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The Role of SMN protein in microRNA biogenesis

Inês do Carmo Gil Gonçalves

Dissertação
Mestrado em Biologia Molecular e Genética

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Resumo em Língua Portuguesa da Dissertação intitulada: "The role of SMN protein in microRNA biogenesis"

A Atrofia Muscular Espinal (AME) é uma doença genética autossómica recessiva, caracterizada por degeneração dos neurónios alfa inferiores localizados no corno anterior da medula espinhal. Indivíduos com AME manifestam atrofia e paralisia muscular progressiva. Sendo uma das doenças mais comuns do sistema nervoso, e a principal causa hereditária de mortalidade infantil, esta doença foi classificada em 4 grupos clínicos: Tipo I (AME infantil – Werdinig-Hoffman), Tipo II (AME intermédia), Tipo III (AME juvenil – Kugelberg-Welander) e Tipo IV (AME adulta).

Uma mutação homozigótica no gene *Survival Motor Neuron-1* (*SMN1*) é a causa determinante da AME. O gene *SMN1* está localizado numa dupla região invertida do cromossoma 5q11.2-13.3, onde é também possível encontrar uma cópia sua, bastante idêntica, designada *SMN2*. Uma transição C-T na posição +6 do exão 7 do gene *SMN2* interfere com o seu processo de splicing, levando à exclusão deste exão. Assim, enquanto o gene *SMN1* codifica na sua totalidade proteína *Survival of Motor Neuron* funcional (FL-SMN), o gene *SMN2* produz 90% de proteína truncada e instável (SMNΔ7) e 10% de proteína funcional (FL-SMN), quantidade esta não suficiente para conferir protecção contra a severidade da doença. SMN é uma proteína de expressão ubíqua, com níveis de expressão particularmente elevados em órgãos como medula espinhal, cérebro e músculos. Em associação com 7 outras proteínas, denominadas Gemins (Gemin2-8), SMN forma um complexo – o complexo SMN. O complexo SMN está envolvido no processo de montagem das ribonucleoproteínas nucleares pequenas (*small-nuclear ribonucleoprotein – snRNPs*), sendo esta a sua principal função. snRNPs são um componente fundamental do spliceossoma, onde são responsáveis pelo reconhecimento da molécula de pré-RNA-mensageiro (*pre-messengerRNA* – pre-mRNA) e remoção de intrões. De igual forma, a proteína SMN desempenha um papel neuro-específico, manifestado através da interferência na expressão e transporte de RNA mensageiro (mRNA) ao longo dos axónios e interferência na síntese proteica que ocorre nas neurites dos neurónios motores.

Os microRNAs (miRNAs) são moléculas endógenas de RNA, de cadeia simples e não codificantes, com importante função na regulação da expressão génica a nível dos processos de transcrição e pós-transcrição. Após a transcrição pela enzima RNA polimerase II, é gerada uma molécula primária (pri-miRNA) em forma de *hairpin*. Esta estrutura é especificamente reconhecida e clivada pela ribonuclease Drosha e o seu co-factor, *DiGeorge syndrome critical region gene* (DGCR8), originando no núcleo uma molécula precursora (pré-miRNA) de ~70 nucleótidos. Seguidamente, esta molécula precursora é transportada pela Exportin5 para o citoplasma, onde ocorre uma segunda clivagem efectuada pela ribonuclease Dicer, que actuando juntamente com outros factores dá origem a um duplex miRNA/miRNA* com ~20 nucleótidos. Uma das cadeias deste duplex, a chamada cadeia guia, é incorporada na proteína Argonauta2 (AGO2), enquanto a cadeira passageira será degradada. AGO2, carregada com a cadeira guia, é então incorporada no complexo RISC, onde por compatibilidade através da região *3'-untranslated region* (3'UTR), se liga ao mRNA alvo, levando ao seu silenciamento por clivagem ou repressão do processo de tradução. A estabilidade da molécula madura de miRNA é controlada por uma série de factores de actuação *cis* e *trans*, formação de complexos proteicos e exposição a nucleases. Em animais, o decaimento de miRNA maduro é controlado pelas exoribonucleases Xrn1 e Xrn2.

Os miRNAs são moléculas expressas em elevado número no sistema nervoso, onde actuam em processos de desenvolvimento, especificação celular, modelação e plasticidade neuronal (plasticidade sináptica). Várias doenças neurológicas estão associadas a uma desregulação na expressão de miRNAs, incluindo AME. Gemin3 e 4 são duas das proteínas Gemin que, para além de constituírem o complexo SMN, estão também ligadas à proteína AGO2 e, consequentemente, ao complexo RISC. Igualmente, várias proteínas que interactuam com a proteína SMN estão envolvidas nos processos de biogénese e decaimento dos miRNAs.

Recentemente foi demonstrada a importância da actividade dos miRNAs para a sobrevivência a longo termo de neurónios motores de medula espinhal *in vivo*. Além disto, foram detectadas alterações na expressão e distribuição de vários miRNAs, destacando-se um aumento constante na expressão do microRNA-183 (miR-183) causado pela perda da proteína SMN.

Assim, uma vez que a proteína SMN actua na regulação e transporte de mRNAs para serem localmente traduzidos, foi colocada em questão uma possível interferência do complexo SMN nos processos de biogénese e decaimento dos miRNAs.

Numa tentativa de compreender qual o mecanismo subjacente à desregulação dos miRNAs causada pela perda de SMN, medimos os níveis de expressão das formas primária e madura do miR-183. Sendo que este miRNA é transcrito de forma única a partir do *cluster* miR-183~96~182, também a expressão das formas primária e madura dos miR-96 e miR-182 foi medida em medula espinhal isolada a partir de um modelo animal de AME, o qual inclui 2 cópias por alelo do gene *SMN2* humano (FVB; P4, $Smn^{-/-}$; $SMN2^{tg/0}$). Os resultados obtidos através de real-time PCR (RT-PCR) mostraram não haver alterações na quantidade de transcritos primários dos 3 miRNAs que formam o *cluster*, enquanto a forma madura do miR-183 e miR-96 apresentaram valores de expressão significativamente elevados. No entanto, miR-182 não revelou qualquer alteração nos seus níveis de expressão. Assim, estes resultados sugerem que existe uma regulação diferencial na biogénese dos miRNAs constituintes do *cluster* miR-183~96~182, e da mesma forma, confirmam que a perda de SMN leva a uma desregulação na biogénese dos miRNAs.

Para perceber o mecanismo molecular através do qual a proteína SMN afecta a expressão dos miRNAs, caracterizámos o processo de biogénese destas moléculas, desde a sua transcrição primária até todos os passos seguintes de processamento de RNA. Para tal, através de RT-PCR quantificámos a expressão de transcritos dos genes envolvidos na biogénese e decaimento dos miRNAs em medula espinal de um modelo animal de AME. Curiosamente observámos uma diminuição na expressão de todos os genes em estudo, com excepção de Drosha e Exportin5, os quais não apresentaram alterações significativas nos seus níveis normais de expressão. Consequentemente, esta regulação negativa observada demonstra que a proteína SMN desempenha um papel na desregulação dos genes envolvidos na biogénese e estabilidade dos miRNAs.

Tendo em conta o papel importante da proteína SMN no processo de splicing, analisámos este processo nos genes envolvidos na biogénese e decaimento dos miRNAs. Especificamente

pretendemos verificar se a perda de SMN provoca alguma alteração no processo normal de splicing dos genes em estudo, levando à ruptura de algum dos transcritos já conhecidos, ou criando um novo variante funcional ou não funcional. Assim, acedemos à plataforma EURASNET, a partir de onde recolhemos toda a informação disponível acerca do splicing alternativo e transcritos existentes para os genes em estudo. Consultando a estrutura genómica destes genes, escolhemos a região mais propícia a sofrer splicing, desenhando pares de primers primers específicos para os exões que flanqueiam a região determinada. Curiosamente, os resultados observados revelaram que o processo de splicing não sofre alterações devido à redução redução de SMN. Para confirmar este pressuposto, os fragmentos amplificados por PCR foram enviados para sequenciação. No entanto, apesar de não encontrarmos alterações no processo de de splicing, foi detectada uma redução na expressão dos fragmentos amplificados nas amostras de de SMA quando comparadas com os controlos. Redução que, através de análise estatística, confirmámos ser significativa para os genes Ago1, Exportin5 e Xrn2.

Depois de demonstrar uma regulação negativa dos genes envolvidos na biogénese e decaimento dos miRNAs, confirmámos se o mesmo se verifica a nível proteico. Para tal, através da realização de Western Blot, medimos a expressão proteica dos genes em estudos. Semelhante ao verificado para a expressão a nível dos transcritos, genes como Dgcr8, Ago1 e Xrn1 apresentaram uma redução significativa nos seus valores normais de expressão. De igual modo, foi também verificada uma pequena redução na expressão dos restastes genes testados, com única excepção da Drosha.

Em conclusão, este estudo demonstra que existe uma desregulação na expressão do *cluster* miR-183~96~182 no modelo animal de AME, sendo provável que esta ocorra a nível pós-transcricional, uma vez que a transcrição do agregado não apresentou alterações. Da mesma forma, observámos que a perda da proteína SMN interfere na expressão dos miRNAs através de uma desregulação dos genes envolvidos na biogénese, estabilidade e decaimento dos miRNAs. Assim, este estudo contribui para o enriquecimento do conhecimento acerca da relação mecanística entre a proteína SMN e os miRNAs, oferecendo informação adicional acerca do papel que os miRNAs desempenham na patologia AME.

The role of SMN protein in microRNA biogenesis

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RESUMO

A Atrofia Muscular Espinal (AME) é uma doença neuromuscular caracterizada pela degeneração dos neurónios alfa motores inferiores, atrofia muscular e perda de função motora. AME é causada por uma mutação homozigótica ou deleção no gene *Survival Motor Neuron-1* (*SMN1*). No entanto, é desconhecida ainda a razão pela qual a perda de função da proteína SMN afecta especificamente os neurónios motores inferiores. Os microRNAs (miRNA) são uma categoria de RNA não codificante expressos em grande quantidade no sistema nervoso. Foi demonstrado que desregulações na expressão dos miRNAs estão associadas a algumas doenças neurológicas, incluindo AME. Especificamente, detectámos um aumento na expressão do microRNA-183 (miR-183) em neurónios com expressão nula da proteína SMN. Desta forma, como objectivo de estudo pretendemos descobrir quais os mecanismos que estão envolvidos na desregulação da expressão dos microRNAs causada pela perda de SMN. Uma vez estando o complexo SMN envolvido no processamento de RNA, foi posto em questão um possível envolvimento deste complexo na regulação da expressão dos microRNAs. Para testar esta hipótese, analisámos o processo de biogénese dos miRNAs desde a sua transcrição primária até aos restantes passos do processamento de RNA, em medula espinal de um modelo animal de AME. A análise de expressão feita ao *cluster* miR-183~96~182 mostrou uma desregulação na regulação do processo de biogénese deste *cluster* em neurónios onde a proteína SMN não é expressa. Da mesma forma, verificámos uma desregulação na expressão dos genes envolvidos na biogénese, estabilidade e decaimento dos miRNAs em medula espinhal de um modelo animal de AME. Assim, os resultados obtidos confirmam que a expressão dos miRNAs é alterada devido a níveis deficientes da proteína SMN.

Palavras-chave: Proteína *Survival motor neuron* (SMN), microRNA, *pathways* de biogénese e estabilidade, expressão, desregulação

ABSTRACT

Spinal muscular atrophy (SMA) is a neuromuscular disease caused by degeneration of alpha lower motor neurons, muscle atrophy and loss of motor function. SMA occurs when *SMN1* (*survival motor neuron-1*) is homozygously mutated or deleted. However, it remains unclear how deficiency of SMN function mainly affects lower motor neurons. microRNA (miRNA) are a sub-set of non-coding RNAs highly expressed in the nervous system. It has been reported that dysregulation of miRNA expression is associated to several neurological diseases including SMA. We found that expression of miR-183 is elevated in SMN deficient neurons. Therefore, we sought to uncover the mechanisms underlying the dysregulation of miRNA expression caused by SMN loss. Since the SMN complex plays a role in RNA processing, we hypothesize that this complex is involved in regulation of miRNA expression. To test our hypothesis, we analyzed miRNA biogenesis from primary transcript to down-stream steps of RNA processing in spinal cord of SMA mouse model. Analysis of the expression of miR-183~96~182 cluster suggests that the biogenesis process for this cluster is dysregulated in SMN deficient neurons. We found that the genes involved in miRNA biogenesis, stability and decay pathways are dysregulated in spinal cord of SMA mouse model. Our findings suggest that SMN deficiency causes dysregulation of miRNA biogenesis and decay pathways.

Keywords: Survival of motor neuron protein (SMN), microRNA (miRNA), biogenesis and decay pathways, expression, dysregulation

INTRODUCTION

Autosomal recessive proximal spinal muscular atrophy (SMA)

Spinal muscular atrophies (SMAs) are a genetically and clinically heterogeneous group of neuromuscular disorders characterized by progressive degeneration of lower alpha motor neurons neurons in the anterior horns of the spinal cord.[1] Affected individuals exhibit autosomal recessive recessive inheritance with proximal manifestation of muscle weakness and atrophy defined as autosomal recessive proximal spinal muscular atrophy (SMA).[2] With an incidence of about 1:6000 to 1:10000 newborns and a carrier frequency of 1:35, SMA is the leading hereditary cause cause of infant mortality.[3,4] Due to the highly variable disease severity, four clinical types of SMA are classified based on the age of onset and achieved motor abilities: Type I SMA (Werdnig–Hoffmann), intermediate Type II SMA, mild Type III SMA (Kugelberg–Welander) and Type IV SMA (adult SMA).[2,4-8]

Survival of motor neuron-1 (*SMN1*) is the disease-determining gene of SMA, leading to the disease due to a homozygous deletion or mutation.[9] This gene is located on the chromosomal region 5q11.2-13.3 in a segment of ~500kb, which includes the telomeric *SMN1* and the similar similar but slightly different centromeric *SMN2*.[10] The human genome contains a copy of *SMN1* *SMN1* and several copy numbers of *SMN2* genes.[11] With 99% of identity, the two SMN copies only differ in 5 base-pair exchange localized within the 3' end of the genes.[9,12] However, only the the C-to-T transition at position +6 of exon 7 (c.840C>T) is localized within the coding region.[13] region.[13] Although it is a translational silent mutation, it severely affects the correct splicing of the exon 7 by disrupting an exonic splicing enhancer (ESE) and generating an exonic splicing silencer (ESS) instead. Therefore, an alternative spliced mRNA isoform that lacks this exon (SMNΔ7) is produced.[12,14] *SMN1*-derived transcripts produce full-length (FL) and functional SMN protein, while nearly 90% of *SMN2*-derived transcripts generate a truncated and instable protein (SMN2Δ7). Although low amount of FL-*SMN2* is also produced, which is equivalent to to ~10% of *SMN1* levels, it is not enough to protect against disease development.[2,15] Since the clinical severity of SMA depends on the amount of SMN protein, the copy numbers of *SMN2* directly impacts disease severity.[16-18]

Survival of motor neuron protein (SMN) and SMN complex

The human SMN protein is a 38kDa protein with 294 amino acids, encoded by 8 exons.[9] Is a ubiquitously expressed protein, with particularly higher levels in spinal cord, brain, kidney and liver. However, in individuals with Type I SMA was detected a 100-fold reduction of SMN levels in spinal cord when compared with the controls.[19] Complete loss of SMN protein in animals causes embryonic lethality, pointing out the crucial role of this protein in early development.[20] The SMN protein is localized in both the cytoplasm and the nucleus. With a disperse distribution in the cytoplasm, the nuclear SMN is found in structures called Gems, for "*Gemini of the coiled bodies*", which are intimately associated with the nuclear coiled bodies (Cajal bodies).[19,21]

SMN is tightly associated with its binding partners and forms the SMN complex. These proteins are named Gemins (Gemin 2-8) due to their localization in nuclear structures, Gems. SMN self-oligomerization creates the backbone of the complex.[23,24] Gemin2, Gemin3, Gemin5 and Gemin7 directly interact with SMN, whereas Gemin4 and Gemin6 are connected with the complex through their interactions with Gemin3 and Gemin7, respectively. The direct binding of Gemin8 with the SMN complex mediates its interaction with the Gemin6/Gemin7 heterodimer, which binds directly to UNR- interaction protein (Unrip).[23-25] The most well characterized function of the SMN complex is the ATP-dependent assembly of the heptameric core of Sm proteins on Uridine-rich snRNAs (U-snRNAs), which are involved in the formation of the spliceosomal small nuclear ribonucleoproteins (snRNPs). SnRNPs are crucial factors within the spliceosome, playing an important role in the recognition and splicing of introns of pre-mRNA in the nucleus.[11,23,26,27] An snRNP molecule consists in one U-snRNAs molecule, a common core comprising a ring of seven common Sm proteins, and several snRNP-specific proteins.[11,23,26] SMA animal models show a reduced U-snRNP assembly activity in the central nervous system, as well as signals of motor axon degeneration due to impaired U-snRNPs assembly.[7,27]

Immunocytochemical studies described an association between SMN and cytoskeletal elements in the axons and dendrites of neurons.[28,29] Endogenous SMN and Gemin proteins co-localized in granules which exhibit rapid, active and bidirectional movement that extends throughout neurites and growth cones of cultured motor neurons.[30] Local translation is known to be implicated in

SMA pathology. The SMN binding partner hnRNP is associated with the 3' UTR of β-actin mRNAs, a well-known mRNA that undergoes local translation. SMN deficient motor neurons show alterations of β-actin protein and mRNA localization in axons and growth cones of developing neurons, which might explain the deficit in axonal actin cytoskeleton organization due to low SMN levels.[23,31] Additionally, it was shown that SMN forms a complex with HuD that binds to the neuritin (cpg15) mRNA and is involved in the transport and deliver of mRNA in motor neuron axons to be locally translated.[32] Thus, it is suggested that SMN plays a neuronal-specific role in axonal mRNA expression/trafficking and local protein synthesis in the neurites [21,32,33] Likewise, it has been shown a neuronal specific role of SMN in axonal outhgrowth and pathfinding.[34,35] Low SMN levels cause alteration in neuromuscular junctions (NMJ) morphology.[36,37] SMN binds to others RNA binding proteins as *Drosophila* homologue of the X mental retardation protein (FMRP), KH-type splicing regulatory protein (KSRP) and FUS/TLS.[38-40]

microRNAs (miRNAs) - biogenesis, stability and decay

microRNAs (miRNAs) are endogenous non-coding single-stranded RNAs (ssRNA) molecules of ~22 nucleotides that play important gene-regulatory roles in animals and plants through transcriptional and posttranscriptional regulation. miRNAs function via base-paring to the 3'-untranslated region (3'-UTR) of messenger RNAs (mRNAs) from target protein-coding genes, leading to gene silencing by mRNA cleavage, translational repression and deadenylation.[41] Functional studies indicate that miRNAs participate in the regulation of a wide range of cellular cellular and developmental processes. Dysregulations of specific miRNAs related pathways are are associated with several human pathologies.[42,43]

The majority of characterized miRNAs are transcribed by RNA polymerase II from independent genes or introns of protein-coding genes resulting in primary transcripts (pri-miRNAs), which fold into a hairpin structure.[42] This structure is specifically recognized by the nuclear ribonucleaseIII (RNaseIII) enzyme Drosha, which together with its cofactor DiGeorge syndrome critical region gene 8 (DGCR8) comprises the microprocessor complex. DGCR8 is a double stranded RNA-binding domain protein that acts as a molecular anchor necessary for the recognition of pri-miRNA. Within this complex, Drosha cleaves the pri-miRNA into a ~70-

nucleotide precursor hairpin (pre-miRNA).[44] Afterwards, this precursor is translocated to the cytoplasm by exportin5 (XPO5) through the nuclear pore complex in a Ran guanosine triphosphate (RanGTP)-dependent process.[45] Once in the cytoplasm, the pre-miRNAs are cleaved cleaved by a second complex comprising Dicer (RNaseIII enzyme) in association with the transactivation-responsive RNA binding protein (TRPB), and the protein activator of the interferon-induced protein kinase (PACT).[46,47] This complex processes the pre-miRNA, generating a ~20bp miRNA/miRNA* duplex. One strand of this duplex is incorporated into the the Argonaute2/ Eukaryotic Translation Initiation Factor 2C (Ago2/Eif2c2) protein as a mature miRNA (guide strand/miRNA), whereas the other strand (passenger strand/miRNA*) is degraded.[42]

Ago2/Eif2c2 is loaded with miRNAs through a specialized assembly called the RISC-loading complex (RLC), which comprises the proteins Ago2, Dicer, TRBP, PACT. Therefore, is induced induced the assembly of a ribonucleoprotein effector complex known as the RNA-induced silencing complex (RISC), which is responsible for the silencing of target genes.[48]. The functional core of RISC is mainly composed by members of the Argonaute family (PIWI1-4 and and EIFC2C/AGO1-4 subfamilies) and glycine-tryptophan protein of 182kDa (GW182). However, only Ago2/Eif2c2 shows intrinsic endonuclease enzymatic activity, being responsible responsible for the mRNA slicer activity in RISC. GW182 proteins act as downstream effectors effectors of repression.[42,43,49,50] Ago2 has an additional independent function in miRNA biogenesis: it generates an intermediate miRNA precursor named Ago2-cleaved precursor (ac-pre-miRNA).[51] Additional proteins associated with RISC have been newly identified such as FMRP; members of the family of DExD/H RNA helicases, as MOV10 and RNA helicase A; R2D2; RNA-binding proteins as HuR; and Gemin3 and 4, which are thought to also have helicase activity.[52]

Transcription, processing and decay of miRNAs are subject to sophisticated control. The multiple steps in miRNA biogenesis seem to be remarkably well coordinated, with the transcription being the major level of control responsible for tissue and development-specific expression of miRNA. Such regulation is carried out by several activators or repressors that interact either with Dicer or Drosha, or binding to pre-miRNA.[42,49,52,53] The stability of mature miRNA is controlled by *cis*- and *trans*-acting modifications, protein complex formation and exposure to nucleases. AGO

proteins also play an important role in miRNA stabilization. miRNA decay is carried out by exonucleases named small RNA degrading nucleases (SDS) that catalyze 3'-to-5' decay in plants, whereas in animals such function is performed by the 5'-to-3' exoribonucleases1 and 2 (XRN1 and XRN2). [53,54]

microRNA in the nervous system

microRNAs are abundantly expressed in the nervous system, where its function affects a large number of neuronal genes. Changing levels of certain ubiquitous and brain-specific miRNAs shape the development and function of the nervous system. miRNAs are mainly involved in developmental process as cell specification, patterning and neuronal plasticity.[55] The involvement involvement in the specification of cell types and maintenance of cell identity was additionally identified as miRNA function.[55] Being expressed in dendrites and axons, miRNA are thought to to be involved in the control of synaptic plasticity and axonal pathfinding.[55] Many miRNAs can can be detected in neurites of primary hippocampal and sympathetic neurons, which suggests an an involvement in the control of local mRNA translation in neurons.[55,56] miRNA-mediated regulation seems particularly well suited in local translational control of synaptic plasticity.[57,58] plasticity.[57,58]

microRNAs and spinal muscular atrophy

Among the Gemin proteins that constitute the SMN complex, Gemin3 and 4 are also binding-partners of AGO2/EIF2C2.[59] Several miRNAs are reported as binding to Gemin3 in human and and murine neuronal cell lines.[60] SMN binding partners as FMRP, FUS/TLS and KSPR are also also involved in miRNA biogenesis and function.[38-40] Therefore, it is postulated that SMN protein protein plays a role in miRNA expression and distribution in neurons to regulate local translation. translation. Recent report shows that miRNA activity is essential for long-term survival of postmitotic spinal motor neuron *in vivo*. In fact, ablation of Dicer leads to a loss of ability to make functional miRNA, leading to manifestation of hallmarks characteristic of SMA. Additionally, embryonic stem cell derived motor neuron from a SMA mouse model show a specific down-regulation of miR-9 and miR9* expression.[61] Such findings suggest miRNA dysregulation due to loss of SMN protein. However, cellular mechanisms involved in SMN mediated miRNA expression and functions are still unknown.

Aims

Since SMN plays a role in the regulation of expression and/or trafficking of mRNAs that are locally translated, was hypothesized that this complex is involved in miRNA biogenesis and stability. To test this hypothesis, SMN expression was knocked down in neurons and the expression of 187 miRNAs was measured.[56] Previous studies and preliminary data showed that that several miRNAs exhibit changes in their distribution and expression due to SMN deficiency. deficiency. The most constantly altered miRNA was miR-183; an increase in overall expression expression of miR-183 was reported in different cells and tissues such as embryonic cortical neurons, spinal motor neurons form SMA animal models and fibroblast cell lines derived from SMA patients (unpublished data).[56] miR-183 is transcribed from a miRNA cluster miR-183~96~182.

The cellular mechanism behind the relationship between the SMN protein and miRNAs is not yet identified. Therefore, the aim of this work is to identify how miRNA expression is dysregulated in SMN deficient neurons: at the transcriptional level, in the biogenesis processes or at stability and decay levels. With this study we (1) measured the expression of primary and mature transcripts from the miR-183~96~182 cluster in spinal cord of 4-days old Taiwanese SMA mice model, which contains 2 copies of the human *SMN2* per allele (FVB, P4, $Smn^{-/-}$;$SMN2^{tg/0}$)[62]; (2) measured the expression of major genes involved in miRNA biogenesis, specifically *Drosha, Dgcr8, Dicer, Ago1/Eif2c1, Ago2/Eif2c2, Xrn1, Xrn2 and Expotin5/Xpo5* in spinal cord of the previously described SMA mouse model; (3) performed splicing analysis to test weather this process is dysregulated in miRNA biogenesis genes; and (4) analyzed the expression of the biogenesis and decay genes at the protein level, performing western blot to measure the protein expression of miRNA biogenesis from spinal cord of the SMA mouse model (FVB, P4, $Smn^{-/-}$;$SMN2^{tg/0}$).

METHODS

Isolation of spinal cord and brain from mice

Spinal cord and brain were isolated from the Taiwanese SMA mouse model (FVB; P4; *Smn*$^{-/-}$; *SMN2*$^{tg/0}$), produced in laboratory of Prof. Dr. Brunhilde Wirth.[62] Both homozygous (FVB; *Smn*$^{-/-}$; *SMN2*$^{tg/0}$) and heterozygous (FVB; *Smn*$^{-/+}$; *SMN2*$^{tg/0}$) contain 2 copies of human *SMN2* in their genome. The strain FVB/NJ was used as wild-type.[62] 4 days old mice were euthanized by carbon dioxide (CO_2) in a closed chamber and, the spinal cord was isolated and stored at -20ºC.

Genotyping

For each animal used in this study, the genotype was checked using the Kapa™ Mouse Genotyping Hot Start Kit (Peqlab Biotechnologies, PB), according to manufacturer's protocol. A polymerase chain reaction (PCR) was performed using specific primer pairs to distinguish the homozygous from the heterozygous (oligo 5'-ataacaccaccactcttactc-3´, oligo 5'-gtagccgtgatgccattgtca-3' and oligo 5'-agcctgaagaacgagatcagc-3') and to confirm the transgene (oligo 5'-cgaatcacttgagggcaggagttt-3' and oligo 5'-aactggtggacatggctgttcattg-3'). The PCR reaction was performed with the conditions recommended by the manufacturer. The PCR products were separated in 1% agarose gel electrophoresis and visualized using ethidium bromide (ApplyChem). Images were taken with ChemiDoc XRS Imaging system (*Biorad*).

RNA isolation from brain and spinal cord

Total RNA was extracted from spinal cord using the *mir*Vana™ miRNA Isolation Kit (Applied Biosystems, AB) according to manufacturer's instructions. Total RNA was quantified using the NanoDrop ND-1000 spectrophotometer (PB). The samples were immediately used, or stored at -20ºC for future use.

cDNA synthesis and real-time PCR for pri-miRNA and miRNA

Real time PCR was performed to measure expression levels of individual primary and mature miRNA forms of miR-182, miR-183 and miR-96. 50ng of total RNA was reversed transcribed using the High Capacity cDNA Reverse Transcription Kit (AB). Pri-miRNA and miRNA

expression was measured by TaqMan® RT-PCR following the same procedure as described before.[63] The RT-PCR was performed with following conditions: an initial incubation stage at 50ºC for 2min, denaturation at 95°C for 10 min and 40 cycles of detection (annealing/extension at 95°C for 15s and 60°C for 1min). All samples were analyzed in duplicated. Amplified signals were collected by 7500 System (ABI) and normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Primers for genes in miRNA biogenesis and stability pathways; expression and splicing

Primer pairs for genes in miRNA pathway were designed using Primer3Plus software and purchased from Metabion. The sequence of the each gene was obtained from NCBI nucleotide data-base. To check splicing variants, the genomic sequences were obtained from USCS genome browser and primer pairs were designed for the exons considered as likely to undergo splicing. Known information about splicing process in these genes was accessed through EURASNET - an alternative splicing network database.

cDNA synthesis and real time PCR for mRNA

100ng of total RNA was reversed transcribed using the High Capacity cDNA Reverse Transcription Kit with random primers (AB). PowerSYBR® Green PCR Master Mix (AB) was used to amplify signals with 20ng of cDNA and 1µM of gene specific primers (**Supplementary Table1**). RT-PCR amplification conditions were: an initial incubation stage at 50ºC for 2min, denaturation at 95°C for 10 min and 40 cycles of detection (95°C for 15s, 60°C for 30s, and 72ºC for 40s). A final dissociation was performed to check if the amplification was correctly performed. All samples were analyzed at least twice. The RT-PCR was performed using 7500 Real Time PCR System (AB). GAPDH was used as internal control.

Polymerase Chain Reaction (PCR) for splicing characterization

To characterize splicing process in the genes involved in miRNA biogenesis and stability, a PCR was carried out with 20ng/µL of cDNA isolated from spinal cord, and 1µM of each gene specific primer pair (**Supplementary Table2).** The amplification was performed with the enzyme *Taq* DNA Polymerase, Recombinant (Invitrogen) and the cycling conditions followed the

manufacturer instruction: an initial denaturation step of 3 min at 94ºC, followed by 43 cycles of 45s at 94ºC, gene specific primer annealing temperature (**Supplementary Table2)** for 30s, and extension at 72ºC for 3min; followed by a final extension step of 10min at 72ºC (Thermocycler: DNA engine Tetrad2 from Mj Research). PCR products were separated in 1% agarose gel electrophoresis and visualized with ethidium bromide (ApplyChem). Images were taken using ChemiDoc XRS Imaging system (*Biorad*).

Gel Extraction

The DNA fragments amplified by PCR were excised from the agarose gel and extracted using the QIAquick Gel Extraction Kit (QIAGEN), following the manufacturer's protocol. DNA concentration was quantified with the NanoDrop ND-1000 spectrophotometer (PB), and sequence was confirmed by sequencing (GATC Biotech). Sequencing results were analyzed using SeqMan Software (Lasergene®).

Protein isolation from mouse tissue

Mouse spinal cord was lysed in 400µL of Lysis Buffer (1M Tris-HCL, Triton X-100, 5M NaCl, 100mM EDTA and ddH_2O) containing protease inhibitor (in a 1:24 dilution). Homogenization was carried out using the T10 basic ULTRA-TURRAX homogenizer. Hereafter, the proteins were sonicated for 10min with the Bioruptor® Plus (Diagenode), and centrifuged (5415 R centrifuge, Eppendorf) for 15min at 13.000rpm and 4ºC. The protein-containing supernatant was collected for further analysis and stored at -20ºC.

Protein quantification

Pierce^{TM} BCA protein assay kit (Thermo Scientific) was used to determine the protein concentration and ensure equal sample loading. We followed manufacturer's instructions for this procedure. The standard curve and concentration values were acquired using the NanoDrop ND-1000 spectrophotometer (PB).

Western Blot

Protein expressions were analyzed by western blots.[64] Proteins were separated in 8% and 10% SDS gel electrophoresis and, transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, pore size 0.45µm) equilibrated with Methanol. Ponceu staining (15s) was carried out to check protein transfer. The membranes were incubated in 5% milk solution for 1h at room temperature to reduce non-specific background bindings (blocking). Subsequently, the membrane was incubated with primary antibody, which the optimum incubation time and concentration were determined empirically: incubation in 5% milk overnight at 4ºC. Before and after the secondary antibody incubation, the membrane was washed 15min in tris buffered saline with tween (TBS-T) buffer for 3 times. The membrane was incubated with secondary antibody for 2h at 4ºC. SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) was used to detected chemiluminescence signals. The signals were obtained and analyzed by the ChemiDOC XRS (Biorad) analysis software.

Antibodies

The following primary antibodies were used: goat anti-Drosha (ab58589, Abcam), rabbit anti-Dicer (SAB4200087, Sigma-Aldrich), rabbit anti-XRN1 (SAB4200028, Sigma-Aldrich), rabbit anti- XRN2 (SAB4500893, Sigma-Aldrich), rabbit anti-Exportin5 (SAB4200003, Sigma-Aldrich), rabbit anti-DGCR8 (SAB4200089, Sigma-Aldrich), rabbit anti-Argonaute (C34C6, Cell Signaling) and rabbit anti-GAPDH (14C10, Cell Signaling). The secondary antibodies used were donkey-α-rabbit-HRP and rabbit-α-goat-HRP.

Statistical Analysis

All image data was collected and analyzed using ImageJ (National Institutes of Health) and Quantity One 4.5.1 (densitometric analysis – Biorad). Statistical analysis was done with Microsoft Excel. The statistical significance was achieved applying the student's *t*-test. ($p<0.05$ = *, $p<0.01$ = **, and $p<0.001$ = ***).

RESULTS

Quantification pri-miRNA and miRNA expression of miR-183~96~182 cluster

Our previous study showed that miR-183 expression is increased in SMN deficient cells, including spinal cord of SMA mouse model and SMN knock down cortical neuron cultures.[56] To To understand the mechanism how SMN deficiency causes the elevated miR-183 expression, we we measured the primary transcript of miR-183 in SMN deficient cells. Since miR-183 is transcribed as a single transcript from miR-183~96~182 cluster, we measured the expression of of all three mature miRNAs as well as primary transcripts in 4 days old SMA mouse model (FVB, *Smn*$^{-/-}$; *SMN2*$^{tg/0}$). Interestingly, while the expression of miR-183 and miR-96 were elevated in spinal cord of SMA mouse, the expression of miR-182 and primary transcripts did not not show any significant changes (**Figure1**).

This data suggest that there is a differential regulation in the biogenesis process among miRNA in the miR-183~96~182 cluster. Likewise, these results suggest that SMN deficiency causes dysregulation of miRNA expression, affecting the biogenesis pathway rather than general transcription.

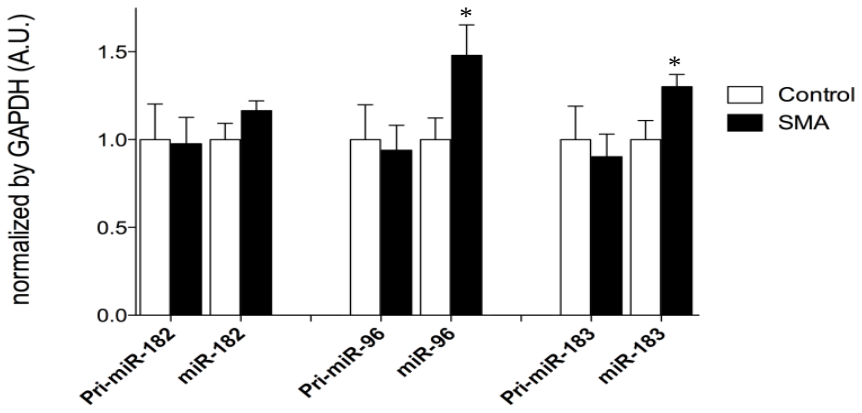

Figure1. The expression of miR-183~96~182 cluster in spinal cords from SMA mouse model (FVB; *Smn*$^{-/-}$; *SMN2*$^{tg/0}$) and control (FVB; *Smn*$^{+/-}$; *SMN2*$^{tg/o}$). In this experiment were used n=12 for SMA and n=8 for control. Primary transcript does not show expression changes in any of the miRNAs. Expression of mature miR-182 does not show any difference, while expression of mature miR-96 and miR-183 have significantly increased. Statistical significance was determined by Student's *t*-test:*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Quantitative real time-PCR analysis of genes involved in miRNA biogenesis and decay pathways

After confirming that miR-183 expression is upregulated in spinal cords from SMA mouse model (FVB, *Smn*$^{-/-}$; *SMN2*$^{tg/0}$), we asked how SMN deficiency caused dysregulation in miRNA expression. We postulated that SMN protein is involved in miRNA biogenesis or stability. To test this hypothesis, we characterized miRNA biogenesis pathway from primary transcription to down-steam steps of RNA processing in spinal cords from SMA mouse model. Therefore, we designed gene-specific primers for each gene involved in biogenesis and decay pathways of miRNAs and performed a RT-PCR to measure its transcript expression levels in spinal cord.

Strikingly, mRNA expression of the many genes involved in the miRNA biogenesis pathway was significantly decreased in spinal cord of SMA mouse model. Only the expression of Drosha and Exportin5 was not altered (**Figure2**). Likewise, we observed a similar substantial reduction in the expression levels of Xrn1 and Xrn2, the exoribonucleases responsible for miRNA decay (**Figure2**).

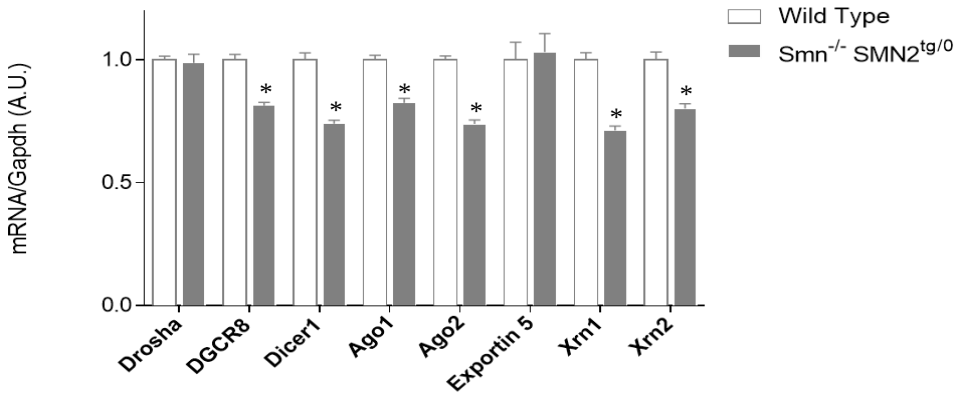

Figure2. Graph shows the expression analysis of genes involved in miRNA biogenesis and decay pathways in SMA mouse model (FVB; *Smn*$^{-/-}$; *SMN2*$^{tg/0}$) and wild type (FVB; *Smn*$^{+/-}$; *SMN2*$^{tg/o}$). The error bars indicate the standard deviation (SD). Drosha and Exportin5 do not show any changes in expression, while a significant decrease is observed in the remaining genes. RT-PCR results show a dysregulated expression of genes responsible for miRNA decay and stability. Data are represented by mean ± SD. N= 14 for SMA and n=10 for WT, except for Exportin5 with n=10 for SMA and WT (Student's *t*-test: *, $p<0.05$; **, $p<0.01$; ***, $p< 0.001$).

Taken together, these results showed that SMN deficiency changes the expression of the genes involved in biogenesis and stability of miRNAs. Accordingly, the observed downregulation intensely suggests that the SMN complex plays a role in miRNA expression through differential regulation of its processing and stability.

Characterization of splicing in genes involved in miRNA biogenesis and decay pathways

The most well characterized function of the SMN complex is its important role in the biogenesis of U-snRNPs complexes in the nucleus.[26] These RNA-protein complexes are the major components of the spliceosome, where its main function is the recognition and removal of introns from pre-mRNA in the nucleus.[65] Since SMN protein is strongly associated with splicing, we aimed to determine if SMN causes effects in splicing process of the genes involved in miRNA biogenesis, decay and stability pathways. Specifically, we intended to uncover whether SMN deficiency leads to a disruption of the gene transcript forms, or creates new functional/nonfunctional splicing variants. To test our hypothesis, we accessed to EURASNET and collected the available information about the alternative splicing events and transcripts of genes under study. Thereafter, according to this information and the genomic structure, we design gene-specific primers flanking the exons considered as likely to undergo splicing, and performed a PCR.

Curiously, we observed that the splicing process was not dysregulated in the genes under test (**Supplementary Figure1A-H**). The amplified PCR-fragments were confirmed with Sanger sequencing. Although we did not find changes in the splicing process of these genes, we could detect significantly lower expression of some genes such as Ago1, Exportin5 and Xrn2 in SMA spinal cord (**Figure3**).

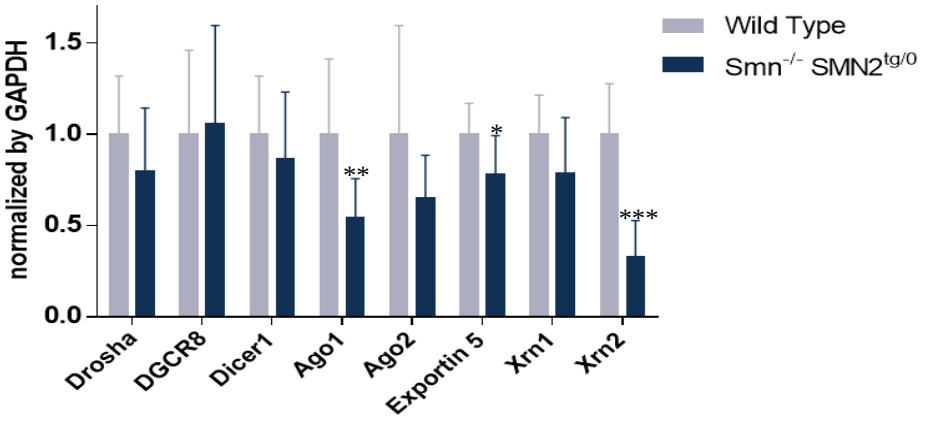

Figure3. Expression of genes involved in miRNA biogenesis and decay in spinal cords from SMA mouse model (FVB; *Smn*$^{-/-}$*; SMN2*$^{tg/0}$) and wild type (FVB; *Smn*$^{+/-}$*; SMN2*$^{tg/0}$). In this experiment were used n=9 for SMA and n=7 for WT, with exception of Drosha. For Drosha we used n=14 for SMA and n=14 for WT. The error bars indicate the SD. Significance was determined using the student's t-test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

This data showed that SMN does not disrupt the splicing process of the genes involved in miRNA biogenesis and decay. However, the decreased expression of some genes confirms that SMN deficiency affects the genes involved in miRNA biogenesis and decay, supporting the hypothesis that SMN is directly involved in the regulation of these pathways.

Analysis of gene expression involved in miRNA biogenesis and decay pathways in protein level

Having demonstrated a downregulation of the genes involved in miRNA biogenesis and decay in the spinal cord of SMA mouse model, we decided to check the expression of these genes at protein level. Therefore we measured the protein expression levels of DROSHA, DGCR8, DICER, EXPORTIN5, AGO family, XRN1 and XRN2 in spinal cord of the SMA mouse model (FVB, P4, *Smn*$^{-/-}$;*SMN2*$^{tg/0}$) and wild type control using Western blots.

As similar to mRNA expression the protein levels of some genes such as DGCR8, AGO and XRN1 were lower in spinal cord of SMA mice compared to the wild type control (**Figure4 and and Supplementary Figure2**). Among the biogenesis genes, while Drosha did not show any

alteration in its protein expression, the levels of the remaining proteins were slightly reduced in in SMA samples (**Supplementary Figure2A-E**). Specifically, the expression of DGCR8 and AGO proteins were significantly reduced (**Figure4**). Moreover, we found a highly significant decrease in the expression of XRN1, whereas expression of XRN2 was not changed by SMN deficiency (**Figure4 and Supplementary Figure 2F-G**).

These findings confirm our hypothesis by showing that SMN deficiency decreases the expression of the genes involved in miRNA biogenesis and decay pathways at protein level. Consequently, such results support that SMN protein regulates miRNA expression via controlling their biogenesis and decay pathways.

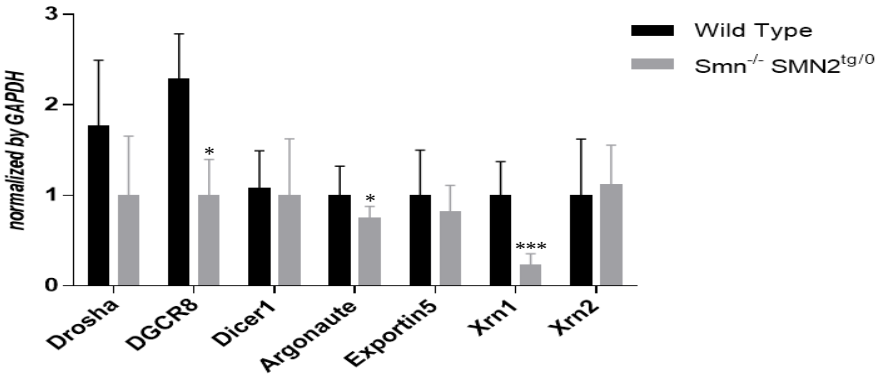

Figure4. Quantification analysis of the protein expression levels of the genes involved in miRNA biogenesis and decay in SMA spinal cord. The error bars indicate SD. Significance was determined using the student's *t*-test: *, p<0.05; **, p<0.01; ***, p<0.001. DROSHA: n=6 for SMA and n=8 for WT; DGCR8: n=3 for SMA and n=3 for WT; DICERr: n=7 for SMA and n=8 for WT; AGO: n=12 for SMA and n=12 for WT; EXPORTIN5: n=8 for SMA and n=7 for WT; XRN1: n=9 for SMA and n=6 for WT; XRN2: n=7 for SMA and n=5 for WT. Results from two independent experiments. GAPDH was used as

DISCUSSION

SMA is a devastating and lethal neuromuscular disease characterized by dysfunction/loss of alpha lower motor neurons in the anterior horns of the spinal cord.[1] Motor neuron degeneration degeneration has been well documented in SMA. Recently, dysregulation of miRNAs such as miR-9 and miR-9* was reported in embryonic stem cells derived motor neurons harboring a mutation causing SMA.[56] The goals of this study were (1) to uncover how SMN deficiency causes increased expression of miR-183 (2) to characterize miRNA biogenesis from primary transcription to down-stream steps of RNA processing in spinal cord of a SMA mouse model.

In this study, we reported dysregulated expression of miR-183~96~182 cluster in spinal cord of SMA mouse model. The predominant mechanism for miR-183 dysregulation seems to be post-transcriptional since the transcription of miR-183 cluster is not changed by SMN deficiency. However, the molecular mechanism underlying the role of SMN protein in the dysregulation of miRNA expression remains to be elucidated.

The incorrect splicing of genes in miRNA biogenesis pathway could explain the aberrant miRNA expression. The major role of SMN protein in splicing and its involvement in the formation of RNPs complex raised the question whether its deficiency could have an impact on the splicing process and machinery.[24,74] To test this hypothesis, we verified a downregulation in the expression of the genes involved in miRNA biogenesis and decay in SMN deficient cells; however its splicing process was not affected. Recently, it was reported an involvement of the SMN complex in the mRNA trafficking and delivery to the axonal compartment for axonal local translation.[32] Thus, it is suggested that the SMN complex may play a role in the formation or trafficking of miRNA-RISC complexes and/or translational machinery. Taken together, we conclude that the SMN deficiency causes dysregulation of the miRNA expression through regulating the genes involved in miRNA related pathways.

Whichever mechanisms are contributing to the dysregulation of miRNA expression, it appears that miRNAs are differentially regulated via their biogenesis and stability pathways. In this study

study we demonstrated that SMN deficiency altered miRNA expression through a dysregulation dysregulation of the genes involved miRNA biogenesis and decay pathways.

As new strategies to uncover the molecular mechanisms underlying this dysregulation we suggest a promoter analysis of the genes involved in biogenesis and decay in SMN deficient cells. Additionally, we will investigate whether neuronal localization of these genes is affected by SMN loss.

Our study provides further knowledge about the mechanistic relationship between the SMN protein and miRNAs, which helps us to understand the role of miRNA pathway underlying SMA SMA pathology.

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The role of SMN protein in microRNA biogenesis

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Supplemental Data

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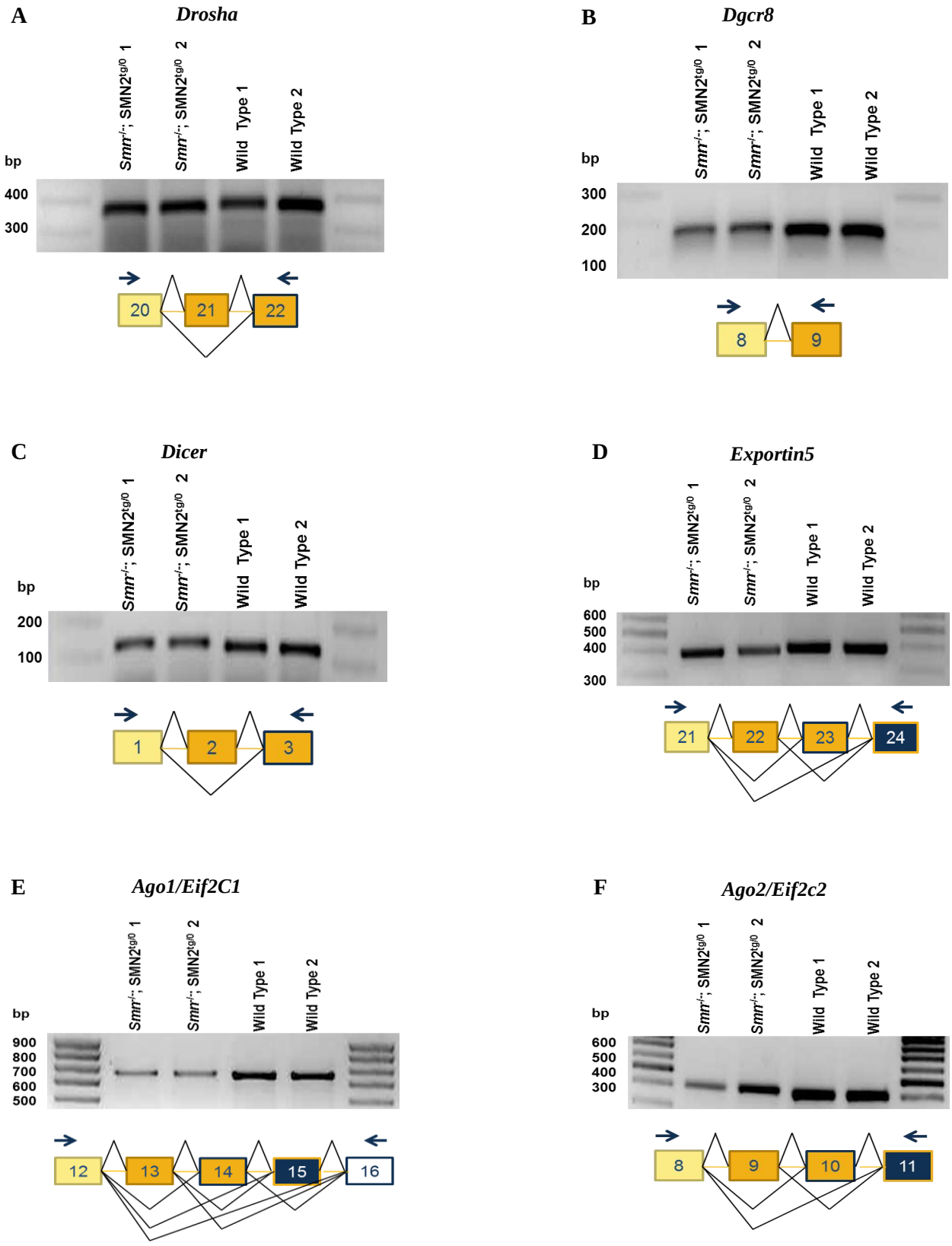
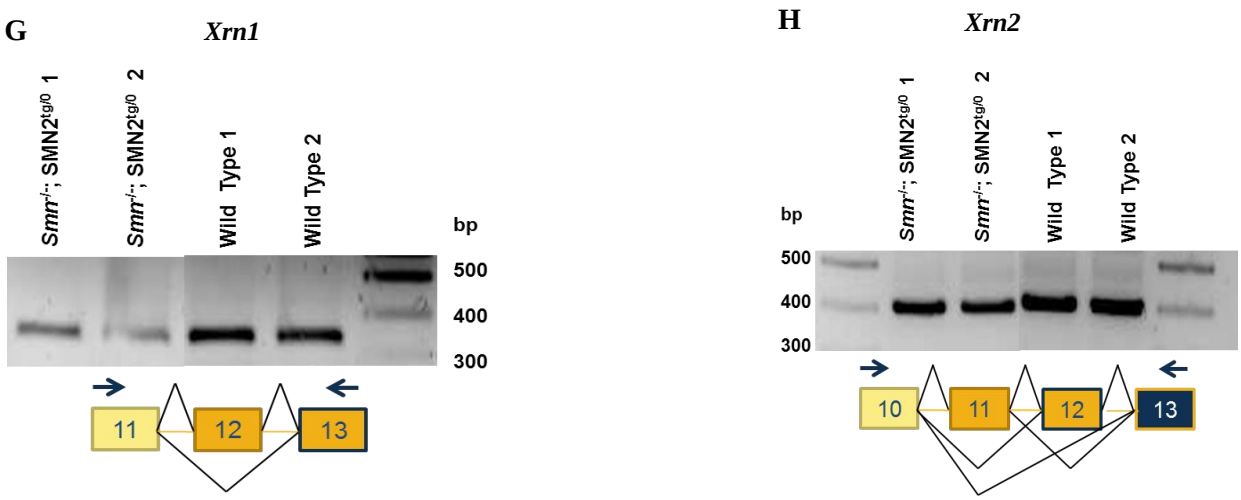
Supplementary Table1. Primer sequences to measure the mRNA expression of the genes involved in miRNA biogenesis and stability in spinal cord from SMA mouse model.

Gene	5' → 3' forward primer	5' → 3' reverse primer	Annealing/ Amplicon length (bp)
mDrosha	GGACCATCACGAAGGACACT	GATGTACAGCGCTGCGATAA	55ºC/197
mDGCR8	GAAACCATGGAATGGGTGAC	CAGAGGTCTCCTGCTTGACC	58ºC/250
mDicer	GTGGAGGGAGACCAGTCAAA	TGGGAAGCTATGGGTTCTTG	55ºC/250
mEif2C1	TCGGAAGATTTCCAAGGATG	GTTGCCATTCCCAAGAGTGT	53ºC/216
mEIF2C2	AAGTCGGACAGGAGCAGAAA	GAAACTTGCACTTCGCATCA	52ºC/182
mXPO5	TTCCTGACTTCCGGCTTAGA	TGGTTGATGACATGCCACTT	55ºC/160
mXRN1	GAGATGAGCGTGGAGTGTCA	CGCAGAAAGGAGAAATCAGG	55ºC/230
mXRN2	TTGAGAAGCAGCGAGTCAGA	CCAGGTGCACTAGCATCAGA	53ºC/217
mGAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA	60ºC

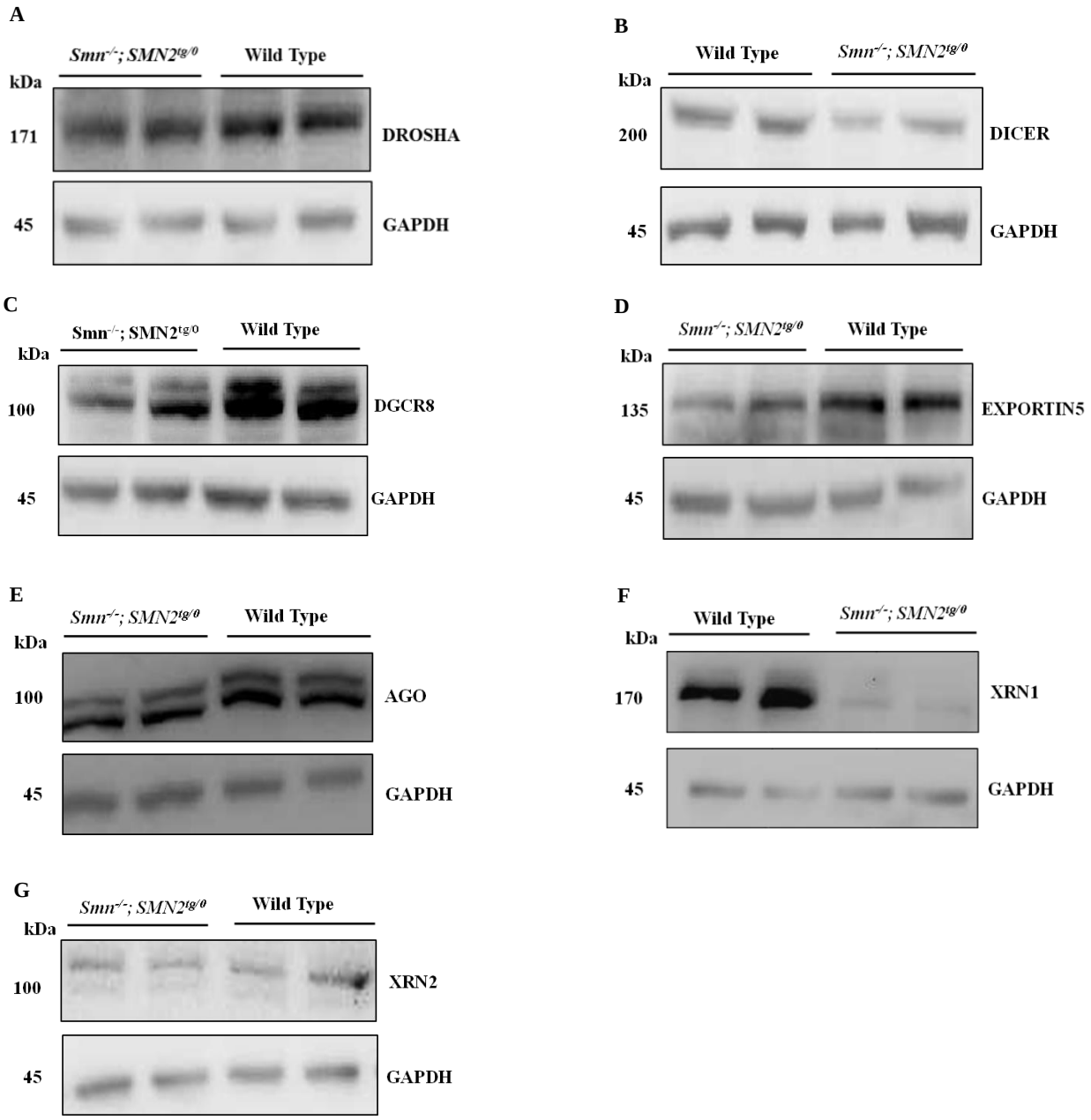
Supplementary Table2. Primer sequences to study the splicing of the genes involved in miRNA biogenesis and stability in spinal cord of SMA mouse model.

Gene	5' → 3' forward primer	5' → 3' reverse primer	Annealing/ Amplicon length (bp)
mDrosha	CATCACATCCGGTACCATCA	GAAGGAGTTGGATCATCTTGG	53ºC/308
mDGCR8	AATCCAAGTGAGCCTTTTGGT	GCTTCTCCTCAGAGGTCTGTTT	60ºC/154
mDicer	TGGGTCCTTTCTTTGGACTG	GAACACGGTCCTTTTTGCAT	58ºC/183
mEif2C1-a	TCGGAAGATTTCCAAGGATG	CGCTCATTCTTGTCAGCACA	55ºC/747
mEif2C1-b	CTGCCATGTGGAAGATGATG	ACTTCCACCTTCAGGCCTTT	58ºC/184
mEIF2C2-a	CGCGTCGGGTAAACCTGT	GATGCGATCTTTGCCTTCTC	53ºC/522
mEIF2C2-b	AAGTCGGACAGGAGCAGAAA	TGAGATGGACTTCTGTACACTGG	53ºC/417
mXPO5	ACCGGAAATGCTAACGAAAA	GAGGTCCAAGGATGGGAGAT	58ºC/420
mXRN1	TTGGGCTGCATTAGACAAAA	CCAGGACTGGACTCCATGAT	55ºC/242
mXRN2	TCTT CCTTCGGCTG AATGTC	ATCCTCAACTTCACCAACTGC	53ºC/374


A
Drosha
Smr-/-; SMN2tg/0 1
Smr-/-; SMN2tg/0 2
Wild Type 1
Wild Type 2
bp
400
300
20
21
22
B
Dgcr8
bp
300
200
100
8
9
C
Dicer
bp
200
100
1
2
3
D
Exportin5
bp
600
500
400
300
21
22
23
24
E
Ago1/Eif2C1
bp
900
800
700
600
500
12
13
14
15
16
F
Ago2/Eif2c2
bp
600
500
400
300
8
9
10
11



Supplementary Figure1. Schematic representation of PCR results from the analysis of alternative splicing of genes involved in miRNA biogenesis and decay pathways. Exons are represented by boxes filled with the respective exon number, introns by yellow lines, patterns of alternative splicing by thin black lines, and arrows indicated the primer-pairs position. Exon skipping was not observed in any of the genes in study. Molecular weight marker size is indicated. (A) *Drosha.* Position of predicted alternative spliced products with exon 21, 22 and 23 inclusion: 360 bp. (B) *Dgcr8*. Primer extension product predicted size is 170 bp (inclusion of exons 8 and 9). (C) *Dicer*. Amplified products have an expected size of 183bp for the inclusion of exon 1, 2 and 3. (D) *Exportin5*. Primer extension product predicted for intron 21, 22, 23 and 24 incluision. (E) *Ago1/Eif2c1*. Amplified fragments with a predicted size of 720 bp (inclusion of exon 12, 13, 14, 15 and 16) (F) *Ago2/Eif2c2*. Position of predicted alternative spliced products with exon 8, 9, 10 and 11 inclusion: 417 bp. (G) *Xrn1*. Fragments amplified by PCR have with predicted size of 250bp (inclusion of exon 11, 12, and 13). (H) *Xrn2.* Position of predicted alternative spliced products with exon 10, 11 and 12 inclusion: 380 bp.



Supplementary Figure2. Western blot analysis of the gene expression at protein level in spinal cord of SMA mouse model. 30μg/mL of protein lysate was loaded for all the samples tested. Primary antibodies were incubated with a 1:350 dilution factor, while secondary antibody was used in a 1:5000 dilution. The expected molecular weights are indicated in the schematic representation of each performed Western Blot. GAPDH was used as internal control to monitor loading efficiency. (A) DROSHA. (B) DGCR8. (C) DICER. (D) EXPORTIN5. (E) AGO. (F) XRN1. (G) XRN2.