ABSTRACT

Autosomal recessive proximal spinal muscular atrophy (SMA) is a devastating neurodegenerative disease and the leading genetic cause of infant death. SMA is caused by homozygous deletion or mutations of the *survival of motor neuron 1* (*SMN1*) gene, resulting in reduced SMN protein levels. Traditionally, SMA is classified as a pure lower motor neuron (MN) disease, causing skeletal muscle atrophy and weakness. However, it has remained elusive, how the deficiency of a ubiquitously expressed protein with housekeeping functions results in this cell type-specific pathology. It is widely acknowledged that lower MNs are particularly vulnerable to low SMN levels; however, numerous studies have revealed that other cell types and tissues, such as skeletal muscle, are also directly affected by low SMN levels in the absence of defective MNs. Moreover, it was not only shown that the selective SMN loss in murine MNs causes a dystrophic phenotype rather than the full SMA pathology spectrum but also that the loss of MN somata from murine spinal cord occurs subsequent to neuromuscular junction (NMJ) pathology. The NMJ is a specialized synapse which provides the link between MNs and skeletal muscles. For the proper conversion of a neuronal action potential into a muscle contraction, this motor unit requires a tight regulation. It is well established that impaired MN function results in defective NMJs and consequently atrophy of the associated muscle. It is important to note that recent studies have also highlighted the role of the muscle itself for functional NMJs. Skeletal muscle is an endocrine organ, and by releasing proteins it not only self-regulates muscle biology but also interacts with its environment. Therefore we hypothesized that secreted factors from intrinsically defective SMA muscle could contribute to the NMJ pathology and MN defects observed in SMA.

An in-depth and differential secretome analysis revealed that SMN depletion in myotubes results in significant secretome changes. Overall, the composition of differentially secreted proteins implies a dysregulation of extracellular matrix-related pathways. The most exciting finding was the reduction of the C1q/tumor necrosis factor-related protein 3 (CTRP3) in the secretome of SMN-depleted myotubes. CTRP3 is a secretory protein, circulating in blood and detected in cerebrospinal fluid. To date, a wide range of beneficial effects of CTRP3 on metabolism, cardiac function, inflammation and survival signalling have been reported in multiple tissues. Considering the known protective and growth factor-like functions of CTRP3, there was strong evidence that CTRP3 deficiency might contribute to the MN and NMJ impairment but also other peripheral pathologies observed in SMA, and therefore led us to further investigate this candidate.

In this study, the analyses of CTRP3 expression levels in the SMA context led to four key findings. Firstly, CTRP3 levels are reduced in the secretome of two SMN-depleted muscle cell lines. Secondly, CTRP3 is expressed in murine muscle tissue and highly reduced in the *tibialis anterior* muscle of SMA mice. Thirdly, circulating CTRP3 in blood plasma of SMA mice is significantly less compared to WT controls. Lastly, CTRP3 expression is reduced in brain of SMA mice compared to WT.

To decipher whether CTRP3 has a functional impact on MNs, MN-like NSC34 cells and primary MNs were treated with recombinant CTRP3 and downstream pathways of CTRP3 were analysed. Remarkably, a large-scale proteomic profiling revealed that CTRP3 stimulates translation- and transcription-related pathways, and in agreement with this, CTRP3 triggered the PI3K/Akt/mTOR pathway, which is upstream of the protein translation machinery. Of note, axonal protein synthesis is known to be essential for neuronal survival, but has previously been

reported to be impaired in SMA. These findings prompted us to evaluate the effect of CTRP3 treatment on the overall protein synthesis rate. Indeed, by performing a SUnSET assay, we have conclusively shown that CTRP3 stimulates protein synthesis. Excitingly, testing for expression changes of specific proteins important for neuronal survival, we found that CTRP3 upregulates protein levels of SMN and the vascular endothelial growth factor (VEGF), while it did not affect *Smn* and *Vegf* transcript levels. It is known that VEGF has neuroprotective functions, and it has recently been reported that VEGF is regulated by CTRP3 in non-neuronal cells. Taken together, our results clearly indicate that CTRP3 has neurotrophic properties in NSC34 cells and primary MNs.

To investigate the physiological role of CTRP3 for MN physiology, we performed an axon outgrowth assay, given that low SMN levels in MNs cause severe defects in axonal pathfinding and outgrowth in SMA animal models. Intriguingly, exogenous CTRP3 treatment dramatically increased axonal outgrowth in early WT and SMA MNs, and almost restored the axon length of SMA MNs to the level of WT MNs.

In summary, this study has shown that SMN-depleted muscle cells differentially secrete proteins, and specifically, reduced levels of CTRP3 - a factor we could assign to novel neurotrophic properties. We propose that a dysfunctional intercellular crosstalk could contribute to the MN and NMJ pathology in SMA. To further elucidate this, I established an *in vitro* system for neuromuscular co-culture in our laboratory to further study the NMJ pathology in SMA, and in this context, the role of CTRP3. Overall, this study highlights that skeletal muscle defects could be more than a bystander in SMA.