusion in many genes and has been implicated in aspects of mRNA regulation.⁵¹ Several studies have shown that knockdown of the PTB protein is sufficient to trigger neuronal-specific alternative splicing in nonneuronal cells.⁵²⁻⁵⁴ To probe the role of PTB in embryonic stem cells, Shibayama et al created homozygous PTB null mouse embryonic stem cells. These embryonic stem cells were viable but did not proliferate normally.⁵⁵

SAM68 is a nucleus-localized RBP that is linked to splicing,⁵⁶ is widely expressed in multiple cell types and its overexpression inhibits neural stem cell proliferation.⁵⁷ RNAi experiments in combination with microarray analysis of altered splicing revealed exons regulated by this splicing factor in mouse neuroblastoma cells.⁵⁸ Chawla et al also demonstrated that knockdown of SAM68 prevents differentiation of mouse embryonal carcinoma cells in the presence of retinoic acid, that SAM68 is up-regulated during neural differentiation and that it affects splicing and/or regulation of genes important to neural phenotype. These signs hint at SAM68 as a powerful regulator of splicing during neural differentiation.

As more RBPs and their motifs are identified with genome-wide techniques, the rules of the RNA splicing code will become clearer. This will facilitate predictive models of RNA splicing and could afford a new level of control over stem cell fate.

CONCLUSION AND PERSPECTIVES

Until recently, studies of stem cell transcriptional regulation have focused on the mammalian genome's many transcription factors. These efforts have revealed powerful stemness-associated transcription factors that can be useful markers for stemness as well as tools for reprogramming cells to a pluripotent state. But as most cell processes are also heavily influenced by posttranscriptional gene expression regulation, refinement of our understanding of stem cell state will rely upon understanding the rules and results of alternative splicing.

The splicing field is currently in a cataloging phase to identify important splice variants and splicing factors that influence splice variant abundance. Shotgun approaches to splice variant detection allow rapid identification of more stem cell-enriched splice variants while techniques such as CLIP allow identification of splicing factors and the functional RNA elements they bind. Initial results demonstrate that differentiation correlates strongly with splicing towards particular splice variants and overexpression of some splice variants is sufficient to dramatically alter stem cell phenotype. As more lineage-specific splice variants are detected, splice variant measurements may provide a highly accurate and sensitive way to determine stem cell state and reveal new avenues for guiding stem cell lineage specification.

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