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General introduction and outline of this thesis

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The human body is not able to effectively regenerate the injured spinal cord following traumatic injury. The failure of recovery is due to the complexity of a spinal injury, including broken vertebrae, bleeding, inflammation and reduced spinal cord perfusion. These clinical challenges can now be well managed surgically. After stabilisation of the lesion site, spontaneous regeneration of the damaged axon tracts is limited. Disruption of the spinal cord tracts results in paralysis below the lesion, affecting mobility and sensation which has profound negative effects on quality of life for the patient. To date, there are no effective treatments that can reliably promote regeneration of spinal cord injuries and achieve restoration of function.

The peripheral nervous system (PNS) is capable of robust regeneration following injury which often results in recovery of function. Conversely, the central nervous system (CNS) exhibits limited recovery following injury. The dorsal root ganglion (DRG) neurons have a single branched axon with one branch in the central nervous system (in the spinal cord) and one branch in the peripheral nervous system. It has been understood for some decades that DRG neurons respond strongly to a peripheral axon injury, which leads to regeneration, but not after a central axon injury, which exhibits limited regeneration. It was shown that a so-called conditioning lesion of the peripheral axon, prior to a central axon lesion of the same DRG neuron, results in considerable sprouting of the central branch into a spinal cord lesion. It can be deduced that the changes the DRG neuron undergoes after peripheral injury are crucial for successful regeneration.

Gene expression profiling of DRG neurons has determined that a large number of regeneration associated genes (RAGs) are differentially regulated following peripheral but not central DRG axotomy. It is likely that this gene expression program is responsible for the regeneration observed following peripheral lesion, and for the sprouting seen after a conditioning lesion and spinal cord lesion. To harness the power of the RAG program after CNS injury, individual RAGs have been overexpressed, but the higher levels of regulation of this so-called 'RAG program' of gene expression, are not well understood. However, a number of RAG transcription factors (RAG TFs) have been identified which may regulate RAG expression. It is thought that multiple TFs will be required to activate RAG expression. Thus, it is the aim of this thesis to identify transcription factor combinations capable of driving the RAG program and promoting regeneration after CNS injury. This was approached in three stages.

A) Identifying TFs and combinations thereof capable of promoting neurite outgrowth (a proxy of axonal regeneration) using *in vitro* screening, B) Delivering TF combinations identified in step A *in vivo* to test their ability to promote regeneration and functional recovery after spinal cord injury when overexpressed in DRG neurons, and C) Investigating the gene expression changes promoted by overexpressing these TFs in DRG neurons *in vivo* to determine how much of the RAG program are they capable of activating.

We hoped that following this experimental strategy would result in an answer to the question posed in the title of this thesis **Can gene therapy with transcription factors drive axon regeneration?** We will return to this question at the end of the **General discussion**, **Chapter 7.**

Chapter 1 is a literature review on the dorsal column lesion model of spinal cord injury and its use in deciphering the neuron intrinsic injury response (primarily in rodents). This review describes the anatomy and function of the DRG axons, surgical approaches towards implementation of the lesion and methods to assess recovery, both functionally and histologically. The review summarises what has been learned about the neuron-intrinsic regenerative response in this model and summarises experiments that have utilized the model to test treatments to increase the neuron intrinsic aspect of regeneration. For completeness, extrinsic interventions to increase regeneration following DC lesion are also described i.e. experiments which target the extracellular environment of the DC lesion site.

In **Chapter 2**, we utilised literature to guide the selection of TFs which are RAGs that were known or predicted to interact with each other physically or functionally. We selected the TFs ATF3, c-Jun, STAT3 and Smad1. We hypothesised that these TFs could potentially promote the regeneration of injured central DRG axons in the absence of a conditioning lesion. Following adeno-associated viral vector (AAV) overexpression (i.e. *gene therapy*) of ATF3 (which had already been shown to have a positive effect on peripheral nerve regeneration), or the combination of ATF3, c-Jun, STAT3 and Smad1 in DRG neurons of rats, we performed either a dorsal root (DR) injury, or a DC lesion. After DR lesion, both ATF3 and ATF3, c-Jun, STAT3 and Smad1 promoted faster regeneration up to the dorsal root entry zone (DREZ) of the spinal cord, but neither were able to pass. Neither ATF3 nor ATF3, c-Jun, STAT3 and Smad1 were able to promote regeneration through a DC lesion nor produce sensory recovery in sensory or sensorimotor tests. In this experiment, the combination of four TFs performed no better than one.

Following the literature guided approach, we implemented a new experimental methodology in **Chapter 3** which utilized a bioinformatics analysis of the RAG program. We performed transcription factor binding site (TFBS) analysis of all genes of the RAG program, then inferred TFs based on overrepresented TFBSs found in RAGs. Nine TFs were selected for screening, ATF3, CEBPδ, c-Jun, KLF7, MEF2C, SMAD1, SOX11, STAT3 and SRF. Seven were known to be involved in regeneration except MEF2C and SRF, which were novel. Using a DRG-

like cell line, these TFs were tested for their ability to promote neurite outgrowth (an *in vitro* proxy for axonal regeneration), either alone or in combination with each other. Screening identified KLF7 and MEF2C as the TF pair which produced the most neurite outgrowth out of all possible pairs, and ATF3, KLF7 and MEF2C as the TF triple combination which produced the most neurite outgrowth out of selected TF triple combinations. Combinations of more than 3 TFs did not exceed the outgrowth produced by this triple combination.

In **Chapter 4** we tested the TF pair KLF7 and MEF2C, the TF triple ATF3, KLF7 and MEF2C and the individual TFs KLF7, and MEF2C for their ability to promote regeneration after DC lesion *in vivo* following AAV overexpression in DRG neurons of rats. Functional recovery was tested over a course of 11 weeks and at 12 weeks animals were sacrificed for histological analysis of lesion sites. Overexpression of the TF pair of KLF7 and MEF2C together, but not individually, resulted in functional recovery in two sensorimotor tests compared to controls. Histological analysis indicated that only KLF7 and MEF2C expressed together promoted sprouting at the lesion site and prevented axonal retraction which was seen in controls and at varying degrees in other TF groups. The triple TF group, ATF3, KLF7 and MEF2C, which promoted higher neurite outgrowth *in vitro* did not appear to have notable regenerative effects *in vivo*.

In **Chapter 5** we investigated the gene expression changes promoted by overexpressing the TFs ATF3, KLF7 and MEF2C alone, KLF7 and MEF2C together, and ATF3, KLF7 and MEF2C together. In order to reliably measure gene expression in rat large diameter DRG neurons expressing multiple TFs we developed a method of identifying these cells by which we co-expressed each TF with a unique fluorescent protein. This allowed visualisation of neurons co-expressing multiple TFs for laser capture microdissection (LCM). We performed RNA sequencing (RNA-seq) on the laser-captured material to reveal the transcriptome following TF overexpression. In a parallel experiment we also generated a gene expression profile in axotomised large diameter neurons using the same LCM method. Weighted Gene Co-expression Network Analysis (WGCNA) indicated that all TF groups, except MEF2C alone, were able to upregulate forty percent of the genes that are strongly upregulated following a peripheral nerve lesion. Finally, a group of 666 genes was only regulated by axotomy and KLF7 with MEF2C. This group of 666 genes, which included a novel group of myosin and muscle function genes, could hold the key to understanding the mechanism behind the positive effects on axonal regeneration and functional recovery observed in **Chapter 4** after overexpressing KLF7 and MEF2C together *in vivo*. The final experimental chapter, **Chapter 6**, is a methodology paper performed in preparation for the *in vivo* experiments of **Chapter 4**. We wanted to determine which spinal level of DC lesion would result in the optimal duration of functional deficit for functional testing, and which sensorimotor tests most efficiently quantify functional recovery following DC lesion. We performed cervical or thoracic level DC lesions in rats and followed their functional recovery for eight weeks with five sensorimotor

functional tests, including one test we developed which we called the *inclined rolling ladder*. Lesions were complete, with axonal sparing excluded by transganglionic tracing with histology of DC axon targets in the brainstem. We determined that a cervical DC lesion produced a more severe deficit than a thoracic lesion (measuring the hind-paw function). In most tests we were able to detect significant deficits with whole time-course comparisons.

Finally, Chapter 7 provides a summary of the results of this thesis and a more in-depth general discussion addressing the new insights gained and the questions which remain. Specifically, we discuss the relative completeness of the knowledge of which RAGs are regulated after injury, as determined by our lab and others. This stands in contrast to the knowledge gaps that still exist in understanding the mechanisms that coordinate the RAG expression program. We describe the discovery of TF combinations to promote RAG expression and our progress translating the application of these TFs into the *in vivo* situation to promote regeneration. We discuss the possibility of directly screening TF combinations *in vivo* in an efficient manner. An emerging theme of research relevant to efforts to recapitulate the RAG program is the role of epigenetics on the success or failure of regeneration. Finally, we highlight some of the technical advances in AAV-mediated gene delivery and *in vivo* quantification of DC lesions and how they might expedite this type of research.

This research on RAG-associated TFs, and analysis of the RNA-seq dataset from Chapter 5 is being continued by Dr Matthew Mason and Prof. Joost Verhaagen of the NIN, Amsterdam. RAG-TFs identified during this research are currently being tested for their effects on regeneration in different *in vitro* and *in vivo* models by our 'ERA-net Neuron' consortium of collaborators in the Centre for Brain Repair, Cambridge, King's College, London, the German Centre for Neurodegenerative Diseases, Bonn and the Slovak Academy of Sciences, Bratislava.