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REVIEW

A SteMNess perspective of survival motor neuron function: splicing factors in stem cell biology and disease

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Abstract **Genome-wide analyses of metazoan messenger RNA (mRNA) species are unveiling the extensive transcriptional diversity generated by alternative splicing (AS). Research is also beginning to identify the splicing factors and AS events required to maintain the balance between stem cell renewal (i.e stemness properties) and differentiation. One set of proteins at the center of spliceosome biogenesis are the survival motor neuron (SMN) complex constituents, which have a critical role in the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs) in all cells. In this review we discuss what is currently known about how AS controls pluripotency and cell fate and consider how an increased requirement for splicing factors, including SMN, helps to maintain an enrichment of stem cell-specific AS events. Furthermore, we highlight studies showing that mutations in specific splicing factors can lead to the aberrant development, and cause targeted degeneration of the nervous system. Using SMN as an example, we discuss the perspective of how stem cell-specific changes in splicing factors can lead to developmental defects and the selective degeneration of particular tissues. Finally we consider the expanding role of SMN, and other splicing factors, in the regulation of gene expression in stem cell biology, thereby providing insight into a number of debilitating diseases.**

Keywords stem cells, splicing, survival motor neuron (SMN), spinal muscular atrophy (SMA)

Introduction

The advent of technologies that enable the deep sequencing of RNA population through-out development has unveiled a wealth of transcriptional diversity generated by alternative splicing (AS). Splicing differences are observed between different cell types, tissues, sexes and species, and now, the extent and the nature of the splicing changes occurring between proliferation and differentiation are being uncovered (Yeo et al., 2007; Wang et al., 2008). Proliferating cells, including stem cells, play a critical role in the development and maintenance of tissues in multicellular organisms. The study of stem cell pluripotency has shown the importance of transcription factor network changes, as well as chromatin remodelling processes, which either maintain stem cell proliferation or drive cell fate decisions (Niwa et al., 2000; Loh et al., 2006; Chen et al., 2008; Dixon et al., 2015). In addition to this, advances are now showing how AS generates stem cell specific mRNA isoforms (or stem cell 'splicing codes'(Barash et al., 2010)) that are required to maintain stem cell identity and, ultimately, function. As cells differentiate, there is need to switch to a daughter cell 'splicing code', which not only shuts down the proliferative capacity of the cell but also drives cell specification (Han et al., 2013). These changes appear to be governed by many splicing factors and RNA binding proteins.

In this review, we briefly describe what is currently known about how splicing changes control pluripotency and cell fate decisions. We describe the splicing factors enriched in stem cells and outline how deficiencies in splicing factors, which are thought to be ubiquitous, can lead to both specific tissue and developmental stage defects. We then go on to consider how the survival motor neuron (SMN) complex, which assembles the core spliceosome components, may control cell maturation and stem cell identity, and discuss how these processes may contribute to the neurological disease spinal muscular atrophy (SMA).

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Splicing and the dynamic spliceosome

The eukaryotic genome can be transcribed into protein coding transcriptional units, or mRNAs, that are required for gene expression. AS of these transcriptional units can occur, allowing varied mRNAs from the same gene to be expressed in different cell types or at different developmental stages. AS is regulated by RNA binding proteins that bind to specific sequences near regulated splice sites. This allows for the recognition and removal of introns through splicing. This processes involves a highly complex molecular machine called the spliceosome, which can comprise of up to 200 polypeptides, making it one of the most complex protein assemblies in the cell (Jurica and Moore, 2003; Valadkhan and Jaladat, 2010). So far, there have been two classes of spliceosomes identified, each characterized by the core components it contains, and the type of introns it removes. The first, the major or U2-dependant spliceosome, consists of the U1, U2, U4, U5, and U6 snRNAs (Will et al., 1999; Ohta et al., 2013; Venables et al., 2013). The second, the minor or U12-dependant spliceosome, consists of the U11, U12, U4atac, U5 and U6atac small nuclear RNAs (snRNAs) (Fischer et al., 2011; Han et al., 2013).The vast majority of introns are spliced by the major U2-dependant spliceosome (Wahl et al., 2009) while only approximately 800 genes in the human genome carry introns that are spliced by the minor spliceosome (Patel and Steitz, 2003; Wang et al., 2008; Sterne-Weiler and Sanford, 2014). Although low in number, genes with minor splice sites are found in many essential genes that are involved diverse cellular processes (Younis et al., 2013).

AS regulates cell pluripotency and differentiation

New techniques such as exon arrays and high-throughput mRNA sequencing (Ozsolak and Milos, 2011) have revolutionised the way we can analyze AS and gene expression. Using these technologies, recent *in vivo* and *in vitro* studies have shown how regulatory networks driven by changes in splicing can promote dynamic remodelling of the transcriptome and drive cell differentiation (Wu et al., 2010). These studies have also coincided with the emergence of both embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) technologies to study pluipotency and differentiation (Feng and Xie, 2013). ESCs are pluripotent cells that have the ability to generate the majority of the cell types in the body, while iPSCs are reprogrammed somatic cells that behave like ESCs. When AS events were examined in human ESCs, splice isoform diversity was shown to be highest in undifferentiated ESCs, while diversity decreased when cell were differentiated into neuronal cell types (Wu et al., 2010). This process, the authors termed '*isoform specialization'*, leads to the expression of a smaller subset of isoforms in differentiated cell types. In turn, it has been shown that when somatic cells are reprogrammed into iPSCs, they go on to express a splicing pattern similar to the ESCs (Han et al., 2013; Ohta et al., 2013; Venables et al., 2013).

Transcriptional networks have been shown to be key in maintaining stem cells *in vitro* and *in vivo* (Loh et al., 2006; Takahashi et al., 2007; Chen et al., 2008; Sousa-Nunes et al., 2010; Dixon et al., 2015). In addition, it now appears that AS-mediated isoform switching is integral to the control of these core transcriptional regulators (Gabut et al., 2011; Graveley, 2011). For example, an ESC-specific isoform of the fork-head transcription factor FOXP1 (termed FOXP1-ES) has been shown to control the expression of downstream transcription factor genes required to maintain pluripotency, including OCT4, NANOG, NR5A2, and GDF3. This is achieved through the addition of an ES specific exon that is located within the 'fork-head' transcription factor domain. This change alters the amino acid composition of the domain and changes the DNA binding properties of the transcription factor. FOXP1-ES expression also leads to the repression of genes that are known to drive differentiation and cell lineage progression.

During myogenesis, myoblasts undergo a series of gene expression changes which drives the generation of the differentiated myotubes (Niwa et al., 2000). In addition, there is a complete switch in the predominant isoform of 330 genes, underlining the importance of isoform switching in muscle differentiation (Chen et al., 2008). One particular AS event that governs myogenesisis that of the Mef2 transcription factor Mef2D (Sebastian et al., 2013). During the myoblast stage, the isoform Mef2Dα1 is expressed almost exclusively. Mef2Dα1 is phosphorylated by active PKA signaling, which in turn drives histone deacetylase (HDAC) recruitment to promoters causing chromatin modifications that lead to the transcriptional repression of the genes associated with muscle differentiation. As muscle cells develop, an additional isoform becomes expressed called Mef2Dα2. Due to the removal of an exon through AS, Mef2Dα2 cannot be phosphorylated and competes with Mef2Dα1 for binding to target genes. Moreover, unlike Mef2Dα1, Mef2Dα2 promotes the recruitment of Ash2L complexes (which have methyltransferase properties (Dixon et al., 2015)) to muscle gene promoters, activating their expression, and promoting differentiation.

These studies along with others (Mayshar et al., 2008; Salomonis et al., 2010) demonstrate how AS events can govern the transcriptional and signaling networks that controls the maintenance of stem cell proliferation and cell fate decisions. As increasing numbers of AS events are discovered in stem cells, the importance of splicing control as a regulator of stem-cell state, cell specialization and differentiation will continue to be underlined.

Splicing factors and stem cell functions

Complementary to the enrichment of AS events and the creation of stem cell specific splicing codes, stem cells require an essential portfolio of splicing factors and spliceosome-associated proteins (Gan et al., 2010; Shirai et al., 2015). These again have been studied in both ESC differentiation and iPSC reprogramming. The spliceosome-associated factor SON, which interacts with pre-mRNA splicing factors during the cell cycle, has been shown to be required for mitotic progression. Its loss leads to spliceosome dysfunction and triggers mitotic arrest deficient 2 (MAD2) dependent mitotic delay at the metaphase-anaphase transition (Huen et al., 2010). Moreover, SON expression is essential for human ESC survival and the maintenance of pluripotency(Loh et al., 2006). In ESCs, SON deficiency leads to the aberrant splicing of the transcripts that encode the cell-cycle associated centrosomal protein TUBG1, as well as the ESC regulators OCT4, PRDM14, MED24 and E4F1, leading to a loss self-renewing properties of the cells (Livyatan and Meshorer, 2013).

During spermatogenesis, the differentiation of germ cell precursors to mature sperm cells coincides with down-regulation of many RNA splicing factors (Gan et al., 2010). To complement this, within the study it was noted that mutations in the differentiation factor *bag of marbles* (*bam*) caused a significant upregulation of the same splicing factors and alternative splicing events. *bam* knockout prevents the differentiation of cells during spermatogenesis and oogenesis, resulting in the over proliferation of undifferentiated germ cells, suggesting that the enrichment of splicing factors and AS events is concordant with the proliferation capacity of mutant germ cells. In a genome-wide RNAi screen in *Drosophila* neuronal stem cells (known as neuroblasts), 27 genes involved in RNA splicing and mRNA metabolism were shown to affect the control of self-renewal, with knockdown of the majority causing a reduction of proliferation, or stem cell loss (Neumuller et al., 2011).

Another example of how two splicing factors can govern the transition between cell proliferation and differentiation was observed in the muscle blind family of proteins MBNL1 and MBNL2 (Han et al., 2013). MBNL1 and MBNL2 are found only at low levels in ES cells, but increase in expression when cells differentiate. Interestingly, AS sites in ES-cell transcripts are enriched with MBNL1 and MBNL2 binding motifs and presence of the proteins control exon choice. When MBNL1 and MBNL2 are overexpressed in ES cells an increase in differentiation-specific AS events occurred, while stem cell specific protein isoforms, such as the ES-cell-specific isoform of FOXP1, are decreased. Conversely, MBNL1 and MBNL2 reduction in differentiated cells induced a switch to ES-type AS patterns. With the identification of DNA binding factors that modify AS in stem cells, an exciting challenge for the future will be to understand how developmentally programmed splicing changes, in conjunction with epigenetic control networks, co-regulate proliferation and differentiation.

In addition to the mitotic divisions observed in stem cells, splicing factors govern the entry into meiosis. Work performed in *Caenorhabditis elegans* has identified a number of splicing factors that control meiotic entry and proliferation in the germline cells (Kerins et al., 2010), with knockdown leading to discovery of subsets of factors that either control germ cell determination, or enhance overproliferation phenotypes. One example, TEG-1, which is involved in snRNP biogeneis, regulates the proliferation versus meiosis decision (Wang et al., 2012) in the germline stem cells. TEG-1, and its human ortholog CD2BP2, function is biogenesis of the U4/U6. U5 pre-snRNP, a component of the major spliceosome (Laggerbauer et al., 2005). *teg-1* mutants enhance overproliferation in the germ line of nematode worm C. elegans, and, depending on the genetic background, can promote the formation of germline tumors (Wang et al., 2012).

Splicing factor deficiencies lead to neuronal and developmental defects

The misregulation of splicing, which contributes substantially to human disease (Feng and Xie, 2013; Sterne-Weiler and Sanford, 2014), can occur in two ways. First, *cis* changes to AS, result from changes at the DNA level. These mutations can affect the canonical 5′ and 3′ consensus sites which are recognized by the splicing machinery. Such changes can lead to exon skipping, intron retention, or create cryptic splice sites that may lead to altered amino acid compositions in proteins, through missense or truncation. The defects caused by *cis* changes in splicing are the subject of a number of good reviews (Faustino and Cooper, 2003; David and Manley, 2010; Feng and Xie, 2013)

The second cause of splicing misregulation involves mutations in the *trans*-acting splicing factors, and the non-coding RNAs that facilitate or catalyze the splicing reactions (He et al., 2011; Sleigh et al., 2011; Jia et al., 2012). These defects can be in the core spliceosomal components (such as the spliceosomal snRNPs), or the proteins that facilitate the coming together of the spliceosome and the mRNAs. Although originally believed to be ubiquitous and highly expressed, many of the spliceosome snRNAs actually display temporal and spatial variation in expression pattern (Forbes et al., 1984; Lund et al., 1985; Sierra-Montes et al., 2005). For example, each class of snRNP has a number of paralogues located around the genome, which can be developmentally regulated (Hinas et al., 2006; O'Reilly et al., 2013). It may be that the precise regulation of snRNP subsets is required for development, and, although there is partial redundancy between many multi-copy snRNPs, deficiencies in individual copies may cause tissue specific or developmental stage specific defects. Interestingly, tissue specific expression patterns of one of the U2 snRNP genes have been observed.

Jia *et al.* demonstrated that one of the multi-copy U2 snRNA genes, *Rnu2-8*, is both spatially and temporally regulated, with the levels of the U2 snRNA being highest in the cerebellum. Indeed, in differentiated neuronal tissues, knock-out in mouse lead to ataxia and neurodegeneration (Jia et al., 2012).

In studies that looked at iPSC reprograming, a reduction in the splicing factors U2af1 and Srsf3 suppressed reprogramming efficiency (Ohta et al., 2013). U2af1(U2 snRNA auxiliary factor 1) and Srsf3 (serine/arginine-rich splicing factor 3) are both non-core components of the spliceosome and although ubiquitously expressed, their reduction had a direct impact on the transition between proliferatory and differentiated cell states. U2af1 aids the recruitment of the U2 small nuclear ribonucleoprotein (snRNP) to the pre-mRNA branch site (Graveley et al., 2001). It is also worth noting that a number of U2af1 mutations have been linked to the hematopoietic stem cell defects associated with myelodysplastic syndromes (Graubert et al., 2012), and other tumors, with loss of the protein affecting the downstream isoform expression of genes in these tissues (Shirai et al., 2015).

Although required in all cells, deficiencies in spliceosome components are being linked to selective developmental brain disorders. The abnormalities in the developmental disease congenital microcephaly are characterized by alterations in the basal cortex of neuroepithelium layers and defects in the neuroepithelium and neuronal stem cell division (Thornton and Woods, 2009). Classically congenital microcephaly has been associated with mutations in centrosome components (e.g., abnormal spindle-like microcephaly-associated protein) that setup the mitotic spindle during cell division; these defects lead to improper chromosome segregation (Wollnik, 2010). Interestingly mutations in the gene encoding the *U4atac* snRNA, a component of the minor U12-dependent spliceosome, are found in individuals with microcephalic osteodysplastic primordial dwarfism type 1 (MOPD1) that present with intrauterine and post-natal growth retardation, abnormal bone growth, and brain growth anomalies (He et al., 2011). MOPD1 is completely co-morbid with microcephaly, along with more wide spread developmental defects suggesting a more general effect of minor splicing. In a parallel study, *U4atac* mutations were also identified in patients with Taybi-Linder syndrome (TALS); a disease characterized by early postnatal sudden death, and severe brain and bone malformations (Edery et al., 2011). In both cases the mutations were believed to be hypomorphic and lead to the defective splicing of sub populations of transcripts containing minor spliceosome introns.

SMN protein and RNP processing

Proteins that are involved in the assembly of the spliceosome have also been shown to have a considerable function in development and, when mutated, disease. The snRNP core components of the spliceosome are assembled in the cytoplasm by the survival motor neuron (SMN) complex that consists of the SMN protein, and a number of accessory factors called gemins. The properties of the gemin proteins have been the subject of comprehensive reviews (Cauchi, 2010; Borg and Cauchi, 2014). Although it has a fundamental function in the assembly of many ubiquitous RNP complexes, loss of SMN causes selective motor neuron defects. Evidence from several studies now shows that SMN activity is not only essential for nervous system set up and maturation, but has a fundamental function in maintaining the proliferatory capacity of stem cells.

Survival motor neuron (SMN) is an essential and widely expressed protein that is evolutionarily conserved from yeast to humans.SMN is enriched in stem cells and embryonic tissues (Barash et al., 2010; Grice and Liu, 2011), and, although constitutively expressed, there is a particular requirement for high levels within a window early in development (Burlet et al., 1998; Gabanella et al., 2005). SMN belongs to the Tudor family of proteins (Maurer-Stroh et al., 2003), whose members are known to interact with methylated arginines or lysines on proteins including histones (Chen et al., 2011). SMN functions in the biogenesis of the spliceosomal snRNPs that are assembled in the cytoplasm and then transported to the nucleus andfunction as a key component of the spliceosome (Fischer et al., 1997; Pellizzoni et al., 1998; Coady and Lorson, 2011) (Fig. 1). The SMN protein performs this task in conjunction with a complex of proteins, called the SMN complex, and loss of SMN has been shown to affect snRNP assembly and splicing (Burghes and Beattie, 2009). In addition to its function in snRNP biogenesis, SMN has been shown to interact with numerous other protein-RNA complexes suggesting a broader association with the processing other non-coding RNA species (Burghes and Beattie, 2009). For example, SMN has been shown to be associated with the processing and transport of small nucleoloar RNPs (snoRNAs (Verheggen et al., 2001)) which are associated with ribosomal RNA (rRNA) processing (Jones et al., 2001; Lee et al., 2008; Krastev et al., 2011), and the RNAs that associate with the Cajal and histone locus bodies (HLBs) (Carvalho et al., 1999; Lefebvre et al., 2002). In addition, components of the SMN complex have been shown to be involved in mRNA processing and transport (Fallini et al., 2012).

SMN localizes both in the nucleus and the cytoplasm (Fig. 1) (Liu and Dreyfuss, 1996). In the nucleus, SMN localizes in distinct nuclear organelles called Cajal bodies and histone locus bodies (HLBs) (Liu and Gall, 2007). In the cytoplasm, SMN locates in U snRNP bodies (U bodies) which contain Sm like proteins (Lsm) and snRNAs. These bodies are found to be enriched in stem cells and undifferentiated neurons (Grice and Liu, 2011). Lsm proteins are involved in pre-mRNA splicing and degradation, small nucleolar RNA, tRNA and rRNA processing, and mRNA degradation (Beggs, 2005), and U bodies could be sites that regulate RNP assembly, in conjunction with other RNA

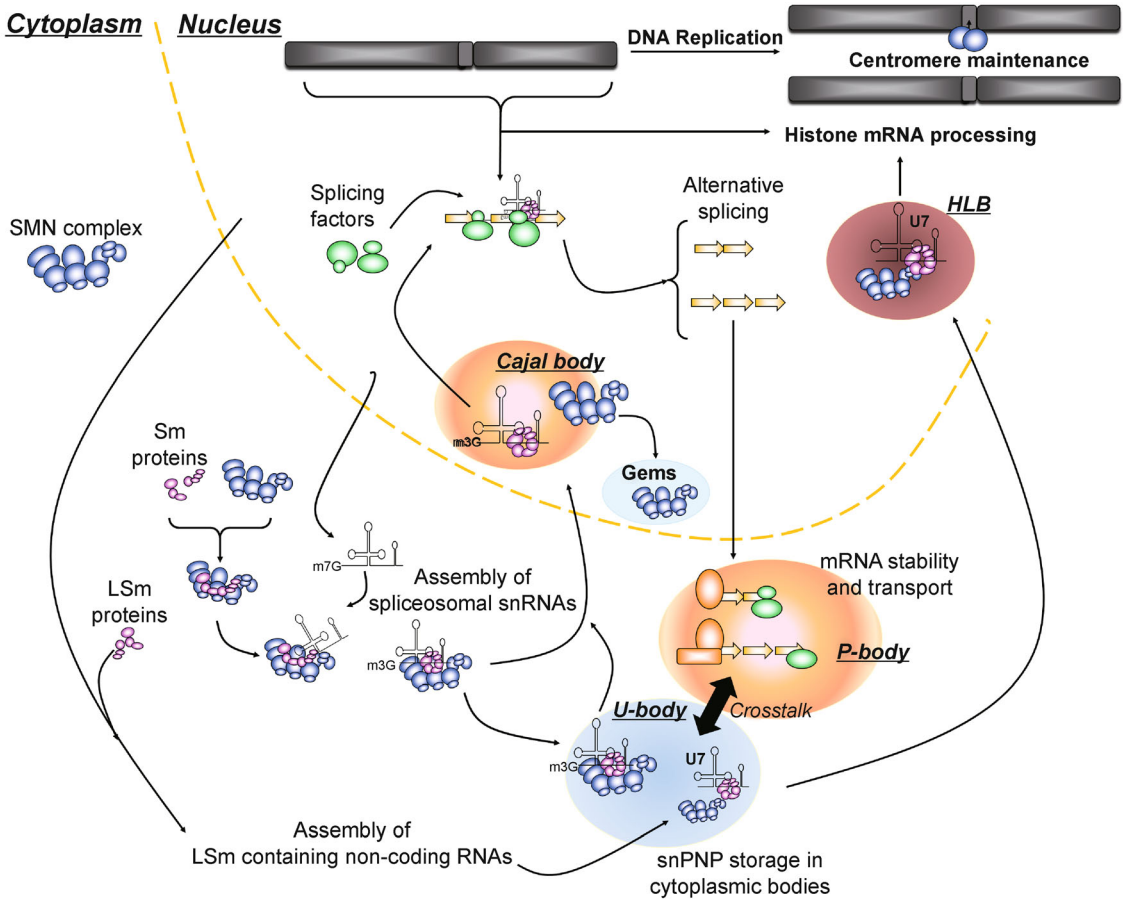

Figure 1 The relationships between SMN and splicing function. The SMN complex is involved in the assembly of spliceosomal snRNPs, and the LSm non-coding RNP associated with histone mRNA processing. The SMN complex with the bound Sm proteins recruits cytoplasmic snRNAs and facilitates the formation of snRNA. The SMN complex, with the snRNPs, is transported into the nucleus. The final processing of the splicosomal snRNPs occurs in the Cajal body, before they are utilized in mRNA splicing. The snRNAs, along with many additional splicing factors facilitate both the removal of the introns and the processing of the mRNA. Cytoplasmic U7 snRNAs are also assembled by an SMN complex, and are also transported to the nucleus. These localize to the histone locus body (HLB). The storage of both the spliceosomal snRNPs and the LSm non-coding RNPs occurs in the cytoplasmic U-body, which is physically and functionally linked to the RNA stability and transport related P-Body.

processing and storage bodies (Lee et al., 2009; Cauchi et al., 2010). To this end, U bodies invariably contact cytoplasmic processing bodies (P bodies) which contain proteins involved in mRNA surveillance and processing, RNA silencing and transport (Liu and Gall, 2007; Lee et al., 2009; Cauchi et al., 2010). Interestingly, knocking-out SMN increases the size of the P body, while, reciprocally, the mutation of P-body components changes the aggregation of SMN in U bodies. This suggests that alterations in SMN proteins can affect how cells control RNA surveillance and downstream translational regulation. In addition, SMN localizes to the Cajal body where it may have a role in additional snRNP maturation and recycling events (Liu et al., 2006; Liu et al., 2009). Finally, SMN was seen to interact with the HLB a dynamic nuclear body that associates with the histone gene cluster. The bidirectional promoter of the histone H3 and H4 genes is the primary seeding site for the assembly of HLBs (Salzler et al., 2013), suggesting that it may function to concentrate processing factors involved in the processing of histone pre-mRNAs that are required during cell division. Furthermore, SMN is required for the biogenesis of the U7 snRNA, which is required for the 3′-end processing of the replication-dependent histone mRNAs (Tisdale et al., 2013), while knockdown of U7 snRNP components leads to cell cycle arrest.

SMN function in stem cell maintenance

Similar to what is seen with isoform usage and splicing factor enrichment, SMN-dependent snRNP assembly is highest during embryonic CNS development, and is substantially downregulated during postnatal development (Gabanella et al., 2005). In addition, SMN protein, and members of the SMN complex, is enriched in both germline stem cells and postembryonic neuroblasts (pNbs, the larval neuronal stem

cells; Fig. 2) of *Drosophila*. These neuroblasts generate both the neurons and glia required for the formation of the adult brain during the later larval stages as well as early pupation. Moreover, SMN protein forms a concentration gradient in the CNS that is inversely proportional to the state of differentiation, with the differentiated neurons appearing to have the lowest levels (Grice and Liu, 2011). Targeted ablation of SMN, using stem cell clonal analysis, reduces stem cell division and leads to stem cell loss – this is seen in both larval neuroblasts and adult germline stem cells. There is also a marked reduction of U2 and U5 snRNAs in the clones generated from the mutant pNbs, as well as dissociation of basally localized mRNP complexes associated with asymmetric stem cell division (Grice and Liu, 2011). Interestingly, SMN protein has also been shown to localize to the centromere regions of the chromosome (Morency et al., 2007). In particular, SMN relocates to damaged centromeres during interphase, in conjunction with the H3K79 methyltransferase DOT1, and may help facilitate the repair of damaged centromeres between cell divisions (Sabra et al., 2013). The fidelity of the interphase centromeres is essential for the proper assembly of kinetochores, and ultimately cell division.

Stem cell defects have also been seen in vertebrate ESCs when SMN is reduced (Chang et al., 2015). ESCs are pluripotent cells that propagate perpetually in culture and can be readily induced to differentiate into various cell types both *in vitro* and *in vivo*. SMN is enriched in ESCs and forms puncta in the cytoplasm analogous to the U body. Both SMN and OCT4 were high in the inner cell mass of the embryo that contains the embryonic stem cells (Fig. 3), but lower in the inner cell mass cells, at the hatched blastocyst stage. SMN knockdown in ESCs causes the downregulation of pluripotent markers, including *Sox2*, *Rex1*, *Klf4* and *Sall4*. It is worth mentioning, however, that the major regulators of pluripotency, – Oct4 and Nanog (Loh et al., 2006), did not change following SMN knockdown in ESCs. However, SMN reduction did activate ERK signaling (Chang et al., 2015) that drives the neuronal differentiation *in vitro*, and *in vivo* (Halfar et al., 2001; Lanner and Rossant, 2010). Activation of ERK signaling blocks self-renewal of ESCs, and induces neuronal differentiation and further transdifferentiation of ESCs into trophoblast stem cells. This suggests that SMN is required to maintain the proliferatory capacity and identity of ESCs, and that SMN reduction can lead to premature differentiation in the mammalian system.

SMN dysfunction in SMA

In humans, loss-of-function and deletion mutations in the *SMN1* gene cause the most common genetic form of infant mortality, spinal muscular atrophy (SMA) (Lefebvre et al., 1995). SMA is a neurological disease that occurs in approximately1:6000 live births and has a carrier frequency as low as 1:40 people (Cusin et al., 2003). In addition to *SMN1*, humans also have a paralogous *SMN2* gene, which, due to a mutation affecting exon 7 splicing generates comparatively low levels of full-length SMN protein (Lorson and Androphy, 2000). *SMN2* copy number varies between individuals, leading to a spectrum of disease severity (Type 1 severe infant onset to Type IV adult onset) that correlates with the levels of *SMN2*-derived SMN protein (Lefebvre et al., 1997).

Since the identification of the disease gene in 1995, there has been considerable debate about how defects in both canonical and non-canonical functions of SMN may lead to the observed motor neuron selectivity (Burghes and Beattie, 2009). In particular, current research identifies two main possible mechanisms linking low SMN protein to for pathology: 1) SMA is caused by defects in subsets of spliced mRNAs that are particularly important for motor neuron development, function, and survival and 2) SMN functions in the assembly and/or transport of non-splicing related RNA species such as the axonally transported mRNA granules that are required for neuronal and synaptic function (Fallini et al.,

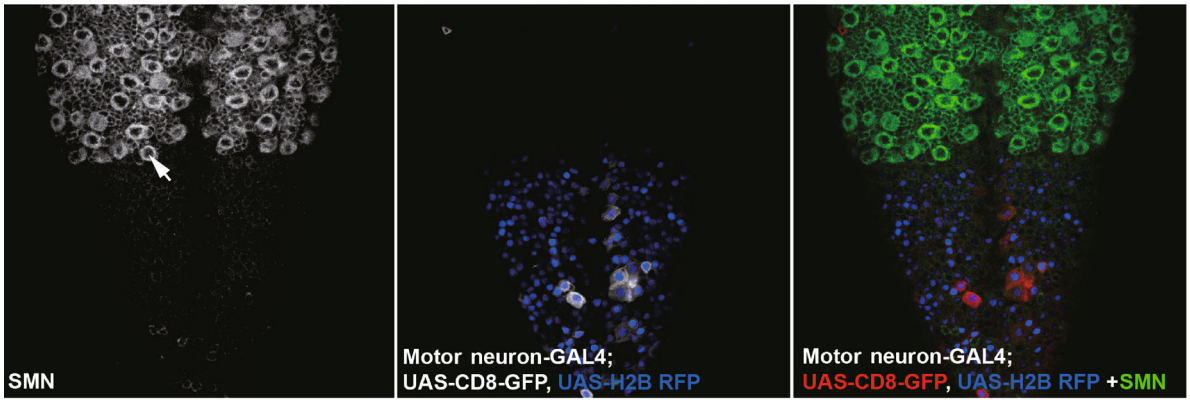

Figure 2 SMN levels in *Drosophila* motor neurons and neuronal stem cells (neuroblasts). Motor neuron-specific GFP (UAS-CD8-GFP, red) and RFP (UAS-H2B-RFP; blue) using D42-GAL4. Cell body levels of SMN (green) in the majority of motor neurons (blue nuclei) are at a basal low level when compared to neuroblasts (arrow) and immature neurons within the CNS.

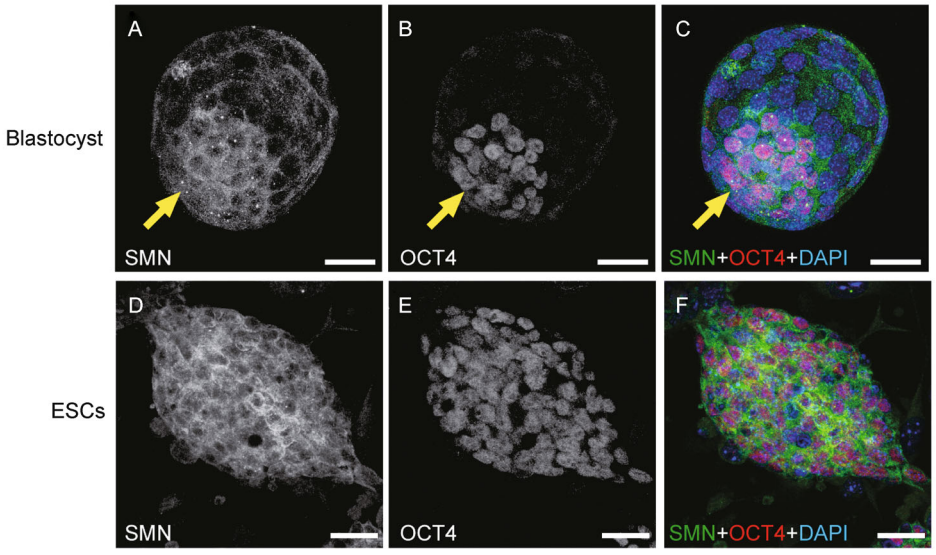

Figure 3 Distribution of SMN in mouse stem cells. (A, B and C) Immunofluorescent staining of SMN at late stage of blastocyst embryos, which produce embryonic stem cells (ESCs). The higher expression of SMN (A, green) is located in the region of inner cell mass (ICM, arrow), which expresses high level of pluripotent marker OCT4 (B, red). (D, E and F) Mouse ESCs with antibodies against SMN (D) and OCT4 (E). Scale bar = 25 μm. Courtesy of Wei-Fang Chang and Li-Ying Sung.

2012). It may be that defects in both processes contribute to the overall phenotype, as the processes are not mutually exclusive. For example, splicing leads to the formation of a *cis*-localization element that, together with the assembled exon junction complex (EJC) on mature mRNA transcripts, controls RNP movement and transport (Ghosh et al., 2012). However, with regard to spliceosome activity, there is evidence for correlation between SMA severity and snRNP activity (Wan et al., 2005; Gabanella et al., 2007). snRNA levels were shown to be broadly reduced in a number of animal models including mouse and *Drosophila* (Winkler et al., 2005; Grice et al., 2011; Sleigh et al., 2011; Praveen et al., 2012; Sleigh et al., 2013). In a number of studies, the minor spliceosome snRNAs are significantly more affected than those of the major spliceosome (Boulisfane et al., 2011; Lotti et al., 2012). Although the minor splicosome splices only less than 1000 genes, they are broadly expressed and have many key cellular functions (Turunen et al., 2013; Younis et al., 2013). Interestingly, minor spliceosome activity is regulated by U6atac abundance, which is rapidly turned over, and U6atacis upregulated in response to p38 mitogen-activated protein kinase activity (Younis et al., 2013). This in turn controls pathways associated with cell stress physiology, cell growth and apoptosis. Moreover, splicing from minor splice sites may govern the expression of genes that act as switches to govern cell growth and organism development (Turunen et al., 2013), and in some species their splicing controls genes intrinsically lined to the switch between stem cell renewal and differentiation (Scamborova et al., 2004). It would be intriguing to think that a subset of these minor spliceosome splice genes are affected when SMN is lost, however the centrality of the minor spliceosome to SMA pathology remains quite contentious, with some studies not showing the increased sensitivity of the U11 and U12 snRNAs to SMN loss (Huo et al., 2014). It is also still not well known to what extent splicing defects (from both major and minor splice sites) occur in patients, and to what extent they cause the selective loss of motor neurons. Only a small number of splicing changes have been identified in presymtomatic animal models, although the great majority of splice change studies were performed on whole spinal cords, and so these studies may lack the resolution to detect individual cell type changes (Bäumer et al., 2009; Zhang et al., 2008). To overcome this, Zhang *et al.* performed RNA-seq on laser captured motor neurons, and the surrounding glial white matter, of an SMA mouse model at presymptomatic postnatal day 1 (P1), and at P4 – P5, a time point when motor neuron pathology is not yet detected (Zhang et al., 2013). This study detected expression changes in proteins associated with synaptic stabilization and pruning (e.g. Z^+ agrin which is integral for neuromuscular development and stabilization; and complement factor C1q, which is involved in synaptic pruning) as well as a number of transcription and splicing factors. Notwithstanding, the mechanisms that cause SMA remains elusive.

SMN deficiency: looking beyond the differentiated motor neuron in SMA

Although classically thought to be neurodegenerative, developmental defects are now seen to be significant

modifiers or even causative mechanisms in SMA, and diseases of the motor system (Sleigh et al., 2014b; Grice et al., 2015). However, in SMA, it is not precisely known which cell type and at which developmental stage the defects first arise. However, increasing evidence implies that defects in the maturation of the neuronal circuitry (Lotti et al., 2012), muscle fibers (Shafey et al., 2005; Bricceno et al., 2014) and glial cells (McGivern et al., 2013; Hunter et al., 2014), as well as the motor neurons, may all contribute to motor neuron loss. This would suggest that multiple pathways are affected, and that these in combination lead to the degeneration of the motor circuitry. First, SMN restoration in motor neurons of SMA model mice results in minimal life-span extension, while motor neuron-specific Smn reduction in wild type mice fails to recapitulate the entirety of the disease phenotype. Expression of SMN in differentiated neurons does however rescue the motor phenotypes in the mouse, showing that there is motor neuron autonomous contribution to SMA (Gogliotti et al., 2012). Secondly, tissues and cell types beyond the nervous system play a significant role in the mediation of SMA (Hua et al., 2011; Hamilton and Gillingwater, 2013). This has led to the belief that SMA is a multi-tissue disorder. In addition, it is thought that cell types and tissues have different sensitivities to SMN loss, leading to tissue-specific defects manifesting on sliding scale of disease severity (Sleigh et al., 2011; Sleigh et al., 2013). A second point of contention is whether SMA arises from neurodevelopmental abnormalities or neurodegeneration. SMA patients and mouse models display limited motor neuron loss in pre-symptomatic stages of development (Monani et al., 2000), indicating that motor neuron loss is a late feature of disease progression. However, Type I SMA patients show motor neuron pathologies, NMJ maturation defects, during fetal development (Martínez-Hernandez et al., 2013). In SMA mouse models, systemic restoration of SMN results in the greatest improvement in motor function and survival when delivered at earlier stages of disease (Le et al., 2011). Collectively, these data suggest that motor neuron dysfunction at least in animal models may arise due to defects set up during the period of nervous system development and early maturation.

Proliferatory defects in the mouse higher brain have been characterized (Wishart et al., 2010) while differentiation defects have been observed in SMN deficient mouse muscle cells (Hayhurst et al., 2012). Gene expression analysis from microarrays of the spinal cord from severe mice models of SMA indicated that normal proliferative pathways may be affected (Bäumer et al., 2009). In wild-type mice, a number of translational changes are observed in the early postnatal period (P1 and P7). These were not observed in severe SMA mouse models suggesting that abnormal, or paused, development might underlie early events in SMA, and may explain general delayed growth in the SMA mice. Developmental pathologies associated with multiple tissue types are prevalent in SMA patients and animal models, and studies from mouse models imply that there is a defined window of SMN requirement that coincides with a rapid period of growth and maturation. It may be that when SMN levels are reduced the timely differentiation of the motor circuit is affected; causing subclinical defects that compound the cell autonomous defects in the differentiated motor system.

SteMNess and SMA?

With the identified enrichment of SMN in stem cells, and the overlaping activity of snRNP in biogenesis proliferating cells, we can now propose a new route to study the pathogenicity of SMN reduction (Fig. 4). It has been shown that SMN enrichment in the neuronal stem cells, and the developing neurons is required for normal neurogenesis. Loss, or a marked reduction, of SMN will have an effect on the stemness, i.e. the properties that define a stem cell. This will impact on the embryonic tissues, as well as the developing nervous system, when SMN levels are reduced sufficiently to affect these systems. SMN also aids the correct functioning of the differentiated motor circuit, and cell autonomous defects may also contribute to the selective loss of these cells (Sleigh et al., 2014a). These differentiated cells have lower levels of SMN. It is also worth noting that as well as reduced levels of SMN1 and snRNP activity, it has been shown that mature motor neurons do not express a particularly high level of SMN2 in humans (Jodelka et al., 2010). This has led to the suggestion that these low levels, along with the knock-on compounding positive feedback effect of increasingly inefficient spicing of the SMN2 modifier due to these low levels, could again contribute to the motor neuron selectivity in SMA (Jodelka et al., 2010; Ruggiu et al., 2012). Because of these low levels the motor system may be particularly sensitive to moderate SMN reduction, and subsets of motor neuron-specific splicing defects may occur. However, at the same time, defects in neurogenesis may occur, rendering the nervous system ill-equipped for normal function (Fig. 4B). How the stem cell splicing code is affected in SMA would be important to study. Do gene expression changes laid down in early development compound the later, motor neuron and motor circuit, defects observed? How these defects modify the parallel mechanisms that govern proliferation and cell fate specification, such as the transcription factor networks and chromatin state changes, would also be of interest. Further to this, multiple tissue defects occur when SMN is reduced to very low levels, and this has been seen in both patients and animal models (Fig. 4C). This will mean that developmental defects in peripheral tissues will also impact on the health of the individual, which may also compound motor defects (Fig. 4C).

Perspective

The control of splicing is essential for the correct develop-

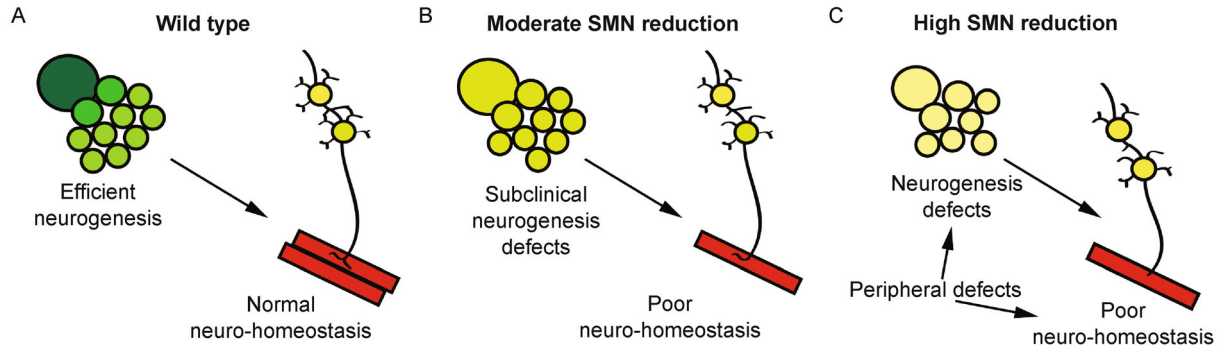

Figure 4 A model for tissue selectivity in SMA. (A) The intensity of the green depicts SMN enrichment in the developing neuroblasts is required for normal neurogenesis. SMN also aids the correct functioning of the differentiated motor circuit, which has lower levels of SMN. (B) The motor system may be particularly sensitive to moderate SMN reduction, while subclinical defects in neurogenesis may occur. During this period the nervous system is ill-equipped for normal function, leading to degeneration over time. (C) Multiple tissue defects occur when SMN is reduced to very low levels. Defects in peripheral tissues also impact on the health of the individual, which may also compound motor defects.

ment of metazoan cells types and tissues. In particular, splicing sits at a key crossroad between transcriptional and translational control in the cellular system. A further characterization of these events, and an understanding of how splicing inter-links with the multilayer regulation of proliferation and differentiation will be invaluable to many fields. With regard to disease, the dysregulation of splicing is a common feature of many diseases. As the control of AS events contribute to the switch between proliferation and cell specialization, it would be interesting to pinpoint if defects in neurogenesis and neuronal differentiation contribute to the maturation defects in the disease SMA, which may be developmental nature. Additionally, it would be interesting to unveil the interplay between stem cell splicing events and the hierarchy of processes that govern proliferation and cell fate specification, such as the transcriptional networks, chromatin modifications, and the action of non-coding RNAs. In turn, this would unveil the expanding role of SMN, and other splicing factors, in the regulation of stemness, as well as gives insights into a number of debilitating diseases.

Compliance with ethics guidelines

Stuart J Grice and Ji-long Liu declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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