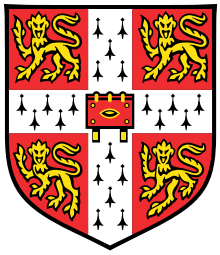
Exploring the methylome and transcriptome of young adult and aged OPCs



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Doctor of Philosophy

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An expert is a person who has made all the mistakes that can be made in a very narrow field.

Niels Bohr

Declaration

I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and Acknowledgements. This dissertation contains fewer than 60,000 words including appendices, bibliography, footnotes, tables and equations and has fewer than 150 figures.

Roey Baror
February 2018

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Abstract

Exploring the methylome and transcriptome of young adult and aged OPCs

Roey Baror

Remyelination is the restoration of myelin sheaths to denuded axons following demyelinating events, which occurs spontaneously in adult mammals, including humans. The principal cells which participate in remyelination are the Oligodendrocyte Progenitor Cells (OPCs). Similar to other regenerative processes, remyelination efficiency declines with ageing. It is still unknown how much of this decline can be attributed to intrinsic changes in the OPCs themselves rather than environmental changes arising from changes in the cellular niche. Thus, we currently have a fundamental gap in our knowledge regarding the basic biology of adult OPCs, and therefore the changes that occur to them with ageing.

In order to address these questions, I have developed a method to reliably isolate all cell types of the oligodendrocyte (OL) lineage from adult rats. This allowed me to identify the specific transcriptome state unique to adult OPCs, which is different to the transcriptome of neonatal OPCs, upon which previous studies have focused. This included genes which support the notion that following the initial phases of developmental myelination, adult OPCs enter a quiescent mode, in a manner similar to other tissue resident stem cells.

Moreover, using a recently established isolation method, I was able to isolate aged OPCs, and develop a transcriptional database that can allow researchers to explore the changes in aged OPCs and identify new targets for enhancing their function.

Lastly, I present in this thesis novel ideas regarding the influence of microglia cell surface molecules on OPC differentiation. I show that changes in the cell surface of aged microglia are inhibitory for OPC differentiation into OLs, and that these changes in microglia are a result of the increase in TGFβ levels with ageing.

In summary, this dissertation introduces new tools and methods that will allow

further in-depth study of adult OPCs, and specifically will help to shed light on the role of adult OPCs in the CNS in homeostasis. Furthermore, I explore the changes that occur within OPCs as they age, and show how such changes reduce aged OPCs ability to efficiently facilitate the process of remyelination.

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Chapter 1

Introduction

1.1 Ageing

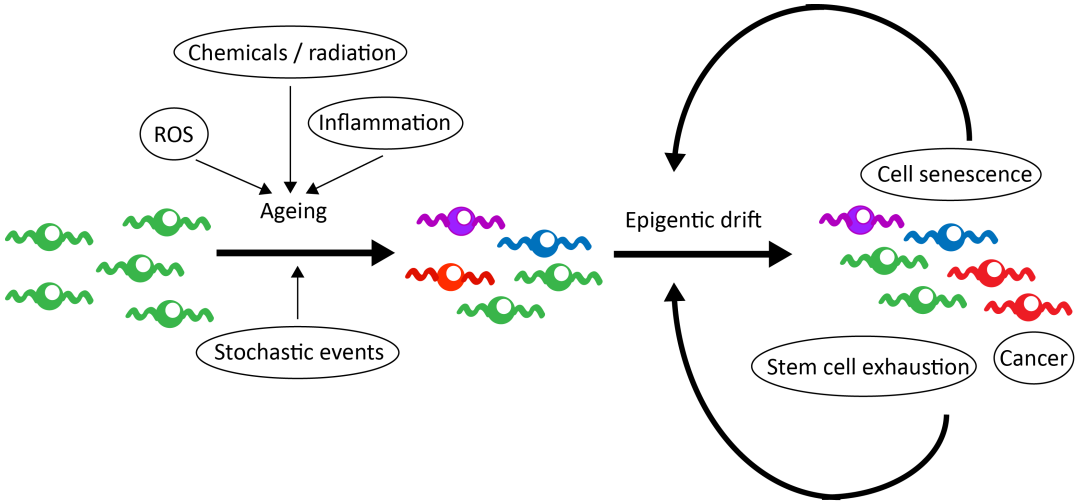
Ageing is the time dependent functional decline shared by all organisms (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013). Among the various phenomena associated with the process of ageing, perhaps the most distinctive one is the decline of the body's endogenous regeneration capabilities. Understanding the factors that govern the ageing process and the search for the secrets of longevity have been sought after by people since ancient times. Today, due to rapid increase in human life expectancy the world's population becomes older (especially in developed countries)(Abajobir et al., 2017), understanding the factors that affect ageing and its associated diseases have become an evermore relevant goal. From an evolutionary point of view, ageing can benefit the population of a specie as a whole, as aged members of the population are replaced by a younger and healthier generation (Kirkwood & Austad, 2000). This theory suggests that ageing is a planned evolutionary mechanism which has evolved through time. Interestingly though, most animals in the wild do not live long enough to grow old. Therefore, it seems that natural selection can only have limited effect on the process of ageing, as natural mortality is usually caused by external factors and occurs mainly in young individuals (Kirkwood & Austad, 2000). In addition to the role of evolution and natural selection in shaping the ageing process, in recent years it has been proposed that the cellular mechanisms that underlie ageing result mainly due to stochastic events, and that cellular damage begins to accumulate from the moment somatic cells and tissues first begin to form (Kirkwood, 2005). As expected, the molecular and cellular processes that underlie ageing are highly complex. The 'hallmarks of ageing' as defined by Lopez-Otin and colleagues (López-Otín et al., 2013) include increasing genomic instability, telomere attrition, altered intercellular

communication, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, stem cell exhaustion, cellular senescence and epigenetic alterations. It is important to note that most likely there is no one clear factor that controls ageing, but rather a combination of the factors mentioned above.
In this thesis I will focus on the epigenetic mechanisms and their contribution to the ageing phenotype, especially in the ageing central nervous system (CNS).

1.2 Epigenetics

The term 'epigenotype' was coined by Waddington in 1942 in order to describe the complex process that lies between the genotype and the phenotype and connects the two (as cited in(Waddington, 2012)). Today the term epigenetics relates to the non-genetic (not encoded in the DNA sequence) mechanisms which control gene expression. Epigenetic mechanisms are studied intensively in various fields of biology, including cancer, various diseases and ageing. Several mechanisms control the epigenome, and the main ones being are the DNA methylation (A. Bird, 2002), histone modifications (for example, acetylation, methylation, phosphorylation and other modifications (Bannister & Kouzarides, 2011)), chromatin remodelling, and non-codingRNA (ncRNA),including microRNA molecules (Sato, Tsuchiya, Meltzer, & Shimizu, 2011). For many years, epigenetic mechanisms have been studied mainly in connection to cancer, as many types cancers exhibit aberrant epigenetic mechanisms that seem to be related to the onset and maintenance of the cancer (Esteller, 2008). This extensive research led to a number of clinical trials which involve epigenetic altering drugs (Nebbioso, Carafa, Benedetti, & Altucci, 2012). Today, more and more studies connect epigenetic alterations to other pathological conditions, including ageing. According to the 'epigenetic drift theory' (Issa, 2014; Martin, 2009), ageing involves the gradual accumulation of epigenetic errors (Huidobro, Fernandez, & Fraga, 2013), as a result of environmental influences (Fraga et al., 2005) and stochastic events (Fraga, Agrelo, & Esteller, 2007; Martin, 2009). This process is schematically illustrated in figure 1.1, which is adapted from Issa et al., (Issa, 2014). As the different epigenetic mechanisms control and affect each other (Fuks, Burgers, Brehm, Hughes-Davies, & Kouzarides, 2000; Fuks, 2005), epigenetic alterations (and DNA methylation in particular) are accumulated in self enhancing cycle with ageing, which ultimately results in chronic changes in the cell transcriptome. In this cycle, DNA methylation stochastic drift (mainly during stem cells proliferation) results in gene expression mosaicism. This leaves the tissue resident stem cell pool depleted. Thus, in the case

of injury which requires the activation of resident stem cells (including proliferation and differentiation), the stem cell response will be inefficient and this can lead to poor regeneration as is usually the result in older age. Other cell populations can harbour epigenetic mutations that can lead to uncontrolled proliferation which will result in cancer. The ageing phenotype promotes further epigenetic alteration by chronic inflammation and uncontrolled proliferation. This way, a vicious self enhancing cycle is created.



Adapted from Issa et al., 2014

Fig. 1.1 **Epigenetic drift in adult stem cells** Illustration of the epigenetic drift cycle. In young age, the tissue resident stem cell population is relatively homogeneous (green coloured cells). Cells can be activated due to normal tissue turnover or as a response to injury. As the organism ages, multiple external forces act on the stem cell population and produce heterogeneity in the population. Thus, the aged stem cell pool is comprised of limited number of 'adequate' stem cells (green cells) which are able to proliferate and differentiate when needed. Other stem cells can become cancerous or enter senescence, thus proving unavailable to to activation.
(adapted from (Issa, 2014))

1.2.1 DNA methylation

DNA methylation is one of the most extensively studied epigenetic markers, and is common to many organisms. In mammalian cells, DNA methylation is mainly performed on a cytosine nucleotide which is located next to a Guanine nucleotide, a sequence usually known as CpG dinucleotide. The addition of methyl group is performed by the DNA Methyltransferase (DNMT) enzymes (Grippo, Iaccarino, Parisi, & Scarano, 1968).

In the vertebrae genome most CpG sites are methylated. In contrast, CpG islands (CGIs), which are regions in the DNA that are enriched in GC content (and usually located around gene promotors - in the human genome, approximately half of the promoters have CpG islands at their 5' end (Deaton & Bird, 2011)), are usually not methylated (Cross & Bird, 1995).

There are two main types of DNMT enzymes: *de novo* DNMTs, which include DNMT3a and DNMT3b which are responsible of creating new 5mC markings on un-methylated DNA strand (Fig1.2A). The second type is the maintenance DNMT, DNMT1. DNMT1 is usually localised around the replication fork during DNA replication. This enzyme is responsible for the heredity properties of DNA methylation, as it methylates the newly formed semi-methylated DNA strand according to the pre-existing pattern (A. Bird, 2002). Thus, after each cell proliferation, the daughter cells will maintain a similar methylome to the parent cell (Fig1.2B).

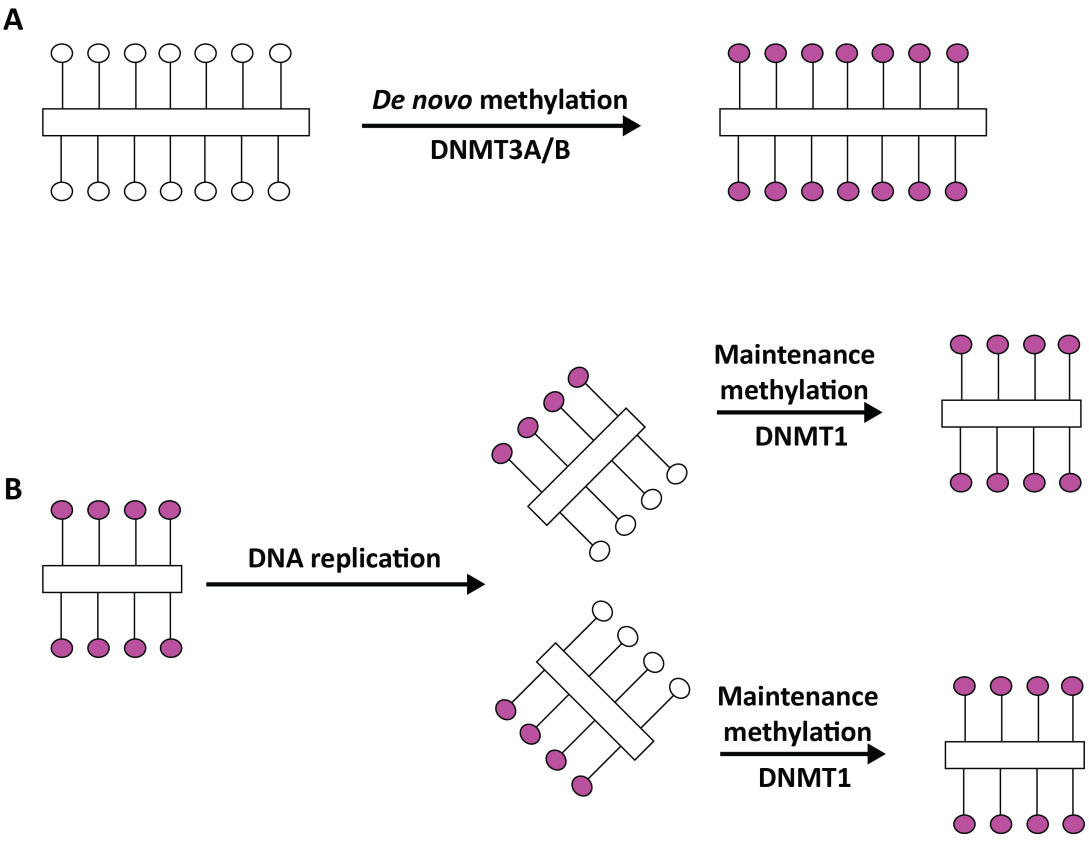


Fig. 1.2 ***De novo*** **and maintenance DNA methylation in mammals** Schematic illustration of the DNA methylation mechanisms in mammalian cells. **(A)** Unmethylated CpGs (marked by white circles) are *de novo* methylated (magenta circles) by DNMT3A/B. Both strands of the DNA are methylated in a similar pattern. **(B)** Following DNA replication (during cell division) the newly made DNA strand is unmethylated, and is then methylated by DNMT1 according to the pattern of the original DNA strand. (adapted from (T. Chen et al., 2003))

DNA methylation is usually associated with gene suppression and this can be done by directly affecting the affinity of transcriptional proteins to the DNA strand (Fig1.3A) or by recruiting repressive complexes (such as, MeCP2 or MBD1). These proteins can then either directly prevent the binding of the transcriptional machinery (Fig1.3C), or recruit histone modifications enzymes that will make the chromatin less accessible to transcription (Fig1.3D) (Nan et al., 1998; Wolffe et al., 2000).

Alternatively, there are some examples of DNA methylation which enhance gene expression. For example, in neural stem cells differentiation, DNA methylation inhibits the inhibitory PcG histone methylation (H3K27Me3) and is vital for the expression of genes which promote neurogenesis (Wu et al., 2010). Another example is the changing binding affinity of CTCF to enhancer regions related to the *Igf2* gene. CTCF is a transcriptional repressor, which has CpG rich binding sites. Methylation of these

binding sites reduces the binding ability of CTCF, and therefore enhances the activation of nearby genes (A. Bird, 2002).

Moreover, some reports claim that genes can be activated while being methylated, as using histone modification inhibitor can activate genes despite their methylation status (Raynal et al., 2012). This suggests a role of epigenetic memory for DNA methylation, and not necessarily active gene silencing.

DNA methylation is considered to play a crucial role in embryonic development, specifically in the control of cell differentiation (Cortese et al., 2011; Oda et al., 2013).

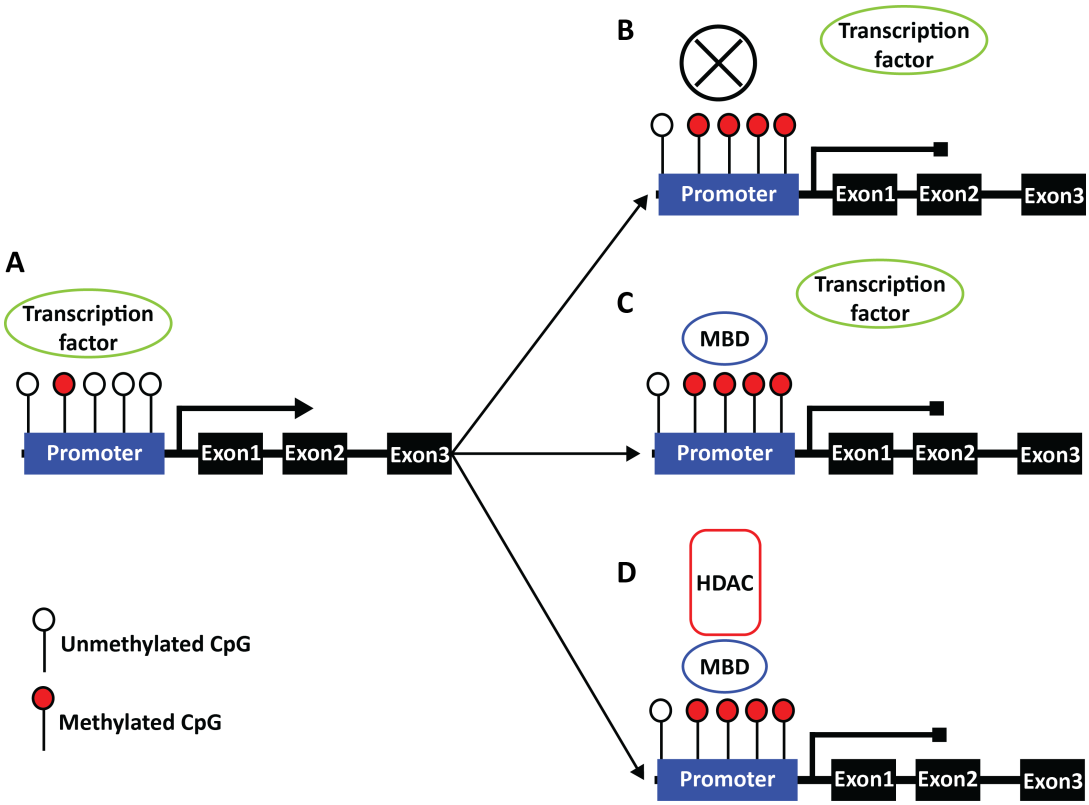


Fig. 1.3 **Transcription inhibition by DNA methylation** Schematic illustration of the mechanisms in which DNA methylation in promoters can inhibit gene transcription. **(A)** Active gene, with low rate of CpG methylation in the promoter region. Following methylation, the gene can be silenced through three main mechanisms:
(B) DNA methylation of CpGs can directly inhibit the binding of specific transcription factors to promoter and enhancer regions. **(C)** DNA methylated regions can be occupied by proteins with Methylation Binding Domains (MBD), thus blocking the binding of transcription factors and/or the transcription machinery itself. **(D)** Methylation of CpGs can recruit HDACs and other histone modifiers which will compact the DNA and make it unaccessible to the transcription machinery.
Adapted according to the following - (Cedar & Bergman, 2009; Fuks, 2005; Nan et al., 1998; Wolffe et al., 2000).

DNA methylation control

The dynamic turnover of DNA methylation and de-methylation is controlled by methylation and recently discovered de-methylation enzymes. In 2009 it has been discovered that enzymes from the TET (Ten-eleven translocation methylcytosine dioxygenase) family (specifically, TET1) can promote active DNA de-methylation by transforming 5mC into 5-hmC (5-Hydroxymethylcytosine). 5-hmC can then be further transformed and ultimately removed by the DNA base excision repair machinery (BER) (Branco, Ficz, & Reik, 2012; Tahiliani et al., 2009). The discoveries of the further modifications of cytosine to 5-hmC might present another layer of genome modifications which is yet to be studied thoroughly.
DNA methylation can be affected by metabolic and mitochondrial processes. For example, Krebs cycle intermediates (such as, succinate and fumarate) are inhibitors of large family of 2-oxoglutarate-dependent dioxygenases (2-OGDO). This family of enzymes includes the TET1 demethylation enzymes (see above). Moreover, TET enzymes demethylation activity is dependent on α-ketoglutarate (another Krebs cycle intermediate) (Salminen, Kaarniranta, Hiltunen, & Kauppinen, 2014).
Other control mechanisms that affect DNA methylation include the involvement of miRNA and other ncRNA in control and guidance of DNA methylation. For example, Di Ruscio and colleagues have discovered that a (l)ncRNA (long non coding RNA) arising from a specific gene can directly regulate the methylation of the same gene by connecting to DNMT1 and inhibiting its activity (Di Ruscio et al., 2013).

DNA methylation and ageing

Epigenetic mechanisms in general and DNA methylation in particular have long been suspected to play a role in ageing, as a complex that can connect between the environmental effects and the genotype in order to express a specific phenotype (Calvanese, Lara, Kahn, & Fraga, 2009). Early studies described a global DNA hypomethylation as organisms age, but more recent studies have shown local DNA hypermethylation in specific genes (and especially CpG island methylation), some associated with tumour suppression (Maegawa et al., 2010).
According to some researchers this accumulations of errors in the DNA methylation patterns globally, will eventually lead to cellular malfunction, whether it is by 'over-silencing' crucial genes, or by not silencing specific genes when needed. Although the specific mechanisms relating DNA methylation and ageing are not clear yet, the accumulations of errors in the methylome of the cell will inevitably result in aberrant transcriptional control. This is especially important for quiescent stem cells, 'waiting'

in tissue niches for activation cues. Changes in these cells methylome, can render them less responsive to external cues and will delay their timely activation, leading to delayed regenerative capacity as the tissue remains damaged (see Fig1.1).

1.2.2 Histone modifications

A key epigenetic process involves histone tail modifications. Some modifications result in transcriptional inhibition, whereas others enhance gene transcription. The following section will survey the biology and key enzymes behind the main modifications which have been shown relevant to oligodendrocyte biology.

- **Histone acetylation** - Histone tails acetylation is carried out by histone acetyltransferases (HATs), whereas the removal of the acetyl groups is carried out by histone deacetylases (HDAC). By adding acetyl groups to lysine residues of histones (both tails and body), HATs neutralise positive charges, thus destabilising the structure of the histone, making it more accessible for transcription.
  HDACs act to remove acetyl groups, thus restoring lysine positive charges and thereby restoring the chromatin compact structure. This usually results in inhibited gene transcription. HDACs are divided to multiple classes, including class III HDACs named Sirtuins (NAD dependent HDACs). This group of enzymes are involved in ageing and longevity in multiple organisms (Pollina & Brunet, 2011).

- **Histone methylation** - Histone methylation is usually performed on side chains of lysines and arginines. Histone methylation alters the charge of the histone protein. Moreover, histone methylation can occur in multiple forms: mono-, di- or tri-methylated. The result of the methylation (suppression or enhancement of gene transcription) depends on the site of methylation, as well as on the number of methyl groups added. Histone methylation can also affect alternative splicing, and recently has been shown to play a role in memory formation in neurons (Ding et al., 2017).

- **DNA methylation and histone modification cross talk** - The cross talk between DNA methylation and various histone marks has been shown multiple times. It is unclear which occurs first. Does DNA methylation induces further repressive histone marks in order to 'lock' silenced gene transcription or whether it is the opposite? (Fuks, 2005).
  In regards to HDAC activity, in some cases histone deacetylation directs DNA

methylation (Cervoni & Szyf, 2001), in which DNA methylation changes state. The state of methylation or de-methylation is controlled by the histone marks around that genomic area. The direct link between DNA methylation and histone modifications can be mediated through methylcytosine-binding protein, such as MECP2 or MBD1/2, all of which have been shown to be able to recruit histone modification enzymes (Cedar & Bergman, 2009).

The epigenetic mechanisms described so far are relevant in multiple regenerative processes in various tissues in the body. In the following sections I will describe the process of remyelination, the regeneration of myelin sheaths in the central nervous system, and the potential effects of epigenetic alterations on this process, with an emphasis on ageing.

1.3 Remyelination

Regeneration processes are an essential during the lifetime of any organism. In mammals, regenerative processes take place in most tissues to some extent, and are usually dependent on the function of specialised tissue resident stem cells. The regenerative capacity changes within the animal kingdom ranges from the remarkable fully regenerative planarian flatworms (Montgomery & Coward, 1974), through full limb recovery achieved by the salamanders (Sanchez Alvarado & Tsonis, 2006) up to the comparatively limited mammalian regeneration abilities (Sanchez Alvarado & Tsonis, 2006; Tanaka, 2003). Even though regenerative processes are limited within the mammalian realm, there are some tissues that can undergo extensive repair following lesion, such as skin and muscle (Muneoka, Allan, Yang, Lee, & Han, 2008). Moreover, even in the Central Nervous System (CNS), which for years has been considered as a non regenerative system, there are examples for regeneration processes that can proceed throughout life. One of these processes, is the process of remyelination, in which demyelinated axons are wrapped again by newly formed myelin sheaths. Neural axons in the vertebrae central nervous system (CNS) and Peripheral Nervous System (PNS) include myelinated and non myelinated axons. Myelin is made by specific cells, Oligodendrocytes (OL) in the CNS and Schwann cells in the PNS. It consists mainly of lipids (75%) together with various proteins, which play a critical role in myelin compaction, as well as in transport of metabolic support to the axons (Morrison, Lee, & Rothstein, 2013; Nave, 2010). Myelination allows the increase of signal transduction velocity in the CNS, while maintaining relatively small axon diameter (Zalc, Goujet, & Colman, 2008). This is achieved by partial myelination of stretches of axons named

'internodes', as they stretch between 'nodes of Ranvier'. The nodes of Ranvier are areas on the surface of the axons which are characterised by high expression of various ion channels. These areas allow the propagation of the neuronal action potential along the axon, in what is known as saltatory conduction. In this way the axon potential can be propagated 5-50 times faster than in a comparable unmyelinated axon, while maintaining relatively small diameter. The increase in signal transduction has enabled the dramatic advancement in the capabilities of the members of the vertebrae kingdom, allowing more complex and efficient neuronal networks.

The role of myelinating glial cells is not restricted only to insulating roles and signal transduction, but they also extend trophic support to the axons. Evidence for this role is shown by studying mice which are deficient in various myelin proteins, such as *Plp-/-* (Griffiths et al., 1998) or *Cnp1-/-* (Lappe-Siefke et al., 2003). These mice show what seems to be complete and intact structure of myelin sheaths, but at the same time show axonal degeneration. Thus, even though the myelin sheaths in these mice serve their structural role as insulators for axons, they cannot give the support needed for axonal maintenance. This phenomena is also expressed in humans who have mutations in the *Plp1* gene, and therefore suffer from the leukodystrophy Peliazeus Merzbacher disease (PMD), which exhibits a similar pathology (Garbern et al., 2002).
Hence, due to the major role myelin serves in both supporting axons, as well as enabling fast signal transduction, demyelination, the pathological process of myelin loss due to OL damage and/or death, in which axons remain nude, can lead to axonal and neuronal degeneration (Y. Lee et al., 2012). In humans, demyelination can result from either genetic mutations in glial cells (for example, leukodystrophies) or from inflammatory damage to myelin and OL cells as in Multiple Sclerosis (MS) (Franklin & Ffrench-Constant, 2008). The leukodystrophies are a group of diseases that arise due to impairment in myelin development and maintenance arising from genetic mutations in various myelin proteins. This includes several diseases, such as X-linked adrenoleukodystrophy (*Abcd1* mutation), Pelizaeus-Merzbacher disease (*Plp1* mutation) and Alexander's disease (mutations in *Gfap*), among others. A common outcome to all of these is axonal damage, caused due to myelin deformation (Mar & Noetzel, 2010).

Inflammation based myelin damage does not usually result from genetic mutations in myelin genes but is usually caused by autoimmunity, of which the most common one is Multiple Sclerosis (MS) (Compston & Coles, 2008) . In this autoimmune disease, OL specific proteins are targeted by the immune system, which leads to immune attacks on the myelin sheaths. This results in myelin loss, and if the myelin is not restored this leads to axonal damage. Remyelination, on the other hand, is the regenerative process

in which myelin sheaths are restored to the axons following a lesion (W. Blakemore, 1974). The main cell type which plays a role in remyelination are the Oligodendrocyte Progenitor Cells (OPC)(Ffrench-Constant & Raff, 1986; Raff, Miller, & Noble, 1983; Zawadzka et al., 2010). These progenitor cells reside in the developing and adult CNS are estimated to constitute 5% - 8% of the cells in the adult brain (Levine, Reynolds, & Fawcett, 2001). While OPCs are the key mediators of remyelination, the major events of this process are orchestrated by a variety of molecules and cells, including CNS resident cells such as microglia (Miron et al., 2013), astrocytes (Talbott et al., 2005), as well as non CNS cells such as circulating monocytes (Kotter, Setzu, Sim, Van Rooijen, & Franklin, 2001) and T cells (Bieber, Kerr, & Rodriguez, 2003; Dombrowski et al., 2017). The different activated cells are all aimed at recruiting OPCs to the lesion, while creating a pro regenerative environment which will enable the OPCs to proliferate and ultimately differentiate into mature myelinating OL in order to remyelinate the axons. The process of remyelination can be divided into multiple phases (see Fig1.4), according the function of the OPCs and the supporting cells. These steps include the activation, recruitment and finally differentiation of OPCs. For the successful completion of each step, an exact combination of cells and molecules are needed.

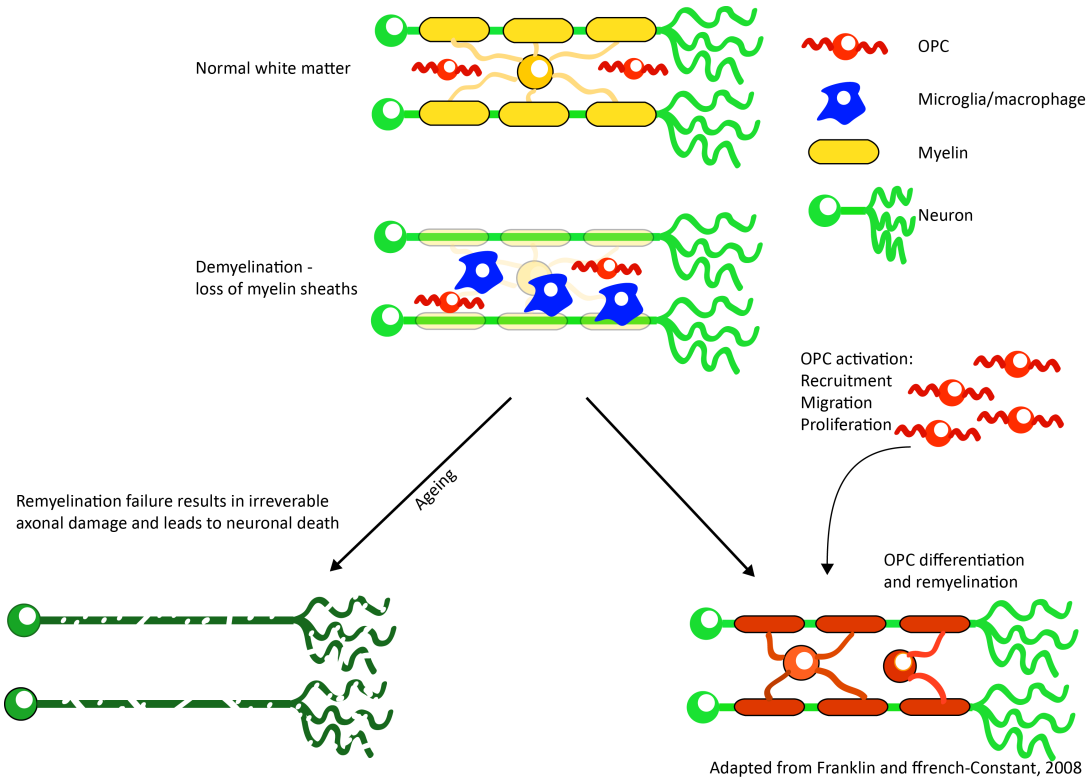


Fig. 1.4 **Remyelination** An illustration of the remyelination process. During homeostasis, axons in the CNS (labeled in green) are myelinated by OLs (labelled in yellow). OPCs (labelled in red) are present throughout the CNS in a non active bi-polar form. Following demyelination, myelin and/or OLs are damaged. This activates a response by microglia (and later on invading macrophages, labelled in blue) which arrive at the lesion area and phagocyte myelin debris. In young mammals this is followed by the activation and recruitment of OPCs to the lesion site where they differentiate into newly formed OLs which then remyelinate the nude axons (marked in orange). In aged mammals, this process fails mainly due the limited differentiation capacity of aged OPCs, thus leading to axonal damage and ultimately neuronal death.

1.3.1 Studying remyelination

Since myelination plays a pivotal role in the normal neuronal activity it is important to study how to enhance the regeneration of myelin (remyelination) after demyelinating lesion, such as in Multiple Sclerosis. For that purpose, multiple animal models have been developed, with the main ones being either toxin based models, or immune mediated models.

- **Toxin induced demyelination models -** These models are based on the injection (or feeding) of specific toxins that mainly target OLs, and thus result in rapid demyelination that does rely on immune system response. In models in

which toxin is injected, it is common to use either Ethidium bromide (EB) or Lysolecithin (Woodruff & Franklin, 1999) injected either to the spinal cord or the cerebral peduncle. The injections are aimed at white matter areas and therefore the toxins kill mainly OL, and this results in a local area of demyelination. Following the injection, a healing process begins following a well defined timetable (according to the species used and its age). This model main advantage are the local and specific lesion and known time scale for regeneration. However, these are not intended to mimic MS as they do not include an immune component . Another toxin based model of demyelination, is the cuprizone model. In this model, animals are fed cuprizone (a copper chelating substance) in their regular diet. This leads to OLs death, which is observed usually in the corpus callosum. Once cuprizone is withdrawn from the diet of the animals, remyelination processes begin (Gudi, Gingele, Skripuletz, & Stangel, 2014).

- **Autoimmune based demyelination models -** As the main demyelinating disease in humans is MS, which is caused to autoimmune attack aimed at OLs (Compston & Coles, 2008), models have been developed to mimic the immune component of the diseases, in addition to demyelinating one which is isolated in the toxin based models. The main model in this category is the EAE model (Experimental autoimmune encephalomyelitis). This model is based on injection of myelin antigens (together with adjuvant) and eliciting immune response in mice, thus activating the immune system against OLs. Unfortunately, the disease course in animal models does not follow the exact same course as in humans, thus making it difficult to use thus model for drug development. (Ransohoff, 2012). Moreover, due to the complexity of the initial immune response, it is difficult to isolate the actual process of remyelination and the causes for its failures.

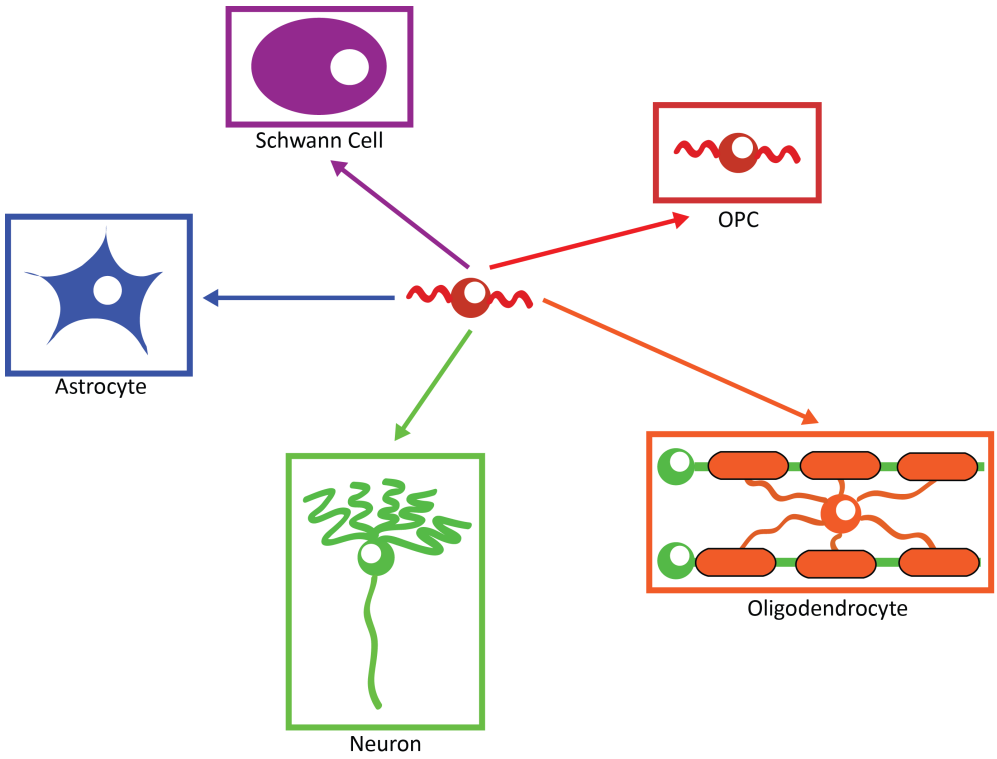
As toxin based models give the advantage of isolating the demyelination process itself without relying on immune response, it is a more common model used when trying to elucidate the changes in remyelination efficiency and the responses of OPCs to demyelinating lesions.

1.4 Oligodendrocyte Progenitor Cells (OPCs)

Ever though the process of remyelination was described several decades ago, (W. Blakemore, 1974), it has been debated what is the origin of the remyelinating cells. Some

researchers have suggested that surviving OL contribute to remyelination, but it is has been shown that the majority (if not all) of remyelination that occurs following demyelination lesions is due to the activity of Oligodendrocyte Progenitor Cells (OPCs) (W. F. Blakemore & Keirstead, 1999; Gensert & Goldman, 1997; Levine & Reynolds, 1999). More recently, using *Mbp* and *Opalin* reporter lines (marking only mature oligodendrocytes) Crawford and colleagues have shown that mature OLs do not contribute to remyelination in toxin induced models (A. H. Crawford, Tripathi, Foerster, et al., 2016).

Earliest descriptions of OPCs were done already in the 1980's (Ffrench-Constant & Raff, 1986; Raff et al., 1983), where they were termed bi-potential oligodendrocyte-type-2 astrocyte (0-2A) progenitor cells, in order to emphasis their dual differentiation potential, as they can differentiate to astrocytes as well as oligodendrocytes. In later years, it has been shown that adult OPCs can differentiate into multiple cell types, including Schwann cells (Zawadzka et al., 2010) and neurons (Belachew et al., 2003), as well as being able to proliferate (Dawson, Polito, Levine, & Reynolds, 2003) (see Fig1.5 for illustrative summary).



Adapted from Crawford et al., 2014

Fig. 1.5 **Multipotency of adult OPCs** Illustration summarising the various cell fates exhibited by OPCs. Adult OPCs have been shown to be able to give rise to mutliple cell types in the CNS (marked in different colours), including oligodendrocytes (orange), astrocytes (blue), Schwann Cells (magenta) and neurons (green), as well as being able to self regenerate (red). Illustration was adapted from Crawford et al., (A. Crawford et al., 2014).

OPCs arise in embryonic stages in multiple generation waves, both in the spinal cord and the forebrain (A. H. Crawford, Tripathi, Richardson, & Franklin, 2016). The first wave of OPCs in the spinal cord arises at embryonic day 12.5 (E12.5) from the ventral ventricular zone and this process is controlled by the expression of Sonic Hedgehog (Shh). Shh has been shown to be both necessary and sufficient for the expression of the bHLH (Basic Helix Loop Helix) transcription factor OLIG2, a central transcription factor in OPC maintenance and differentiation (Lu et al., 2000). The second wave of OPCs arise on E15.5 from the dorsal VZ, and is Shh independent (Cai et al., 2005), and these will eventually make up approximately 20% of the total OPC population.

In the developing forebrain, the first wave of OPCs arises at day E12.5 in the medial ganglionic eminence (MGE) and anterior entopeduncular area in the ventral telencephalon (forerunner of the forebrain). These cells will be largely eliminated by

the time of birth. The second wave of OPCs (at day E15.5) arises from lateral and caudal ganglionic eminences (LGE and CGE), and the final wave arises at the time of birth at the cortical VZ (A. H. Crawford, Tripathi, Richardson, & Franklin, 2016). In recent study it has been shown that OPCs which arise from different developmental origins differ in their roles and remyelinating capabilities in adulthood and old age (A. H. Crawford, Tripathi, Richardson, & Franklin, 2016). Further studies into the differences between OPCs of differing origins are currently underway, with emphasis on the role of OPCs in normal brain state (Forster 2017, personal communications).

It is unknown exactly what is the role of OPCs in adulthood, once developmental myelination is completed (around the age of two months in rodents), but as described above they play a pivotal role in recovery following demyelinating lesions. OPC response to demyelination is a complex process which can be divided into several phases.

The first stage includes the activation of OPCs. This includes a switch from a quiescent state to more proliferative and responsive state. OPC activation is triggered by the actions elicit by microglia and blood-borne macrophages which infiltrate the CNS and populate the lesion area, as well as by activation of astrocytes. The release of multiple inflammatory molecules following demyelination, activates the OPCs and induces their re-entry into cell cycle (Franklin & Ffrench-Constant, 2008; Rhodes, Raivich, & Fawcett, 2006) (a more comprehensive description of the roles of the immune system in remyelination is found in section 1.4.1). The activation of OPCs includes the up-regulation of multiple genes, including *Cspg4* (NG2) (Levine & Reynolds, 1999), *Olig1/2*, *Nkx2.2* (Fancy, Zhao, & Franklin, 2004) and *Sox2* (Zhao et al., 2015) among other factors (full description of transcription factors involved in OPC differentiation can be found in section 1.4.2).

Following their activation, OPCs migrate to the lesion area by following various chemokines and growth factors released by the immune cells which colonise the lesion. Upon arrival to the lesion site, OPCs will undergo extensive proliferation (Redwine & Armstrong, 1998), thus more OPCs than required will be generated. Multiple factors drive OPC proliferation, including PDGF, FGF and IGF-1 (Hinks & Franklin, 1999) -

- **PDGF** (platelet-derived growth factor) is secreted mainly by astrocytes, and acts in OPCs through its type A receptor, PDGFRA (expressed mainly by OPCs in the CNS). *In vitro* administration of PDGF promotes OPC proliferation (Wolswijk & Noble, 1992). Similarly, over expression of *Pdgf in vivo* in transgenic mice results in increased numbers of OPCs in demyelination lesions (Woodruff, Fruttiger, Richardson, & Franklin, 2004)) (but does not improves remyelination

in aged animals).

- **FGF-2** (fibroblast growth factor) is another growth factors of which its levels are increased during the early stages of remyelination (Hinks & Franklin, 1999). FGF indirectly effects OPC proliferation by modulating PDGF dependent pathway (McKinnon, Matsui, Dubois-Dalcq, & Aaronsont, 1990). FGF-2 production is upregulated in astrocytes by the CNTF (Albrecht et al., 2003), and CNTF itself promotes the survival of OPCs *in vitro* (Talbott et al., 2007).

- **IGF-1** (Insulin-like growth factor 1) is suggested to have an important role in both proliferation and the survival of OPC, as *Igf1r-/-* mice show poor remyelination following cuprizone induced demyelination (Mason, Xuan, Dragatsis, Efstratiadis, & Goldman, 2003).

Besides these growth factors which are secreted mainly by astrocytes, various cytokines released by immune cells present in the lesion (mainly activated microglia and macrophages) play a crucial role in the activation and proliferation of OPCs.

Following their rapid proliferation, in order to complete the remyelination process, OPCs must switch from proliferative cell type to a differentiating one, thus exiting the cell cycle (Caillava et al., 2011) and activating a set of genes that will eventually induce the production of myelin sheaths. A timely withdrawal of pro-proliferation growth factors is essential for the successful exit of cell cycle and the beginning of OPC differentiation (Hinks & Franklin, 1999, 2000; Tokumoto, Durand, & Raff, 1999). Thus, any delay in arrest of growth secretion can cause a delayed differentiation, which can result in axon degeneration.
The process of OPC differentiation includes both morphological changes, which are accompanied by intrinsic changes in gene expression. Figure 1.6 illustrates both the morphological changes that occur along OPC the differentiation of OPCs, as well as shows some of the markers frequently used to identify the different phases in differentiation.

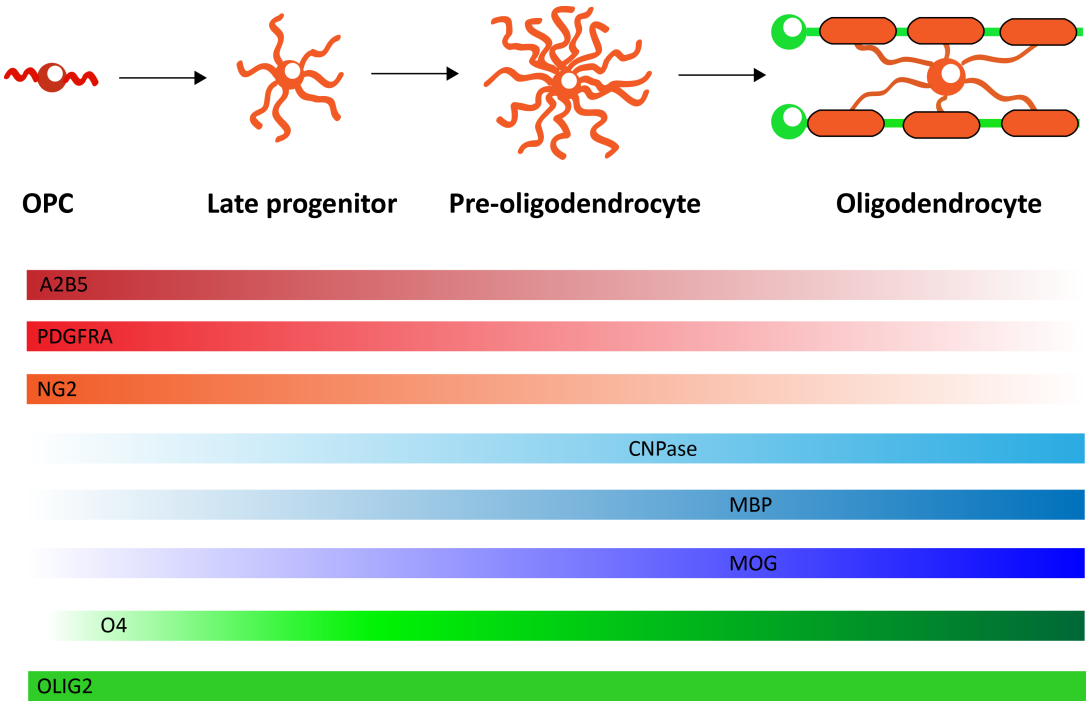


Fig. 1.6 **OPC differentiation** Illustration of the differentiation process of OPCs. During the differentiation into oligodendrocytes, OPCs exhibit morphological and transcriptomic changes. Changes in morphology include the transition from a bi-polar cell (left hand side) to a multi process cell, and ultimately the generation of myelin membranes and axon ensheathment. The lower panel of the figure shows important markers and their expression patterns during OPC differentiation. OPC early markers are labelled in red, while OL markers are labelled in blue. Green labelled markers are expressed throughout the lineage.
Adapted from (Fancy et al., 2009).

Thus, the differentiation process in OPCs is governed by both extrinsic factors (secreted by other cell types as well as other OPCs), and intrinsic mechanisms. In the following sections (1.4.1 and 1.4.2) I will review the key external components and and internal processes that govern OPC differentiation as known from current literature.

1.4.1 Extrinsic factors in remyelination and OPC differentiation

separate

Innate immune system in remyelination

The process of remyelination relies of a complicated orchestra on cells types with multiple divergent roles. One of the most important cell types in the process of remyelination, are the cells of the innate system, and more specifically the microglia

cells, the resident mononuclear phagocytes of the CNS (Chan, Kohsaka, & Rezaie, 2007).

First described as separate cell type by Pio del Rio-Hortega in 1919 (Kettenmann & Verkhratsky, 2008) the origins of the microglia in the CNS have remained a controversial for many years. Unlike other glia cells types (astrocytes, OL and OPCs) and neurons which all arise from the neuroepithelial layer, it has been disputed whether or not microglia also rise from the neuroectoderm or do they derive from ancient mesodermal origin (similar to other tissue resident macrophages) (Cuadros & Navascués, 1998). In 2010, an extensive study by Ginhoux and colleagues seems to have ended much of the dispute (Ginhoux et al., 2010). According to multiple lineage tracing essays presented in the study, microglia cells originate from primitive myeloid precursors. These precursors differentiate in the yolk sac of mammals before the onset of blood circulation, and thereby arise before embryonic day 8 in the mouse. Throughout adulthood, the microglial cell pool is mainly self renewed and does not rely on blood circulating monocytes, which is the case in other tissue resident macrophages (Ajami, Bennett, Krieger, Tetzlaff, & Rossi, 2007; Ginhoux et al., 2010; Mildner et al., 2007).

Microglia play important roles during development, as well as during normal homeostasis in the brain (Paolicelli et al., 2011). In normal adult conditions, microglia posses a ramified morphology and are distributed evenly throughout the CNS. Microglia constantly survey their environment and detect new threats, as well as playing a role in maintaining brain homeostasis (Nimmerjahn, 2005). The constant surveillance allows microglia to quickly respond to any event in the CNS and restore homeostasis by phagocyting cellular debris. There are many differences between microglia and infiltrating macrophages, both transcription wise (Lavin et al., 2014), as well as evidence for the distinct role in states of inflammation, such as in MS (Yamasaki et al., 2014). Due the similarities of microglia and infiltrating macrophages, only recently tools have been developed that allow simple and reliable distinction between the two populations (Bennetta et al., 2016).

Microglia play a crucial role during remyelination, and are thought to play an important role in the beginning of pathological process in MS which can be exhibited in two main forms studied extensively in past studies:
Firstly, microglia are responsible for clearing myelin debris from the lesion site (Lampron et al., 2015). Microglia are the first cells which migrate to the lesion and the removal of debris after injury/lesion is crucial step for a successful regeneration of the lesion. Efficiency of myelin debris clearance can determine the efficiency of the whole remyelination process as myelin debris is inhibitory to OPC differentiation both *in vitro* (Robinson & Miller, 1999), and *in vivo* (Kotter, Li, Zhao, & Franklin, 2006).

This is also evident in older animals in which myelin clearance is slower and therefore inhibits OPC differentiation, although this can be reversed (Natrajan et al., 2015; Ruckh et al., 2012). It has been proposed that higher efficiency of myelin clearance in the PNS (in comparison to the CNS) is one of the crucial factors for which PNS axonal regeneration is more successful in comparison to the CNS (Vargas & Barres, 2007).

The second role of microglia is the secretion of pro regenerative factors that at the first step will induce OPC migration to the lesion site, and later will promote their differentiation (Miron et al., 2013). Microglia express and secrete a variety of molecules which can have either pro or anti regenerative effects. The microglia expression profile depends largely on the their type of activation. Classical studies have largely divided microglia activation states into two distinct modes: pro-inflammatory (classical activation, also known as M1) and anti-inflammatory (termed as alternative activation, M2). These activation modes, have been linked to *in vitro* settings. M1 macrophages are generated by activating microglia with Lipopolysaccharides (LPS) and this imitate primed inflammatory microglia/macrophages *in vivo*. Similarly, activation of microglia/macrophages using IL-4 directs them to a M2 phenotype, which mimics an alternative activation mode. Classical notion holds that as a general rule, pro-inflammatory microglia (M1), and inflammation in general are inhibitory to regeneration in general and remyelination specifically, whereas M2 microglia are supportive of regeneration and remyelination specifically (for example, (Miron et al., 2013)). More recent studies have challenged both of these claims.
Firstly, the artificial divide between M1 and M2 microglia/macrophages, and the notion that LPS/IL-4 *in vitro* activation mimics the *in vivo* phenotype is heavily disputed by many researchers in the field (Martinez & Gordon, 2014; Ransohoff, 2016). This is supported by studies which use advanced genomic techniques that show that there are profound differences between *in vitro* M1 microglia and their *in vivo* counterparts they are suppose to mimic - primed microglia from inflamed tissue. (Holtman et al., 2015) Another perhaps oversimplified assumption which is has been challenged in recent years, is the assumption that inflammation (and therefore M1, or pro inflammatory microglia/macrophages) are inhibitory to regeneration and remyelination in general, whereas a M2 phenotype (alternative activation) is a more pro regenerative one. For example, Butovsky and colleagues have shown that conditioned media from LPS activated microglia inhibits Neural Stem Cells (NSCs) differentiation, both into neurons as well as into OL. In comparison, conditioned media from IL-4 activated microglia, have enhanced NSCs differentiation *in vitro* (Butovsky et al., 2006). Thus, it seems that this clear cut distinction regarding the protective role of anti-inflammatory microglia versus the pro-inflammatory counterparts is not accurate.

Several reports have shown that pro-inflammatory cytokines play a crucial role on remyelination, including TNFα (Tumour Necrosis Factor alpha) and IL1-β. TNFα (a potent pro inflammatory cytokine) is beneficial and required for successful proliferation of OPCs and remyelination (Arnett et al., 2001). In contrast, TNFα has also been shown to play a role in the actual process of demyelination by inducing cell death through TNF receptor 1 (*Tnfr1*) and thus causing OL death directly, and this is without the contribution of BBB breakdown (Akassoglou et al., 1998). Despite this, TNFα null mice show lower rates of remyelination, which suggests that TNFa beneficial effects outweigh its deleterious effects (Arnett et al., 2001). Similarly, IL-1β has also been shown to regulate OPC proliferation and promote their activation (Arnett, Wang, Matsushima, Suzuki, & Ting, 2003; Mason, Suzuki, Chaplin, & Matsushima, 2001; Vela, Molina-Holgado, Arévalo-Martín, Almazán, & Guaza, 2002).

Past studies have shown that microglia do not undergo major changes in their transcriptome in the transition between demyelination and remyelination in cuprizone model (Olah et al., 2012). According to Olah et al., microglia do not exhibit significant global changes in their transcriptome upon transition from demyelination stage to remyelination. This is in contrast to later studies, such as by Miron el al (Miron et al., 2013) which show a significant switch in microglia phenotype during remyelination. The discrepancies between the studies can arise from multiple technical reasons as the two studies used different types of toxin induced demyelination (cuprizone vs focal injections of lysolecithin/ethidium bromide). The models differ in processes and development of the disease. Moreover, Olah et al have used a microarray which does not encompass the full transcriptome (but only 9000 genes) whereas Miron et al used specific M1/M2 markers to signify the transition that microglia undergo during remyelination.

The role of neuronal activity in remyelination

The role of neuronal activity in myelination (as well as remyelination) is the subject of intense research. Early studies have claimed that OPC proliferation *in vivo* is depended on electrical activity in axons in developing rat optic nerve (B. a. Barres & Raff, 1993). In these studies, lack of activity in in the optic nerve (either due to transection of the nerve or by using TTX toxin) resulted in decreased proliferation of OPCs, which could be overcome by the addition of PDGF. More recent studies have strengthened the notion that axonal activity play a role in OPC activation by showing that OPCs express NMDA receptors (Káradóttir, Cavelier, Bergersen, & Attwell, 2005) which are activated in the event of ischaemia. Moreover, in the adult CNS a recent report has

shown neuronal activity enhances myelination and that this myelination in the adult CNS is required for some types of learning (Gibson et al., 2014). Other molecules which have been shown to be released from neurons and affect OPCs are Semaphorins, which usually act in directing axonal growths cones. In particular, class 3 Semaphorins have been shown to exert various effect on OPCs migration and activation, in which Semaphorin 3A acts to repel OPCs (Syed et al., 2011), whereas Semaphorin 3F acts as a chemotactant and attracts OPCs (Piaton et al., 2011).
While these studies support the notion that myelination (both in the developing CNS, as well as in the adult CNS) is dependent on axonal and synaptic activity, *in vitro* experiments using artificial nano fibres show that OPC innate myelination capabilities are robust and do not require signals from axons. In these experiments even though OPCs are cultured in presence of inert nano fibres and receive no electrical/biochemical signals from the fibres, quickly after differentiation they start wrapping the fibres (S. Lee, Chong, Tuck, Corey, & Chan, 2013). Following these insights, a study by Redmond and colleagues have shown that OPCs in culture can wrap around other structures which are not necessarily fibres, such as cones. Using a comparison between transcriptome data from axons and dendrites Lee and colleagues identified JAM2 as a surface antigen expressed mainly on dendrites and which inhibits myelination by OPCs (Redmond et al., 2016). It is important to note though that these experiments were performed using neonatal OPCs, which by definition will be highly active and responsive cell type, as they are isolated from a brain undergoing intensive myelination and are not necessarily identical to adult OPCs, which are the cells which will be functional in remyelination processes (Moyon et al., 2015). This experiment also gives a good example to the importance of surface antigens and their potential effects on OPCs and remyelination.

Extracellular matrix and surface molecules effects in OPC differentiation

When OPCs arrive at the location of the lesion, they encounter multiple cell types which constantly shape the microenvironment, not only by the factors they secrete (as described in above sections) but can also affect OPCs through direct cell to cell contact. The following section will discuss some of the more relevant molecules expressed on the surface of various cell types and contribute to the ECM in the lesion area. I have chosen to focus on the molecules which have been shown to affect OPC differentiation in demyelination lesions, and/or in *in vitro* settings and are relevant to ageing as will be describe in further chapters.

- **Chondroitin sulfate proteoglycans (CSPGs)**
  CSPGs are a large family of proteoglycans, which are composed of a protein core and a chondroitin sulfate side chain. In mammals, CSPGs are encoded by eight genes, marked *Cspg1-8*. Many CSPGs have been shown to inhibit remyelination (Lau et al., 2012). One of the CSPGs which has been shown to inhibit OPC differentiation, is NG2, encoded by *Cspg4* (Pendleton et al., 2013). *Cspg4* is mainly expressed by OPCs, and is upregulated upon their activation (as described above). Larsen et al. have shown that up regulating MMP-9 can oppose the inhibitory effects of NG2 by removing NG2 deposits (Larsen, Wells, Stallcup, Opdenakker, & Yong, 2003).
  Another, more recent study from the Yong lab, (Keough et al., 2016), further explored the detrimental role of CSPGs in inhibiting OPC differentiation and remyelination. The authors show that in the presence of CSPGs, none of the therapeutic drugs previously reported to enhance OPC differentiation were able to do so. This paper clearly shows the critical role of the lesion microenvironment, with emphasis on the various components of the ECM in modulating OPC differentiation. The authors further show that inhibition of CSPGs production in astrocytes can reverse the negative effects of astrocyte cell surface antigens on OPC differentiation *in vitro*.

- **Fibronectin**
  Fibronectin is glycoprotein, encoded in humans by the gene *Fn1*. Fibronectin is up regulated in active MS lesions, both by macrophages and endothelial cells (Sobel & Mitchell, 1989). A recent study has claimed that fibronectin aggregation in MS lesions inhibits remyelination (Stoffels et al., 2013). *In situ* hybridisation showed *Fn1* expression in macrophages/microglia, as well as in astrocytes in demyelinating lesions, but very little co-localisation with cells of the OL lineage. Further experiments show that fibronectin aggregates from astrocytes directly inhibits OPC differentiation *in vitro* and remyelination *in vivo* (shown by injecting fibronectin directly into toxin induced demyelinating lesions).

- **CD44**
  CD44 is glycoprotein expressed by multiple cell types throughout the body. CD44 plays a role in multiple cell processes related to cell - cell and cell - matrix interactions, including proliferation, adhesion and migration. The main ligand

of CD44 is Hyaluronan (HA), a polymer which can be found in various sizes in the body. The molecular weight of HA seems to play a crucial role in its function (Jordan, Racine, Hennig, & Lokeshwar, 2015). In the CNS, CD44 is expressed mainly by astrocytes and in low levels in microglia (Zhang et al., 2014). In MS lesions, demyelinated lesions show high levels of HA. HMW HA can also directly inhibit OPC differentiation and remyelination *in vivo* and are associated with high levels of CD44 expression (S. a. Back et al., 2005).

1.4.2 Intrinsic factors that govern OPC differentiation

Selected transcription factors and signalling pathways in OPC differentiation

The process of OPC differentiation is not only controlled by the extrinsic cues described, but is also governed by a complex intrinsic network.
Research in past years has led to the discovery of the bHLH transcription factors, OLIG1 and OLIG2. OLIG2 is essential and required for for the normal development of OL (Takebayashi et al., 2002). *Olig2* is expressed in high levels throughout the OL lineage (Zhang et al., 2014) and it role in differentiation seems to come through the regulation of several other important transcription factors, such as NKX2.2, NKX6.2, TCF7L2 (also known as TCF4), MyTI, SOX10 and SOX17 (reviewed in (Boulanger & Messier, 2014)). The following section will elaborate on some of the more notable pathways and transcription factors (TFs) that play a role in OPC biology.

- **SRY-box containing gene (SOX) family**
  Several TF from the SRY-box containing gene family play important roles in OPC differentiation, in particular *Sox2*, *Sox10* and *Sox17*.

  SOX2 has been studied extensively in multiple stem cells, and is considered a master gene in ESCs and is required for maintenance of pluropotency and self renewal (Avilion et al., 2003). It is also one of the 'Yamanaka factors' required for the reprogramming of Induced Pluripotent Stem Cells (IPSC) (Takahashi & Yamanaka, 2006). In OPCs, *Sox2* is expressed during development (Zhao et al., 2015), but not in adult OPCs. *Sox2* expression reappears in OPCs following demyelination and OPC activation. SOX2 is required for the regulation OPC proliferation and it ensures that sufficient numbers of OPCs are generated in the lesion site before differentiation begins (Zhao et al., 2015).

  SOX10 is a crucial transcription factor in glial development in general, and in OPC differentiation specifically., as well as in Schwann cell development in

the PNS (Kuhlbrodt, Herbarth, Sock, Hermans-Borgmeyer, & Wegner, 1998). *Sox10* is expressed throughout the OL lineage and changes the levels of its expression throughout the stages of OL lineage (Kuhlbrodt et al., 1998). *Sox10* deficient mice show a significant decrease in the expression of PLP and MBP in the spinal cord (Stolt, 2002). *In vitro* experiments have also shown the importance of SOX10, as *in vitro* inhibition of *Sox10* using siRNA results in decrease OPC differentiation, whereas over expression of *Sox10* results in the expression of NKX2.2, OLIG2, MBP and PLP (Z. Liu et al., 2007). *Sox10* can exert its effects synergistically with other transcription factors (such as *Olig2* in OL development, or POUdomain protein Tst-1/Oct6/SCIP in Schwann cell development (Kuhlbrodt et al., 1998)). It can also regulate MBP expression directly as it can bind to three binding sites in the MBP promoter (Nicolay, Doucette, & Nazarali, 2007).

One of the direct targets of SOX10 in OL is *Myrf*, whereas SOX10 binds to *Egr2/Krox2* in Schwann cells(Hornig et al., 2013; Lopez-Anido et al., 2015). *Myrf* null mice have pre myelinating OL, but they exhibit extreme deficits in the expression of myelin proteins. Similarly, over expression of *Myrf in vitro* results in enhanced expression of myelin genes (see further description in later sections).

SOX17, a SOX F type transcription factor, plays a role in OPC maturation, as its down regulation with siRNAs in cultured OPCs results in reduction of O4+ cells in comparison to increase in A2B5+ cells. Further more, over-expression of *Sox17* in cultured OPCs results in increased expression of MBP, MAG and CNP. Direct infection of *Sox17* reduces the percentage of proliferating cells *in vitro*, thus promoting OPC differentiation by inducing cell cycle exit (Sohn, 2006).

- **NKX-homeodomain factor** -

  Multiple NKX factors are involved in OPC differentiation, including NKX2.2 and NKX6.2 (Cai et al., 2010).

  NKX2.2 is rapidly up regulate in upon OPC activation, and is highly expressed in proliferating OPCs (Zhang et al., 2014; Boulanger & Messier, 2014; Fancy et al., 2004), but is down regulated upon the transition to mature OL, thus it seems to play an important role in early differentiation, but not in the mature myelinating OL.

  NKX6.2 is thought to play a role in OPC differentiation as it expressed in parallel to MBP and PLP (Awatramani et al., 1997). NKX6.2 is a DNA binding protein and can bind multiple sites within the promoter regions of both *Plp* and

Mbp (Awatramani et al., 1997).

- **basic Helix Loop Helix (bHLH) factors** -
  The main bHLH factors described in relation to OPC biology are OLIG1 and OLIG2. **OLIG2** is expressed throughout the OL lineage, including OPCs and OLs. OLIG2 is required for for normal development of OPC (Takebayashi et al., 2002). *Olig2-/-* mice do not survive, making it difficult to fully understand the role of OLIG2 in OPC differentiation. But what it is known is that OLIG2 is required for maintaining OPCs in the OL lineage, as treating OPCs with *Olig2* targeted siRNA inhibits their differentiation into OL *in vitro* (Cheng et al., 2007).

  Unlike OLIG2 which is essential for both developmental myelination, as well as remyelination, **OLIG1** is not required for developmental myelination, but is necessary for remyelination, since *Olig1* null mice fail to remyelinate in response to induced demyelination, but display small differences in developmental myelination (Arnett et al., 2004; Cheng et al., 2007). During OL maturation, OLIG1 is shuttled out of nucleus in immature OL to the cytoplasm in myelin forming OL. Similar to the experiments described above regarding *Olig2*, treating OPCs *in vitro* with siRNA against *Olig1* results in reduction of OPC differentiation (Cheng et al., 2007).

- **Other selected transcription factors**
  More transcription factors which directly induce differentiation and myelin formation, include the zinc-finger protein Myelin transcription factor 1 (MYT1) which is thought to regulate and fine tune the transition between proliferation and differentiation phases (Nielsen, Berndt, Hudson, & Armstrong, 2004; Vana, Lucchinetti, Le, & Armstrong, 2007).

  MyTI promotes OPC maturation, as over expression of *Myti in vitro* promotes OPC differentiation, measured by the expression of CNP (using luciferase reporter essay). Moreover, infection of OPCs with truncated MyTI (in order to interfere with the normal activity of MyTI) results with dramatic reduction in differentiation capabilities of OPCs (Nielsen et al., 2004).

  MYRF (Myelin Gene Regulatory Factor) is another transcription which is necessary for OPC differentiation and OL maturation. MYRF is a DNA binding proteins, and its targets include many myelin genes (Emery et al., 2009). Inhibition of *Myrf* using siRNA results in reduction of OPC differentiation and removing exon coding for DNA binding domain from *Myrf* transcript prevent myelination (Emery et al., 2009).

Cell intrinsic pathways involved in OPC differentiation

- **The Mammalian Target of Rapamycin (mTOR) pathway**
  mTOR is a serine threonine kinase which regulates cell growth in response to PI3K/Akt stimulation, and is also involved in nutrient sensing. mTOR is active through two distinct signalling pathways, which are characterised by the complex which are formed: mTORC1 and mTORC2 (characterised by the presence of the adaptor proteins RAPTOR or RIPTOR, respectively). Tyler et al. (Tyler et al., 2009), have shown that mTOR is an essential pathway in OPC differentiation, and that inhibiting mTOR prevents OPC to transition from preOL to fully myelinating OL. The addition of Rapamycin *in vitro* in this study resulted in decrease of OPC differentiation and increased expression of multiple transcription factors which are associated with OPC early activation (e.g. *Nkx2.2*), as well as genes associated with OPC proliferation (e.g. *Id2/4*), which inhibit OPC premature differentiation (Kondo & Raff, 2000; Wang, Sdrulla, Johnson, Yokota, & Barres, 2001).

- **Wnt signalling pathway**
  Using an ISH expression array of transcription factors, Fancy et al., have uncovered an important role for Wnt signalling in OPC differentiation (Fancy et al., 2009), focusing on the role of TCF4 in this process, as TCF4/β-catenin complex inhibits OPC differentiation. Furthermore, APC (β-catenin inhibitor) is upregulated in differentiating OPCs, and mice that lack APC exhibit reduced remyelination. Moreover, AXIN2 which is a target of the Wnt pathway (acting as negative feedback by degrading β-catenin) is required for OPC differentiation and its stabilisation can accelerate OPC differentiation (Fancy, Harrington, et al., 2011). Wnt pathway is also inhibited by HDAC1/2 activity (Ye et al., 2009). The authors further identify TCF7L2 (Transcription Factor 7 Like 2, also known as TCF4) as a link between HDAC1/2 activity to Wnt pathway and OPC differentiation. HDAC1/2 compete with β-catenin to interact with TCF7L2. When HDAC1/2 interact with TCF7L2 it becomes an activator of OPC differentiation. More recently it has been shown that Wnt signalling inhibits OPC differentiation by working in cooperation with the BMP signalling (Feigenson, Reid, See, Crenshaw III, & Grinspan, 2011). BMP signalling is known to inhibit OL development and instead promote astrogenesis, both in OPCs (Neumann 2017, personal communication), as well as in neural stem cells (Bond, Bhalala, & Kessler, 2012).

- **Notch pathway**
  Notch pathway have been described as a differentiation inhibitor, as it has been shown Jagged1 (NOTCH receptor primary ligand) inhibits OPC differentiation *in vitro* through the activation of two bHLH factors, HES1/5 which are down stream targets of the Notch pathway. HES1/5 promote OPC proliferation and inhibit cell cycle exit, thus preventing OPC differentiation. Adult unmyelinated axons expresses JAGGED1, thus preventing their remyelination (Wang et al., 1998). This view was contested by a more recent paper by Stidworthy et al., which have shown that in rodents, remyelination is not affected by NOTCH signalling, as *Notch-/-* mice show no difference in remyelination, suggesting that the NOTCH pathway role in inhibiting OPCs is limited to developmental myelination.

Epigenetic mechanisms in OPC differentiation

- **Histone deacetylases (HDACs)**
  As discussed previously (see section 1.2.2), HDAC are a group of enzymes which play a important role in histone modifications. HDACs reverse the activity of HATs (histone acetyltransferases), by removing acetyl groups from histones lysine tails. This generally leads to chromatin compaction , thus reducing transcription of specific genes. HDAC role in OPC differentiation in development have been shown to be critical in both zebrafish (Cunliffe & Casaccia-Bonnefil, 2006) as well as in rodents (Shen, Li, & Casaccia-Bonnefil, 2005). In the developing rat brain, there is progressive decrease in acetylation of specific histones (namely H3) in OL lineage cells (marked by SOX10 expression). Moreover, administration of various HDAC inhibitors (such as, Valporic acid)specific resulted in hypomyelination. This effect was limited to a specific time window, after which the cells were immune to the effects of HDAC inhibitors, Thus, HDAC are required specifically for the differentiation of OPCs, but not for the maintenance of myelin once OL have been formed (Shen et al., 2005).

- **Histone methyltransferases (HMTs)**
  Using ChIP-sequencing targeted at H3K9me3 and H3K27me3, Liu and colleagues revealed an increase in H3K9me3 markings in OLs in comparison to OPCs. Genes regulated by this mark included *Pax6, Lhx1, Grip1* and *Dcx* - all genes related to neuronal fate modulation (J. Liu et al., 2015). As H3K9me3 is known as a repressive histone marker, one can assume it is used to specify OL fate in progenitor cells.

- **Micro RNA (miRNA)**
  Knocking out DICER under the *Plp1* promoter results in demyelination, suggesting that miRNAs play a role in the maintenance of OLs through maintenance of lipids and redox reduction (Shin, Shin, McManus, Ptáček, & Fu, 2009). Further studies examined the various miRNAs differentially expressed in OPCs and OLs. Most up regulated miRNAs were expressed in OL, and the authors have pointed specific roles for miR-338, miR-318 and specifically miR-219 in OPC differentiation. Further study revealed that miR-219 inhibits key OPCs specific genes, including *Pdgfra, Sox6, Foxj3* and *Zfp238* (Dugas, Cuellar, Scholze, & Ason, 2010).

- **DNA methylation**
  Unlike the role of other epigenetic mechanisms in OPC differentiation, very few studies have studied the role of DNA methylation in the OL lineage. Early studies from the 80's have shown that injection of 5-azacytidine, a nucleoside inhibitor of DNMTs, during developmental myelination resulted in decrease of myelination (Ransom, Yamate, Black, & Waxman, 1985). The injection affected mainly OL and not astrocytes, which suggests that DNA methylation plays a role in differentiating cells and fully mature ones. Moreover, examination of the optic fibres several days after the injection has shown small increase in myelination, meaning the effects of AZA were limited to the currently differentiating cells. Using more advanced methods Moyon et al. (Moyon, Huynh, et al., 2016) studied the role of DNA methylation in OL development. The authors show an increase in 5-mC in OLs in comparison to OPCs, as well as an increase in the expression of DNMT3A in OLs. As expected, DNMT1 levels decreased with OPC differentiation, as this enzyme is mainly active in cell division and DNA replication (as explained above). ERRBS analysis revealed an increase levels of methylation in promoters of multiple genes which related to progenitor state in OLs, such as *Nkx2.2* and *Pax6*. As expected, myelin related genes (such as, *Mog*) were hypomethylated in OLs in comparison to OPCs. *Dnmt1* KO under the promoter of *Olig1* resulted in hypomyelination and decreased survival of the mice. Interestingly, knocking out *Dnmt3a* under the same promoter did not lead to hypomyelination. The phenotype exhibited by the *Dnmt1-/-* mice was not due to reduce progenitor numbers, but was concluded to be due to reduced differentiation capabilities of the OPCs. This was due to alternative splicing events, caused from the hypomethylation phenotype.
  In adult OPCs, unlike neonatal ones, DNMT3A and not DNMT1 seems to play

a more important role in OPC differentiation during remyelination (Moyon et al., 2017). This is expected, since differentiating adult OPCs will need to methylate specific gene sets in order to initiate a transcription network necessary for differentiation.

1.5 Remyelination failure in ageing

While the process of remyelination is quite remarkable in its efficiency, exemplified by the ability of rats to quickly recover from toxin induced demyelination lesions (W. Blakemore, 1974), this is not always the case. Even though first studies have concluded that remyelination does not decline with ageing (W. Blakemore, 1974), more recent studies have clearly shown that remyelination efficiency does decline with age (Ruckh et al., 2012; Shields, Gilson, Blakemore, & Franklin, 1999; Shen, Liu, & Li, 2008; Shen, Sandoval, et al., 2008), similar to other regeneration processes. The decreased efficiency of remyelination can be a result of decline in each of the processes leading to remyelination, i.e. OPC activation and migration to the lesion, OPC proliferation, OPC differentiation and final remyelination.
In aged animals OPC recruitment and differentiation was implicated to be delayed (Sim, Zhao, Penderis, & Franklin, 2002), but in many cases it is the last step of OPC differentiation which is rate limiting. For example, over expression of PDGF-A in astrocytes (under the *Gfap* promoter) resulted in an increase of OPC numbers (i.e. increased OPC proliferation), albeit, this enhanced proliferation does not translate into increase OPC differentiation in aged mice (Woodruff et al., 2004). This suggests that indeed the failure in remyelination does not stem from low numbers of OPCs, but from lack of OPCs which can successfully differentiate in myelinating OLs.

Further evidence for the major role of OPC differentiation failure as a main reason for remyelination failure is postmortem autopsies on MS patients. Multiple studies have revealed that MS lesions contain sufficient number of OPCs but limited OPC differentiation and remyelination (Chang, Tourtellotte, Rudick, & Trapp, 2002; Kuhlmann et al., 2008; Wolswijk, 1998). More specifically, some reports have described multi process pre-myelinating cells that express PLP protein, but do not engage and enwrap axons (Lucchinetti, 1999). This strengthens the notion that:

1. OPCs are present in MS in sufficient numbers

2. OPC differentiation failure is a major rate limiting step for remyelination in MS patients

Some reports, such as by Boyd et al (Boyd, Zhang, & Williams, 2013), point out that at least in a minority of lesions (approximately 37%), failure in efficient recruitment of OPCs seems to be a critical factor in remyelination failure.

Since remyelination failure in MS is mainly present in chronic lesions, coupled with the fact that chronic MS lesions numbers rise with age together with higher incidences of neurodegenerative phases (Ransohoff, 2012), it becomes evident that ageing is a contributing factor for remyelination failure in MS, not by limiting the number of progenitor cells, but by affecting intrinsic and extrinsic factors that render OPCs less likely to differentiate successfully. Therefore it is clear that there is a need to describe and understand how do the effects of ageing affect OPCs and the other cells active in demyelinated lesion environment.
The effects of ageing on the process of remyelination can be divided into two main groups: the first, effect on the lesion microenvironment. For example, changes in the activities of microglia/macrophages and astrocytes, as these cells are essential in shaping a pro-remyelinating lesion environment in which OPCs can differentiate and remyelinate the nude axons in in efficient and timely manner. The second effect resulting from ageing, is the intrinsic changes in OPCs and how they contribute to the decline in their differentiation capacity. In the next few sections I will review the changes in ageing microglia (section 1.5.1) as well as the changes known in ageing OPCs (section 1.5.2).

1.5.1 Ageing microglia

The changes in the microglia transcriptome with ageing have been described in multiple studies (Hickman et al., 2013; Holtman et al., 2015). The classic view of the changes in the microglia was to classify them as changing toward a more pro-inflammatory profile (Norden & Godbout, 2013). It has been suggested that the priming of microglia is induced through long term exposure to low levels of inflammation through life and especially in ageing. It seems that this is an oversimplification. Using advance genomic techniques and WGCNA, Holtman and colleagues demonstrate that 'primed' microglia (isolated from aged mice, as well as from mice models of various neurodegenerative conditions) show a completely different expression profile in comparison to *acutely* activated pro-inflammatory microglia (by using i.p. LPS injection). Therefore, as mentioned before, it is critical to view the microglia specific roles in remyelination and how the efficiency of microglia activity changes with ageing.
The most striking effect of ageing on microglia, is the dramatic decline in microglia phagocytosis capabilities (Njie et al., 2012). Young microglia and blood derived

macrophages exhibit high capacity of myelin phagocytosis, and this is critical for successful remyelination to occur (H. Neumann, Kotter, & Franklin, 2009). The efficiency of the phagocytosis process and myelin debris clearance is significantly reduced in ageing microglia/macrophages (Natrajan et al., 2015), and this (at least partly) have been shown to contributes to the failure of remyelination in aged animals (Ruckh et al., 2012). This was shown in a study using heterochronic parabiosis (Ruckh et al., 2012). In this study old and young mice blood circulation are connected. This way, young mice serum could flow into the old mice, including circulating cells, such as monocytes. Using GFP mutant, the authors were able to detect which cells crossed from the young to old animals and aided in remyelination following toxin induced lesions. Exposure to young milieu enhanced the proliferation of OPCs in the lesions of the old animals, as well as their differentiation capabilities. This ultimately led to enhanced remyelination in old animals which were paired with young animals. Most of the invading cells (GFP+) in the lesions were macrophages. This led to the conclusion that the improvement in remyelination could be due to young macrophages invading the lesions in the old animals. The contribution of the young macrophages was deemed limited to their enhance myelin clearance capabilities, as no differences were noted in the production of key factors known to enhance OPC differentiation. It is important to note that only a few factors have been tested, thus it is likely that the beneficial effect is not only limited to the enhanced myelin clearance capability. Nonetheless, this study shows that aged OPCs have the potential to regenerate to certain extent by modulating the environment and exposure to specific serum factors. But this rejuvenation was not complete, so it is clear that aged OPCs also harbour intrinsic changes which need to be discovered in order to completely overcome the effects of ageing on remyelination. Another method shown to reverse the decreased phagocytosis efficiency was by activating RXR (Retinoid X Receptor) using RXR agonists, such as bexarotene. Bexarotene has also been shown to enhance the phagocytosis capabilities of MS patients derived monocytes (Natrajan et al., 2015), and is currently being tested in clinical trials.

The changes relating the secretome profile of microglia and their effect on remyelination are less clear. Studies have shown that aged microglia exhibit delayed recruitment to the lesion site. They have also shown that levels of multiple pro-inflammatory secreted molecules (such as iNOS, TNFa, IL-6 and IL-1b) are present in demyelinated lesions for longer periods of time in aged animals (Hinks & Franklin, 2000; Zhao, Li, & Franklin, 2006). It is logical to conclude that sustained high levels of several inflammatory cytokines will inhibit remyelination, as some of these molecules (such as, IL-1β and TNFα) have been shown to play a role in OPC proliferation

(see section 1.4.1). This way, OPCs are recruited to the lesion site, where they can proliferate, but do not receive the correct set of differentiation signals in a timely manner, thereby missing a critical time window for remyelination, therefore the axon is left demyelinated and vulnerable to further degeneration, due to the lack of trophic support by OL (as described before). Therefore, according to this model, the delayed differentiation of OPCs in lesions is at least somewhat due to lack of timely differentiation signals from the resident microglia/macrophages. There has not been a full transcriptome analysis which compares the responses of aged and young microglia in remyelination, thus it is impossible to to test whether only pro-inflammatory cytokines remain in higher levels, or this is also true for other types of molecules that might be released by the microglia/macrophages in the lesion and will potentially delay the OPC differentiation process.

Chapter 5 in this thesis describes the experiments I conducted using both young and aged acutely isolated microglia. These experiments begin to explore the changes in both the secretome of aged microglia (using conditioned media experiments), as well as investigating the changes in microglia surface antigens and their effects on OPC differentiation capabilities.

1.5.2 Intrinsic changes in aged OPCs

Due to the technical difficulties in isolating and culturing aged OPCs, we have little knowledge regarding the intrinsic changes in OPCs that occur throughout ageing. Even so, some studies have shown intrinsic changes within OPC function which render them less effective. A direct evidence for the intrinsic changes in ageing OPCs was provided by Shen et al., in two studies from 2008 (Shen, Sandoval, et al., 2008; Shen, Liu, & Li, 2008). These studies show loss of epigenetic markers (specifically histone acetylation and methylation) in aged animals. The loss of these modifications results in higher activity of specific differentiation inhibitory genes, such as *Hes5* and *Id4*. These studies also show that the recruitment of HDAC1/2 in OPCs is essential for successful OPC differentiation, as inhibiting HDAC activity using pharmacological inhibitors results in reduced OPC differentiation. Moreover, the authors show a reduced recruitment of HDACs in aged animals which leads to reduced differentiation capabilities. Examination of RNA-seq database (Zhang et al., 2014) shows very little differences in HDAC expression through OPC differentiation. Combining the two results leads to the conclusion that it is not simply a matter of up regulating HDAC which required for differentiation, but it is their correct recruitment to specific genomic locations, mainly to inhibit differentiation inhibitory genes. This must be depends on a

correct expression of various transcription factors which are needed to direct HDAC to correct genomic locations.

Furthermore, since heterochronic parabiosis aged animals did not completely rejuvenate, coupled with the fact that OPCs were not transferred between the animals, suggests that there intrinsic changes in aged OPCs that needs to be reversed in order to restore full remyelination capabilities in aged animals (and humans) (Ruckh et al., 2012).

In conclusion, even though OPCs and remyelination have been studied for many years, there is little knowledge about the unique effects of ageing on OPCs and how this is related to their decline in regenerative capacities. Most of our knowledge regarding OPC biology comes from studying neonatal OPCs, which display very different characteristics than aged ones, both *in vitro* and *in vivo* (B. Neumann, Baror, Dietmann, Wijngaarden, & Franklin, n.d.)

Therefore, there is an apparent need to describe and create a complete database of the aged and young adult OPCs, in order to obtain the relevant targets for potential therapeutic interventions. In chapters 3 and 4 I describe the generation of two main databases: the first, a database which describes the epigenetic and transcriptomic changes between the different cell types that compose the OL lineage in adulthood. The second compares and describes the changes in the transcriptome and methylome of ageing OPCs.

A further neglected area of study is the interaction between the different cell types that play a role in remyelination and how this interaction is affected by ageing. In the final chapter of this study I will bring examples for this kind of interaction between microglia and OPCs, while addressing potential ageing effects.

1.6 Aims and objectives of this study

Although it is accepted that adult stem and progenitor cells differ from their neonatal counterparts, the intrinsic properties of adult OPCs and other cells in the OL lineage have not been characterised, and most studies rely on findings based on studying neonatal cells. Moreover, the importance of ageing in the decline of the regenerative capacity of OPCs is of great clinical relevance, but it is not yet known what are the intrinsic changes in OPCs that render them less capable of responding in the case of demyelination. Similar gaps of knowledge hinder our understanding of changes that occur in ageing microglia, with emphasis on non immune activities. Therefore, in this thesis I set out to fulfil the following objectives:

1. Explore the intrinsic changes and mechanisms are in play during adult OPC differentiation. This was achieved by establishing a novel dataset of the methylome and transcriptome of the different cells in the OL lineage in the adult rat.

2. Discover intrinsic changes in aged OPCs in order to explain the decline in efficiency of differentiation presented by aged OPCs by establishing a full transcriptome and methylome of aged and young OPCs.

3. Explore the changes in aged microglia with emphasis on both the expression of surface antigens and secreted molecules and their potential contribution to decline in remyelination with ageing. This was done by using a novel *in vitro* method to co-culture microglia and OPCs, which allows to isolate the effects of microglia extracellular matrix molecules.

Chapter 2

Material and methods

2.1 Animal husbandry

Only Sprague Dawley rats were used for this study. Rats were bred and raised at the Innes Building Animal Facility at the University of Cambridge. All animals were fed standard diet and were kept under 12 hours cycles of light and darkness. All animals used for experiments were sacrificed by schedule 1 approved methods, according to the requirements and regulations set by the United Kingdom Home Office.

2.2 Cells immunohistochemistry

At the end of each *in vitro* experiment, cells were fixed by adding 4% PFA to each well and incubating in RT for 10 minutes. Cells were then washed twice using PBS (5 minutes each wash, RT, shaking). Cell nuclei were labeled with 2µg/ml Hoechst 33342 (Sigma). Before applying primary antibodies, cells were blocked using 5% normal donkey serum (NDS) for 20 minutes, RT. For intracellular stainings, 0.1% Triton was added to the blocking solution. Primary antibodies (see table 2.1) were diluted in PBS + 5% NDS and samples were incubated overnight in 4°C. Following incubation, samples were washed twice with PBS (10 minutes, RT, shaking) and secondary antibodies (see table 2.1) were added accordingly. Secondary antibodies were diluted in PBS + 5% PBS, and samples were incubated in RT for 2 hours. Following incubation, samples were washed 3 times with PBS, 10 minutes each time. Second wash included Hoechst 33342 for nuclear staining (2ng/ml). Image acquisition was performed using a Leica-SP5 microscope (Leica) and LAS software (Leica) or a Zeiss Observer A1 inverted microscope (Zeiss) and Zeiss Axiovision software. For each well (in 48 well plate) 3-4 images were taken in randomly selected areas. Further image processing

and analysis was performed using the ImageJ software package. Images quantification was done using CellProfiler software (Broad institute, Harvard University). In total, for each condition a minimum of 200 cells were counted.

Table 2.1: **List of antibodies**. All primary antibodies were IgG subclass antibodies unless specified otherwise.

Antibody	Dilution	Vendor	Catalog#
Primary antibodies			
Mouse anti-A2B5 (IgM)	1:300	Millipore	MAB312
Mouse anti-CD11b	1:300	Serotec	MCA275R
Mouse anti-CNPase	1:300	Abcam	ab6319
Mouse anti-O4 (IgM)	1:1000	R&D Systems	MAB1326
Mouse anti-NG2	1:300	Milipore	AB5320
Rabbit anti-Ki67	1:500	Abcam	Ab166667
Rabbit anti-NG2	1:300	Millipore	MAB5320
Rabbit anti-Olig2	1:1000	Millipore	AB9610
Goat anti-Iba1	1:500	Abcam	ab5076
Goat anti-Olig2	1:500	R&D Systems	AF2418
Chicken anti-GFAP	1:1000	Abcam	ab4674
Rat anti-MBP	1:300	Serotec	MCA4095
Goat-anti-Mouse-MOG-Biotinilated	1:300	R&D systems	BAF2439
Secondary antibodies			
Alexa 488 donkey anti-mouse	1:500	Invitrogen	A21202
Alexa 488 donkey anti-rabbit	1:500	Invitrogen	A21206
Alexa 488 donkey anti-goat	1:500	Invitrogen	A11055
Alexa 488 donkey anti-mouse IgM	1:500	Invitrogen	A21042
Alexa594 donkey anti-mouse IgG	1:500	Invitrogen	A21203
Alexa594 donkey anti-rat IgG	1:500	Invitrogen	A21209
Alexa594 donkey anti-rabbit IgG	1:500	Invitrogen	A21207
Alexa647 donkey anti-rabbit IgG	1:500	Invitrogen	A31573
Alexa647 donkey anti-mouse IgG	1:500	Invitrogen	A31571
Alexa647 donkey anti-Goat IgG	1:500	Invitrogen	A21147

Table 2.1 Antibodies used for IHC

2.3 Isolation of adult OPCs

Note: all centrifugations were conducted in room temperature, unless specified otherwise.

2.3.1 Single cell suspension

Neonatal (P0-P20), young adult (P30-P90) and aged (12 -27 months) rats were decapitated after lethal injection of phenobarbital or sacrificed by inhalation of increasing CO2 concentrations. Brains were removed shortly after death (including cerebrum and cerebellum) and placed into ice cold HALF isolation medium (Hibernate A Low Fluorscence; see table 2.2 for full list of ingredients), supplemented with 2% of B27. Meninges, olfactory bulbs and remains of spinal cord (if present) were removed and brain tissue was chopped mechanically into 1mm pieces using sterile scalps. The minced tissue was spun down in 100g for 2 minutes in RT. Brain tissue was then suspended in dissociation solution (34U/ml papain (Worthington), 20µg/ml DNAse Type IV (Gibco) in HALF) for 30 (neonatal) to 40 (adult) minutes in 37°C, on a shaker set to 55 RPM. Digestion was stopped by adding cold HBSS- (Hank's Balanced Salt Solution, no magnesium / calcium; Gibco). HBSS(-) was used in order to minimise the possibility of cell attachment to tubes, as magnesium and calcium are necessary for cell attachment. The tissue was centrifuged for 5 minutes, in 250g. Supernatant was completely aspirated and tissue was re-suspended in neutralising solution (HALF + 2% B27 + 2mM sodium-pyruvate) for 5 minutes in RT. In order to create a single cell suspension, cells were first titurated using a 5ml serological pipette and subsequently three fire polished glass pipettes in decreasing opening sizes. After each trituration step the tissue suspension was allowed to sediment and the supernatant, containing the cells, was transferred into a fresh tube, while being passed through a 70µm cell strainers to ensure single cell suspension. After removing the supernatant each time, 2ml of fresh neutralising solution were added to the remaining tissue. After the whole tissue was passed though the cell strainer, a 90% pre-filtered (20µn filters) isotonic Percoll solution (GE Healthcare, diluted in 10xPBS pH7.2 (Gibco)) was added. Final Percoll concentration of 22.5% was achieved by adding phenol-red free DMEM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; Gibco). The single cell suspension was then centrifuged for 20 minutes in 800g. This resulted in separation of debris and cells. Myelin debris was removed and the cells were washed using HBSS-. Cells were then incubated for 1 minute in Red Blood Cell lysis buffer (RBC lysis buffer; R7757; Sigma-Aldrich) in order to remove contaminating red blood cells. Solution was then topped up by HBSS- and centrifuged again. Cells were suspended in HALF based washing buffer (HALF WB) which included 2mM EDTA, 2mM sodium-pyruvate, 0.5% BSA and 10ng/ml human recombinant insulin (Gibco) for antibody staining according to the protocols described in the following sections of this chapter.

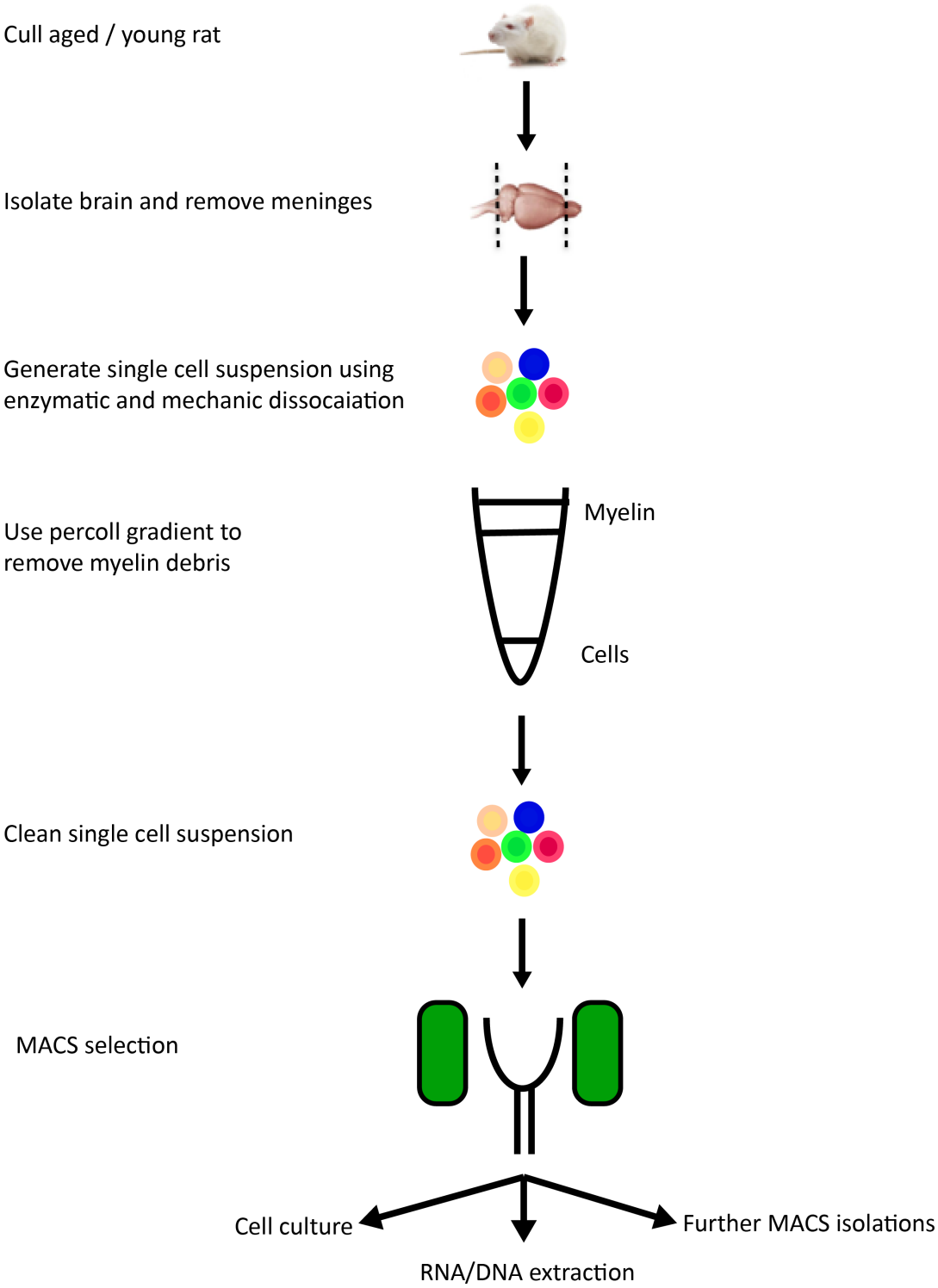


Fig. 2.1 **MACS isolation of glial cells from aged and young rats** Schematic illustration of isolation protocols used for isolation the various glial cells used in this study. Young or aged rats were culled using lethal injection of Pentobarbital, followed by dissection of whole brain. Brains were mechanically dissociated using scalpels and then enzymatically dissociated using Papain digestion. Cells suspension was then subjected to Percoll gradient in order to remove all myelin debris. Clean single cell suspension was then labelled with relevant antibodies and MACS isolation was performed as described in the following relevant sections.

Chemical	µM	MW	mg/5l
Amino acid			
Glycine	400	75.07	150.1
L-Alanine	22	89.09	9.8
L-Arginine hydrochloride	483	174.2	420.7
L-Asparagine-H2O	5.5	150.13	4.1286
L-Cysteine hydrochloride-H2O	7.7	313.2	12.06
L-Histidine hydrochloride-H2O	200	209.6	209.6
L-Isoleucine	802	131.2	526.1
L-Leucine	802	131.2	526.1
L-Lysine hydrochloride	798	146.2	583.3
L-Methionine	201	149.2	149.9
L-Phenylalanine	400	165.2	330.4
L-Proline	67	115.13	38.569
L-Serine	400	105	210
L-Threonine	798	119	474.8
L-Tryptophan	78	204.2	79.6
L-Tyrosine disodium salt dihydrate	398	181.2	360.6
L-Valine	803	117.2	470.6
Vitamins			
Choline chloride	28	139.62	19.55
D-Calcium pantothenate	8	238.27	9.53
i-Inositol	40	180.2	36
Niacinamide	30	122	18.3
Pyridoxine hydrochloride	20	206	20.6
Thiamine hydrochloride	10	337	16.9
Inorganic Salts Ferric Nitrate	0.25	404	0.5
Potassium Chloride	5360	74.55	1997.9
Sodium Bicarbonate	880	84	369.6
Sodium Chloride	89000	58	25810
Sodium Phosphate dibasic	906	120	543.6
Zinc sulfate	0.67	287.56	0.9633
Other Components D-Glucose (Dextrose)	25000	180.2	22525
Sodium Pyruvate	227	110.04	124.9
MOPS	10000	269.3	13465

Table 2.2 Formulation of isolation medium (HALF; Hibernate A Low Fluorescence)

2.3.2 Magnetic Activated Cell Sorting (MACS)

Cells were counted using a regular hemocytometer and 2.5µg mouse-anti-rat-A2B5-IgM antibody (Millipore; cat#MAB312) were added for every 10 million cells (all antibodies used for MACS are listed in table 2.3). Cells were incubated in 4°C for 25 minutes. After incubation, solution was topped up to 8ml of Miltenyi Washing Buffer (MWB; 2mM EDTA, 2mM Na-Pyruvate, 0.5% BSA in PBS, pH 7.2-7.4) in order to wash unattached antibodies. Solution was centrifuged (300g, 5 minutes) and the pellet was re-suspended in 80µl of HALF WB supplemented with 20µl rat-anti-mouse-IgM magnetic beads (Miltenyi; cat#130-047-302) per 10 million cells. Cells were incubated in 4°C for 15 minutes. Secondary antibody was washed with 8ml of MWB and solution was centrifuged (300g, 5 minutes). Cell pellet was resuspended in 1ml of HALF WB and MACS was performed according to supplier instructions (Miltenyi). In short, MS column was placed on a magnet, pre-wet with 500µl of MWB. Cell suspension was then loaded to the column. The column was then washed three times using 500µl of MWB (allowing each time the full amount of buffer to fully run through the column). Finally, column was removed from the magnet allowing A2B5 positive cells to be washed off the column using 1ml pre-warmed, CO2 pre-equilibrated OPC medium (see table 2.4 for full components list). On average, 10^6 cells were isolated per rat (young or aged).

2.3.3 Fluorescence Activated Cell Sorting (FACS)

Cells were re-suspended in 100µl of HALF WB per 10 million cells. The following antibodies were then added:

- Mouse-anti-Rat-A2B5-IgM (Alexa 488 conjugated) (Millipore; cat#MAB312RX)
- Mouse-anti-CD11b-IgG2a (APC conjugated) (Biolegend; cat#201809)

For each antibody a single stain was made using a small fraction of the original cell population (100K per single stain). Cells were incubated with antibodies for 25 minutes in 4°C. Antibodies were washed twice using 1ml of HALF WB. Second wash included Hoechst 33342 (2µg/ml; Sigma) to detect live/dead cells. Cells were flowed using BD AriaIII Cell Sorter.
Isolation strategy was as follows: Detect live/dead cells, isolate CD11b positive cells (microglia), detect A2B5 positive cells (OPCs). Staining thresholds and autoflourscences were set for each isolation using single stain samples. For all isolations, cell viability was more than 90%. A illustration of the isolation strategy is shown in chapter 4.

Antibody	Dilution	Vendor	Catalog#
Primary antibodies			
Mouse anti-A2B5 (IgM)	2.5µg per 10^7 cells	Millipore	MAB312
Mouse anti-CD11b-Microbeads	10µl per 10^7 cells	Miltenyi	130-105-634
Mouse anti-O4 (IgM)	2.5µg per 10^7 cells	R&D Systems	MAB1326
Goat-anti-Mouse-MOG-Biotinilated	2.5µg per 10^7 cells	R&D systems	BAF2439
Secondary antibodies			
Mouse-anti-Biotin magnetic micro beads	20µl per 10^7 cells	Miltenyi	130-105-637
Rat-anti-mouse-IgM magnetic beads	20µl per 10^7 cells	Miltenyi	130-047-302

Table 2.3 Antibodies used for MACS

2.4 Adult OPC culture

Cells were plated onto 48 well plates (VWR) pre-coated with PDL (Sigma). OPC medium (OPCM) was used for experiments unless stated differently. Cells were plated in low density, 10K cells per well (48 well plate). After seeding, cells were left to recover overnight in media supplemented with b-FGF and PDGF (Peprotech) according to age of the animal (neonatal 20ng/ml; young adult and aged 30ng/ml). For aged cells, after two days media was changed to promote proliferation (OPCM + 20ng/ml b-FGF and PDGF) or differentiation (OPCM + 40ng/ml T3). During both differentiation and proliferation, media was changed every other day.

Table 2.4: **OPC medium formulation**. The medium was mixed, sterile filtered and kept at 4°C.

Component	Final Conc.	Stock Conc.	Vendor	Catalog#
DMEM/F12	1X	1X	Gibco	31330-038
NAC	60µg/ml	6mg/ml	Sigma	P4333
Insulin	10µg/ml	4mg/ml	Gibco	12585014
Sodium pyruvate	100µM	100mM	Gibco	11360070
Apo-Transferrin	50µg/ml	5mg/ml	Sigma	T2036-100MG
Putrescine	16.1µg/ml	1.61mg/ml	Sigma	P5780
Na-Selenite	40ng/ml	0.5µg/ml	Sigma	S5261E
Progesterone	60ng/ml	6µg/ml	Sigma	P8783

Table 2.4 OPC medium formulation

2.5 Isolation of aged and young Mononuclear phagocytes (MNPs)

Following OPC (aged or young) MACS isolation, A2B5 negative cells were centrifuged (300g, 5 minutes) and cell pellet was re-suspended in DMEM-F12 supplemented with 10% FBS (Fetal Bovine Serum) for ghost experiments, or OPC modified media for conditioned media experiments. Cells were plated densely in untreated suspension plates for overnight recovery. Next day, cells were detached from the suspension plates using ice cold HBSS- supplemented with 5mM EDTA. Cells were incubated in detachment solution in 4°C for 15-20 minutes and then removed using a pipette. The cell suspension was then centrifuged (300g, 5 minutes) and re-suspended in 90µl HALF WB with 10µl anti-Rat-CD11b magnetic micro beads (Miltenyi; cat#130-105-634) for every 10 millions cells for 15 minutes in 4°C. Cells were washed using cold 8ml MWB and then pelleted again (300g, 5 minutes). Cells were then loaded to a MS column as described above.

2.5.1 Ghost protocol

CD11b positive cells (microglia) were plated densely in 48 well plates, 40K cells per well in microglia media (DMEM-F12 + 10% FBS). Cells were let to recover for 48 hours before media was completely removed and ddH_2O. Plate was immediately placed in dry ice for instant freeze. Plates were later kept for short term in -20°C, or long term in -80°C. Before use, plates were defrosted and the water was removed completely before new cells were plated on top of the ghosts.

2.5.2 Conditioned media

Cells were plated in density of 40,000 cells per well onto 24 well plates using OPCM. After overnight recovery, media was completely removed and replaced with fresh OPCM supplemented with either TGFβ (Peprotech) or TGFβ inhibitor (SB-431542, Tocris). Cells were cultured for 48 hours, afterwards media was removed, filtered using 22µ filters to remove any cell debris and was frozen immediately using dry ice. Conditioned media was stored in -80°C until used.

2.6 Isolating OL lineage cells

Single cell suspension was achieved as describe above. Cells were re-suspended in 100µl of HALF WB with 2.5µg of Goat-anti-Mouse-MOG-Biotinilated (RD systems; cat#BAF2439) antibody for 25 minutes in 4°C. Cells were washed with 8ml of MWB and centrifuged (300g, 5 minutes). Cell pellet was re-suspended in 80µl HALF WB supplemented with 20µl Mouse-anti-Biotin magnetic micro beads (Miltenyi; cat#130-105-637) for 15 minutes in 4°C. Cells were washed with 8ml of MWB and centrifuged (300g, 5 minutes). MACS was executed as describe above. MOG negative fraction was centrifuged (300g, 5 minutes) and MACS A2B5 was performed as described above. A2B5 negative cells were centrifuged (300g, 5 minutes) and cell pellet was re-suspended in 100µl of HALF WB with 2.5µl Mouse-anti-O4-IgM (R&D systems; cat#MAB1326) and incubated in 4°C for 25 minutes. Cells were then washed using 8ml of MWB and then centrifuged (300g, 5 minutes). Cell pellet was re-suspended using 80µl HALF WB with 20µl rat-anti-mouse-IgM magnetic beads (Miltenyi; cat#130-047-302) and incubated for 15 minutes in 4°C. Cells were washed using 8ml of MWB and centrifuged (300g, 5 minutes). MACS was then performed as describe above. Following MACS, cells were plated in 48 well plates (VWR) for culturing (same conditions as for adult OPCs) or were lysed immediately for DNA and RNA isolation. Number of cells isolated per animal changed according to the age of the animal (see chapter 4), but for young adult rats (2-3 months old) which were used for DNA/RNA isolation, approximately 10^6 OLs and OPCs were isolated per rat, and 3×10^5 POLs.

2.7 RNA sequencing

2.7.1 RNA isolation

Following A2B5 MACS sorting, cells were pelleted (1000g, 5 minutes) and immediately lysed using RLT Plus Lysis Buffer (Qiagen). For young and aged OPC isolation, approximately 10^6 cells were used on average per sample. RNA isolation was performed using Qiagen RNeasy Micro Kit (Qiagen), including on column DNA digestion step. Following elution, RNA was frozen immediately using dry ice and was stored at -80°C until library preparation.

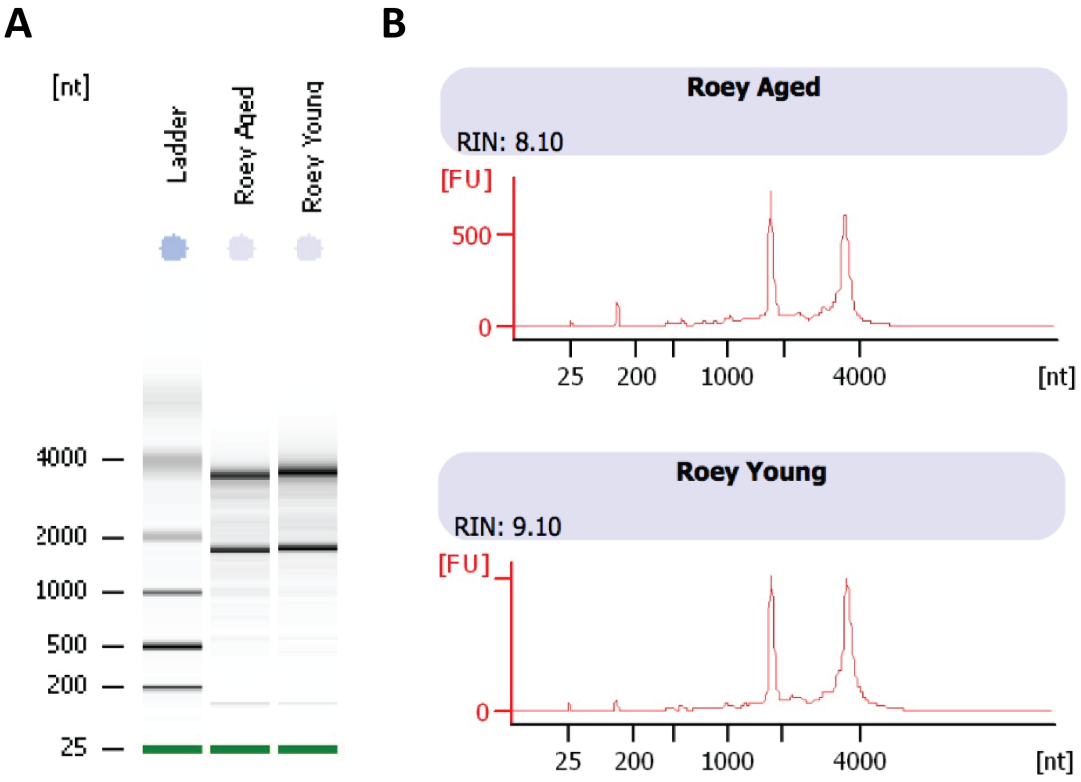


Fig. 2.2 **RNA quality control** Example for successful RNA QC, using a Bioanalyzer. Before library preparation, each sample was subjected to QC using a Bioanalyzer (Agilent). Minimal required RIN (RNA Integrity Number) was 8. **(A)** Electrophoresis run of two samples and a ladder, showing high bands in 4000 and 2000 nucleotides bands. **(B)** Graphs showing peak readings at 4000 and 2000 nucleotide for a young and aged OPCs RNA samples.

2.7.2 Library preparation

RNA quality was assessed by Qubit measurement and posterior RNA nanochip/picochip Bioanalyzer. Only high quality samples were used for library preparation and sequencing (RIN > 8, see figure 2.2). Ribosomal RNA was depleted with rat-specific oligos (InDA-C technology). Sequencing libraries were prepared using 10-100ng total RNA and the Nugen Ovation RNA-Seq Systems 1-16 for Model Organisms Kit (0349-32).

2.7.3 Full transcriptome sequencing and downstream analysis

Sequencing was performed on the Illumina HiSeq4000 in a pair-end 150 base pair format. Adapter sequences were removed and reads were quality-trimmed using TrimGalore. Trimmed reads were aligned to the rat reference genome (RGSC6.0/rn6) by using TopHat2 (http://ccb.jhu.edu/software/tophat, version: 2.0.13) guided by

Ensemble gene models. Raw counts per gene regions were obtained by featureCounts. Replicates were evaluated, counts were normalised and differential expression of transcripts was evaluated by the R Bioconductor DESeq2 package. Expression levels were further normalised by transcript length (per kB). Transcript annotations were based on Ensemble (Release 82). GO analysis was done using GOrrila (Eden, Navon, Steinfeld, Lipson, & Yakhini, 2009). Ingenuity pathway analysis was done by IPA (Qiagen). Principal Component Analysis was performed using Clustvis (Metsalu & Vilo, 2015).

2.8 DNA methylation sequencing

2.8.1 DNA isolation

Following FACS isolation, A2B5 positive cells were pelleted (1000g, 5 minutes) and DNA was extracted using Qiagen DNeasy Blood Tissue Kit, including RNA digestion step, using Rnase A on column digestion, according to manufactures instructions.

2.8.2 Whole Genome Bisulfite Sequencing (WGBS) and downstream analysis

Whole Genome Bisulfite Sequencing (WGBS) was performed on Illumina HiSeq4000. Reads were trimmed with TrimGalore, with default parameters. Reads were aligned and de-duplicated using bismark. Methpipe was used to calculate DNA methylation levels in all regions (Song et al., 2013). Window size for promoter methylation analysis was 1000bp. For aged and young OPCs, approximately 5×10^5 cells were used for each sample.

2.9 qRT-PCR (OPCs, microglia)

RNA was isolated from acutely purified OPCs and microglia or from cultured OPCs and microglia using Qiagen RNeasy Micro Kit, or Directzol RNA MicroPrep Kit (Zymo Research; cat#R2061). Isolated RNA was immediately frozen using dry ice and was further stored in -80°C. RNA quantities were measured using Nanodrop 2000 (Thermo Scientific). cDNA was generated using Qiagen QuantiTect Reverse Transcription Kit according to the instructions of the manufacturer (Qiagen; 205310). For RT-qPCR, primers were acquire from KiCqStart SYBR Primers (SIGMA-ALDRICH) and used at a concentration of 300µM. cDNA, primers, and the Syber Green Master

Mix (Qiagen; 204141) were mixed as instructed by the manufacturer, and RT-qPCR and melting curve analysis were performed on Life Technologies Quantstudio 6 Flex Real-Time PCR System. Fold changes in gene expression were calculated using the delta delta Ct method in Microsoft Excel using TATA box binding protein (*Tbp*) as a control gene. Statistical significance was determined using two-tailed unpaired t-tests assuming equal variances.

2.9.1 Primers used for qRT-PCR

See table 2.5 for full list of primers used in this study.

Gene name	Sequence
Ascl1	5' - AAACAAGGGAAGAGGAAAAG - 3' 5' - CATTGAATCTAAGTCCTGGTG - 3'
Cd44	5' - AAGATTTTATCTCCAGCACC - 3' 5' - CTGTCTATATCAGTCATCCTGG - 3'
Cspg4	5' - ACAAGCTCAAGAATTTCCAC - 3' 5' - TGAAGTTCCCTGTAGTGTAAG - 3'
Cnp1	5' - TTTCAAGAAAGAGCTTCGAC - 3' 5' - TAAGATCTCCTCACCACATC - 3'
Enpp6	5' - AATTTGTCTCTCCTTTGACC - 3' 5' - CTTTCTGGACATCAGATAGC - 3'
Fn1	5' - AAGCCAATAGCTGAGAAATG - 3' 5' - AAGTACAGTCCACCATCATC - 3'
Il10	5' - TCTCCCCTGTGAGAATAAAAG - 3' 5' - TAGACACCTTTGTCTTGGAG - 3'
Pdgfra	5' - GAGATTATGAATGTGCTGCC - 3' 5' - TTTCTCGTGAACAGAAATGC - 3'
Sox6	5' - TCCCAATTTTTCCACATGAC - 3' 5' - GTTATCACCTGGCTTGTATG - 3'
Tbp	5' - CATCATGAGAATAAGAGAGCC - 3' 5' - GGATTGTTCTTCACTTTGG - 3'
Tnf	5' - CTCACACTCAGATCATCTTC - 3' 5' - GAGAACCTGGGAGTAGATAAG - 3'

Table 2.5 List of primers

2.10 OL lineage sequencing

Following MACS isolation, cells were pelleted (1000g, 5 minutes) and DNA and RNA was isolated using ZR-Duet DNA/RNA MiniPrep (Zymo Research, D7001)

according to manufacture instructions. Thius, RNA and DNA were isolated from the same cells. RNA isolation included on column DNA digestion using DNase I. Further sequencing and analysis was performed as described above. Number of cells used changed between cell types. Number of OLs and OPCs were approximately 10^6 cells per sample, whereas for POLs only 3×10^5 cells were isolated from each sample.

2.11 Statistics

Statistical analysis was performed on GraphPad Prism (GraphPad Software, Inc.), excluding one sample T-Tests which were performed using SPSS (IBM). Immunohistochemical staining were counted using CellProfiler (as described above) and results were compared using suitable statistical tests. If more than two groups were present, a one-way analysis of variance (ANOVA) test was performed, followed by appropriate post test in order to compare individual groups. For all statistical analysis, differences between groups were considered significant at p-value < 0.05.
Graphs were produced using GraphPad Prism or RStudio.

2.12 Flow cytometry

Cells were collected immediately after MACS isolation, pelleted (1000g, 5 minutes) an re-suspended in 500µl of 4% PFA. Solution was incubated on ice for 20 minutes. Following incubation, cells were washed twice using PBS and finally re-suspended in 100µl PBS. Before flow cytometry, cells were were blocked with 5% NDS for 20 minutes in RT. Primary antibodies were added according to table 2.1 for 30 minutes in RT. Following primary incubation cells were washed twice using PBS and then incubated for 20 minutes with appropriate secondary antibodies (see table 2.1). Cells were washed twice using PBS, and re-suspended in MWB for flow cytometry analysis. For every flow cytometry experiment, appropriate single stain controls were obtained. Flow cytometry was performed on Attune NxT Flow Cytometer (Thermo Fisher Scientific). Results were analysed using FlowJo (FlowJo, LLC).

Chapter 3

Transcriptomics and epigenetics of OL lineage in the adult rat

3.1 Introduction

A key step in successful remyelination is the differentiation of OPCs to mature myelinating OLs. OPC differentiation is a complex process, which includes both morphological and transcriptomic changes. The morphological transition is characterised by the transition from a bi-polar cell type, to a multi process cell, and eventually the creation of multiple myelin sheaths. This morphological transition is accompanied by various changes in gene expression, which ultimately lead to the expression of multiple myelin proteins. Previous studies have characterised both the transcriptomic and methylomic changes in the neonatal OL lineage (Moyon, Huynh, et al., 2016; Zhang et al., 2014). Studying neonatal cells limits the ability to draw conclusions regarding adult cells behaviour and biology. This is especially true for OPCs, as during the rodent neonatal stage these cells are highly active, as myelination takes place in this time frame. This is in contrast to OPCs which reside in the adult brain, where *de novo* myelination is rare and might be restricted mainly to learning processes (Mckenzie et al., 2014; Young et al., 2013). Therefore, similar to other tissue resident stem cells one would expect cells to be in a more quiescence state, which will have different properties than the activated neonatal state (Cheung & Rando, 2013).

As remyelination is usually studied in the context of the adult/ageing organism, I set out to study the adult counterparts of the OL lineage. For that I developed a method that can reproducibly isolate cells from distinct phases in OPC differentiation, and then continued to perform a full transcriptome and methylome analysis in order to explore the critical changes between OPCs and differentiated OLs, hopefully to shed

more light of the distinct functions of adult OPCs, the mechanisms that govern their differentiation in the adult CNS. Moreover, it is currently unknown what role do adult OPCs play in normal brain function (if any). The dataset presented in this chapter will perhaps enable researchers to begin exploring the specific roles OPCs might play outside the remyelination paradigm.

3.2 Experimental strategy

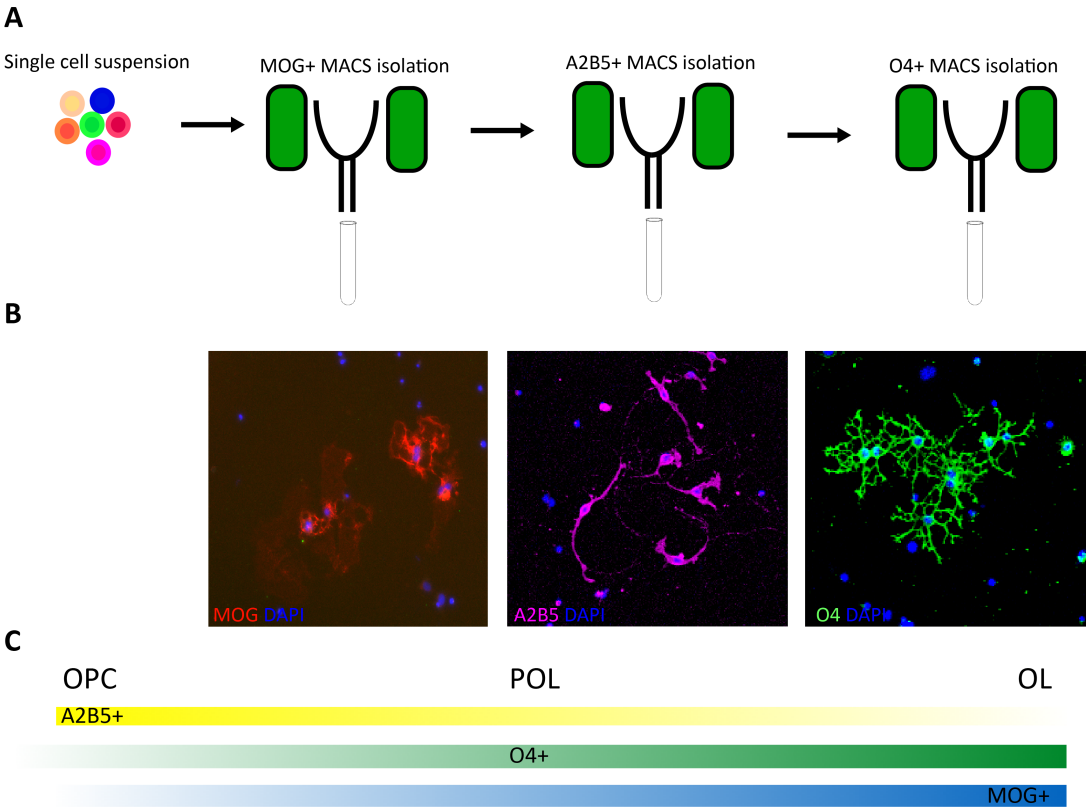


Fig. 3.1 **OL lineage triple isolation method** A summary of the method used to isolate cells from the different stages of the OL lineage. **(A)** Schematic presentation sequential MACS steps used for the isolation of OL lineage cells. **(B)** Set of images showing the various markers used to discriminate between the various cell types within the OL lineage. The sequential steps used in the isolation are also marked, as MOG+ cells were the first to be isolated, followed by A2B5+ cells and finally O4+ cells. **(C)** Illustration of the expression levels of the markers used for the cell isolation within the OL lineage cells.

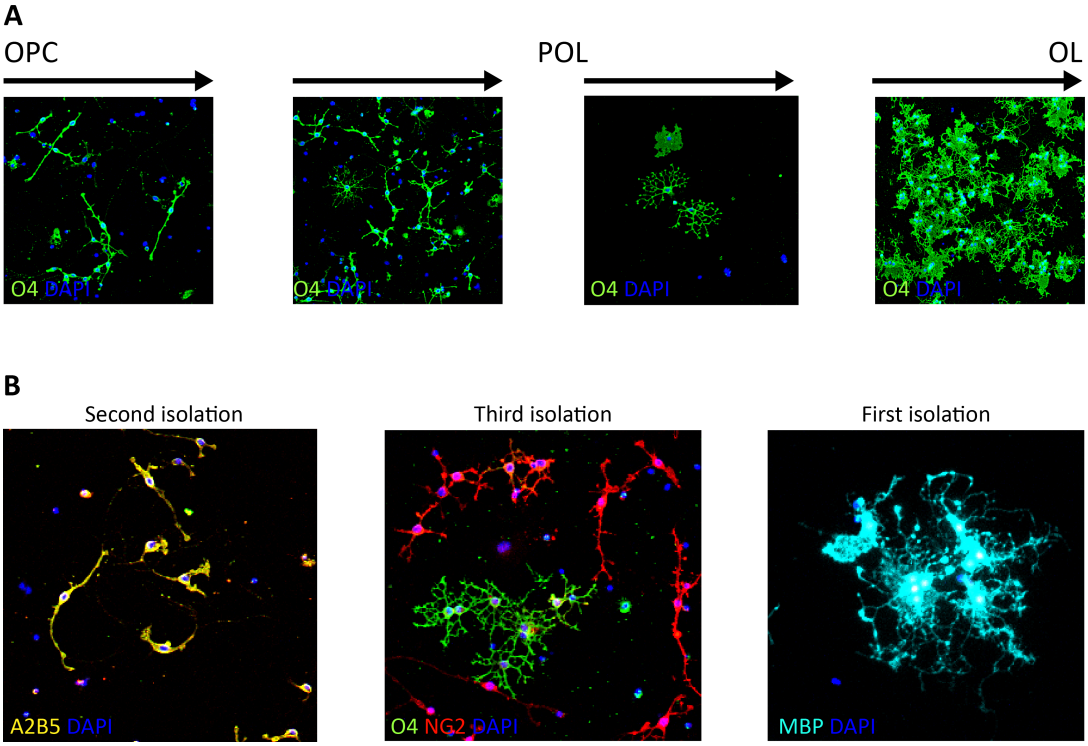


Fig. 3.2 **OL lineage cells** A summary of the the differences exhibited by the different cells types isolated using the method described above (fig3.1). **(A)** Morphological changes exhibited by the various cell types, all stained using O4 antibody (green). This exemplifies the growing comlexity exhibited by more differentiated cells (right hand side of the panel). **(B)** Set of images showing selected markers for each stage of differentiation. Cells were cultured *in vitro* for 48 hours after isolation.

In order to develop a isolation strategy, I based my work on the adult OPC isolation protocol previously developed in the lab (see Materials and methods). I decided to base the isolation on sequential MACS sortings, each time removing a specific population in the lineage. The distinction between the three populations are shown in figure 3.2, as well as the isolation sequence. Panel A in figure 3.2 shows the changes in morphology during OL differentiation and maturation *in vitro*, from a bi-polar cell to membranous cells type. Panel B shows key markers which are associated with the various phases in OL differentiation. Panel C identifies the markers used for the lineage isolation, which was done as follows:

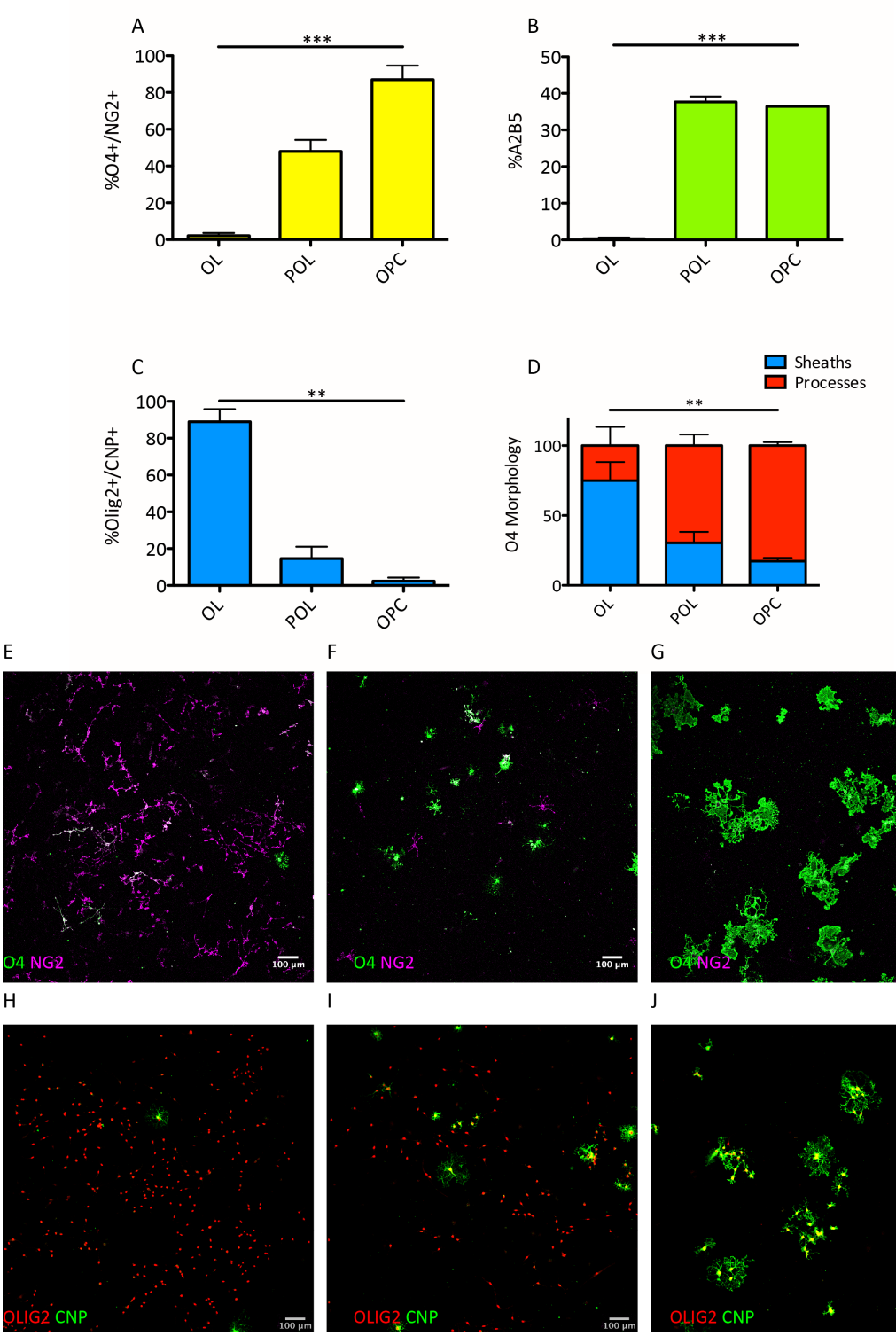
Oligodendrocytes (OLs) were isolated first using anti MOG antibody. OLs are recognised both by the expression of MBP (a mature OL marker) after short time in cell culture, as well as by exhibiting a distinct membranous morphology, expressed by O4 staining in the upper panel of the figure. MOG negative were then be sorted by A2B5 MACS to isolate OPCs (MOG-/A2B5+). OPCs were characterised by expressing

progenitor markers and the lack of mature OL markers. A2B5 positive cells were also distinct from the OLs, by the high percentage of bi-polar cells present in culture, and the lack of mature membranous, sheath-like morphology (see section 3.3.1 for more details). A2B5 negative cells were then labeled by O4 antibody to isolate cells that are pre oligodendrocytes (POL; MOG-/A2B5-/O4+). O4 positive cells exhibit mainly multi-polar morphology, and only few of them showed bi polar morphology or membrane like morphology.

3.3 Results

3.3.1 Isolation and *in vitro* characterisation of OL lineage cells

To characterise the differences between the various cell population isolated in the Triple MACS protocol, I cultured each cell type in identical conditions and assessed their differentiation capabilities. All cells were plated in similar densities, and were cultured in identical media. Following isolation, cells from P20-30 neonatal rats were plated in PDL coated, 48 well plates, in a density of 10,000 cells per well. Juvenile cells were used as they are easier to maintain in culture. As A2B5 is expressed in both neonatal and aged OPCs and used for isolating OPCs in various ages (see chapter 4 as well as (Windrem et al., 2004)), I could use the same antibody for OPCs from all ages. Cells were cultured for 24-36 hours in OPCM supplemented with 20ng/ml of FGF and PDGF before switching to differentiation media (OPCM with 40ng/ml of T3). Differentiation was assessed after a total of 72 hours in culture, from which 24 hours were in differentiation conditions (OPCM with 40ng/ml T3). I chose a short time scale for assessment of differentiation for two main reasons: firstly, the media I used was made in order to answer the specific needs of OPCs, a progenitor cycling cell, and not OLs - a fully differentiated cell type which might require different culturing conditions. Therefore, I did not want my results to be skewed by high proportions of cell death. Secondly, from past experiments and the experience of other members in the lab, I knew that OPCs do not significantly differentiate within 24 hours in culture. Therefore, I could be certain that cells that show differentiated morphology and appropriate markers were in fact freshly isolated OLs.


A
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%O4+/NG2+
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80
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Fig. 3.3 ***In vitro* culture of cells from different phases of the OL lineage** OL lineage cells were isolated as described above and were cultured for 72 hours in OPCM. **(A)** Proportions of NG2 positive cells out of all O4 positive cells (all cells in the OL lineage) in order to assess progenitor proportions. Using this double labelling has shown that almost none of the OLs expressed NG2 (2.05%), whereas approximately 50% (47.99%) of POL express both markers and 86.9% of OPCs expressed both markers. These changes were highly significant (one way ANOVA, n = 4, p-value < 0.0001). **(B)** Percentage of A2B5 positive cells (out of total cells). This staining also revealed a significant difference (one way ANOVA, n = 2, p-value = 0.0001) between the different populations, where OLs do not express the progenitor marker in comparison to OPCs (36.44%) and POLs (37.66%). Supporting the notion that the OL fraction contained very small number of contaminating progenitor cells. **(C)** To test differentiation capacities of the different cells, I used CNPase staining to mark differentiated OLs. The figure shows the percentage of CNPase positive cells out of the total OLIG2 positive cells (total OL lineage). Differences between the cells groups were significant (one way ANOVA, n = 2, p-value = 0.0029), as 88.95% of the OLs were labeled double positive, in comparison to 14.66% of the POLs and only 2.25% of the OPCs. **(D)** O4 staining was used to further assess differentiation by analysing cell morphology. Cells were identified as either having processes (marked in red,'Processes') or membranous sheaths (marked in blue, 'Sheaths') using CellProfiler software. This analysis yielded a significant difference in proportions between the various cell groups 74.97% of O4 expressing OLs exhibited a cell morphology which showed distinct sheaths, only 17.24% of the O4 positive OPCs have shown the same morphology and 30.365% of the POLs (two-way ANOVA; interaction p-value = 0.017 and column factor p-value = 0.0482).

Cells from different phases in the OL lineage, can be defined according to expression of specific antigens and according to unique morphologies. Figure 3.3 summarises *in vitro* experiments in which cells from all groups were cultured at the same conditions. I observed significant differences in both gene expression as well as morphologies between the different cell types. OPCs and POLs expressed higher degree of NG2 and A2B5, both markers for early stages of OL lineage (fig3.3A-B), whereas, OLs showed significantly higher percentage of CNPase positive cells (fig3.3C). Changes in morphology (fig3.3D) were also significant, as cells in the OL group showed significantly high proportion of membranous and sheath like morphology in comparison to cells from the other groups.

The high percentage of CNPase positive cells in the OL cell group (88.95%), as well as their complex morphology, implies that these are in fact acutely isolated oligodendrocytes, and are not progenitors cells which differentiated rapidly in culture.

Using flow cytometry analysis of cell from the triple isolation, I could verify both the purity of the samples, as well as the proportions of progenitor cells. Figure 3.4

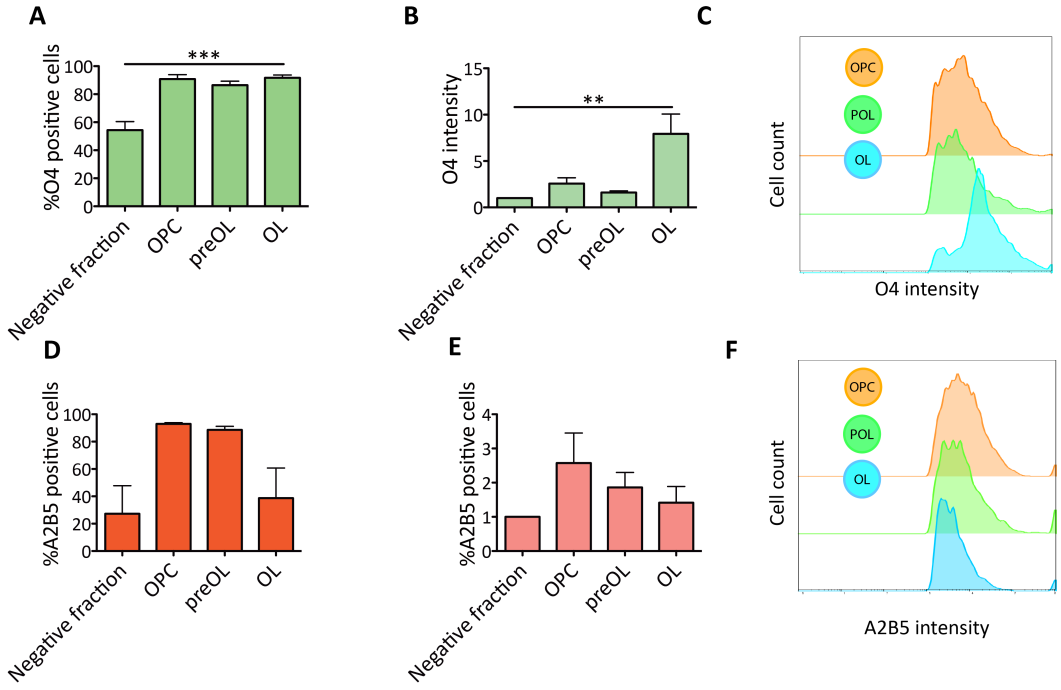

Fig. 3.4 **Flow cytometry analysis of OL lineage cell types** To acutely assess the purity of the cells in the various groups, I used Flow Cytometry analysis. For flow cytometry analysis cells from juvenile rats were used (P20). Cells were isolated according to the triple isolation method described before and were then fixed immediately in suspension. I used O4 to identify cells from the OL lineage and A2B5 to identify OPCs specifically. **(A)** Percentage of O4 positive cells out of all cells in suspension. For all cell groups, percentage of positive cells was above 80%, and this was significantly higher than the percentage in the negative fraction (one way ANOVA, n = 4, p-value < 0.0001). **(B)** Measuring the intensity of the O4 staining revealed a significant higher staining intensity in the OL fraction in comparison to the OPC and POL groups (one way ANOVA, n = 4, p-value = 0.0034). **(C)** A representative histogram of intensities used to calculate medians of O4 staining intensity. Intensity was calculated in arbitrary units. **(D)** Using A2B5 antibody I tested the percentage of progenitor cells in each cell group. Higher proportions of A2B5 positive cells were detected in both OPC and POL groups in comparison to OLs (one way ANOVA, n = 2, p-value = 0.007). **(E)** Comparing A2B5 intensity in all cell groups, revealed a decrease in intensity in POLs and OLs in comparison to OPCs (one way ANOVA, n = 2, p-value > 0.05). **(F)** A representative histogram of intensities used to calculate medians of A2B5 staining intensity. Intensity was calculated in arbitrary units.

describes these experiments. Cells from all groups show high proportion of O4 staining (above 80%), a known marker for OL lineage. As expected, O4 intensity significantly increases in OLs in comparison to OPCs/POLs, according to their position in the lineage. Using A2B5 antibody, I could assess the percentage of progenitors in each group. As expected, I observed a steady decline (yet not significant) in both the percentage of positive cells (p-value = 0.07), as well as their intensities. Intensities for both O4 and A2B5 were calculated using the median intensity of positive cells. OPCs exhibited high levels of A2B5 positive cells (92.95%, 1.344%), supporting the this a valid method to isolate OPCs.

3.3.2 Developmental changes in OL lineage *ex vivo*

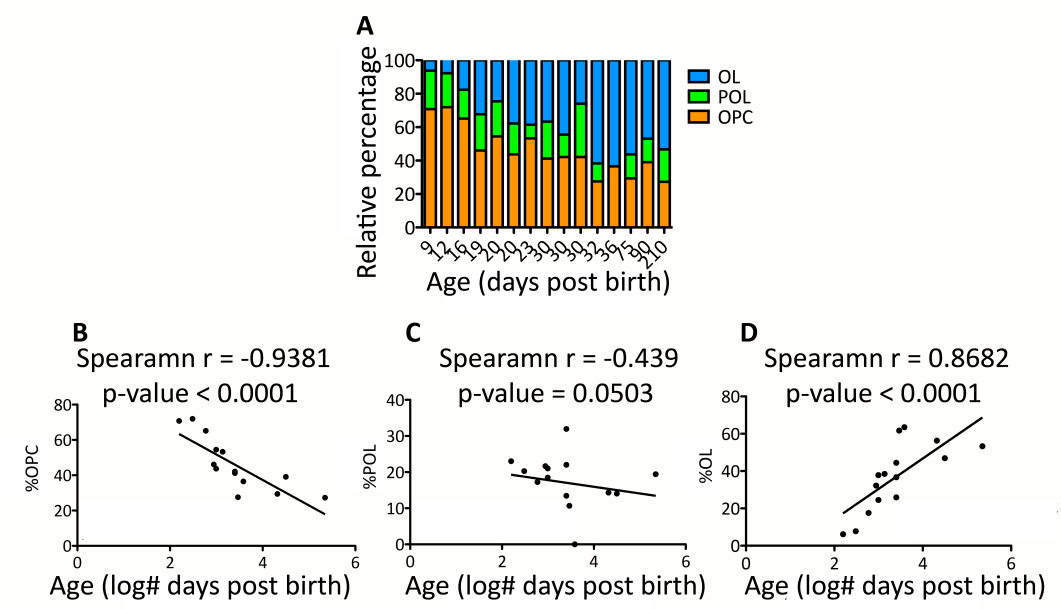

Fig. 3.5 **Changes in OL lineage cells in development** Total cell number was counted in each sample after percol gradient step, and in the end of each isolation step. **(A)** A summary of each fraction percentage out of the total cells isolated. OLs are marked in blue, POLs in green and OPCs in orange. There is a clear transition from a predominantly progenitor based population in early neonatal stages, to a more terminally differentiated population in later developmental stages. **(B-D)** Linear plots describing the change in proportion of each cell group with time. R^2 values and p-value are stated for each figure. The linear plots provide evidence for the changes within the OL lineage, as the proportion of OLs significantly increases on the expense of the OPC fraction.

Summarising the cell numbers from each triple isolation shows changes in in the proportions of progenitor and differentiated cells within the OL lineage total population.

I chose to calculate the changes in proportions as percentage of OL lineage cells isolated, and not total cells in the brain (as counted at the beginning of each isolation following the percol gradient), in order to avoid the data being skewed by changes in total cell number which can sometimes change between isolations, irrelevant to the final numbers. This calculation allowed me to assess the changes in relative ratios the different cell populations.

As seen in figure 3.5A, the more developed the animal is, there is an increase in the relative proportions of OLs in expense of OPCs. In order to further explore the cause behind the changes in the cell proportions, I plotted the percentage of each cell type across time, as seen in figure 3.5B-D. In these graphs, the Y axis represents the change in cell percentage as a proportions of the total OL lineage isolated, while the X axis shows the log age of the animal in days. Figure 3.5B shows the significant increase in OL proportions as a percentage of the total OL lineage with development, while figure 3.5D shows the opposite trend for OPCs.

3.3.3 Transcriptome analysis of adult OL lineage cells

After verifying that I was able to successfully isolate distinct populations of the OL lineage which exhibit the correct characteristics of the various steps in OPC differentiation (section 3.3.1), I moved on to isolate cells from adult rats (n = 4, 2 males, 2 females) aged 2-3 months old in order to isolate both RNA and DNA for transcriptomic and epigenetic analysis. t-SNE analysis provided evidence for successful separation of three distinct populations (fig3.6A). t-SNE is a machine learning based algorithm which was developed specifically for dimensionally reduction of high-dimensional datasets (developed by Geoffrey Hinton and Laurens van der Maaten). Using this method, I was able to divide my samples according to two components as determined by the analysis. Plotting changes in volcano plots (fig3.6B-D) show multiple genes with expression change between the different phases of the OL lineage. Comparing OLs and OPCs resulted in 1925 differentially expressed genes, while comparing between OLs and POLs resulted in 1083 differentially expressed genes. OPCs and POLs showed 1243 differentially expressed genes. This is expected, as OPCs and OLs are the most 'distanced' population on the OL lineage.

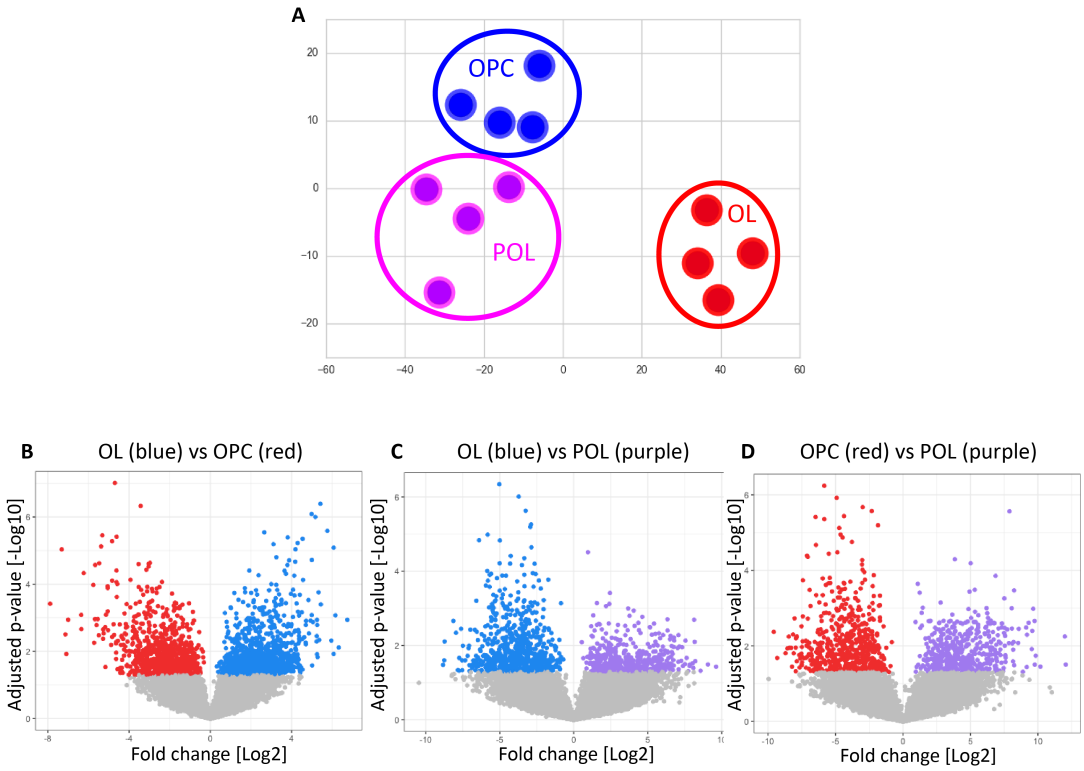

Fig. 3.6 **Transcriptomic analysis of adult OL lineage cells (A)** Full RNA sequencing of adult OL lineage cells, isolated into three distinct groups: OLs, POLs and OPCs (n = 4 for each group). **(A)** A t-SNE analysis of the three groups according to their gene expression profile. This resulted in a clear distinction between the three groups according to two components. Each group marked in different colour, as specified. **(B-D)** Volcano plots presenting changes in gene expression between the different cell populations. In each plot, genes which show significant changes (adjusted p-value < 0.05) are marked in colour according to the cell type (OL -> red, POL -> purple, OPC -> blue)

The following tables show the GO analysis of enriched processes in each cell type in comparison to the other cells (GO analysis was generated using GOrrila). GO analysis show the expected upregulation of myelin genes in the OL cell population in comparison to the OPCs and POLs. OPCs show upregulation of processes related to cell activation, detection of stimulus and immune system regulation. POL enriched processes were mainly regarding the detection of stimulus.

OL vs OPC	
GO process	**Enrichment p-value**
Axon ensheathment	1.25893E-11
Ensheathment of neurons	1.25893E-11
Myelination	3.16228E-11
Glial cell differentiation	1.58489E-06
Oligodendrocyte differentiation	1.51356E-05

OL vs POL	
GO process	**Enrichment p-value**
Myelination	0.00017378
TNFSF11 mediated signalling pathway	0.000190546
Axon ensheathment	0.000281838
Ensheathment of neurons	0.000281838
Ether lipid metabolic process	0.000316228

Table 3.1 Top GO processes enriched in OLs vs OPCs and POLs

POL vs OPC	
GO process	**Enrichment p-value**
Amino acid transport	0.000194984

POL vs OL	
GO process	**Enrichment p-value**
Regulation of response to stimulus	5.37032E-05
B cell chemotaxis	0.000204174
Positive regulation of cystein-type endopeptidase	0.000338844
Regulation of motor neuron apoptopic process	0.000691831
Regulation of response to external stimulus	0.000758578

Table 3.2 Top GO processes enriched in POLs vs OPCs and OLs

OPC vs OL	
GO process	**Enrichment p-value**
Regulation of immune system process	3.16228E-15
Positive regulation of immune system	1.58489E-14
Cell activation	3.98107E-12
Regulation of immune response	6.30957E-12
Immune system process	7.94328E-12

OPC vs POL	
GO process	**Enrichment p-value**
Protein refolding	4.16869E-06
Detection of chemical stimulus	3.16228E-05
Negative regulation of inclusion body assembly	3.46737E-05
Detection of chemical stimulus involved	3.54813E-05
Regulation of inclusion body assembly	5.88844E-05

Table 3.3 Top GO processes enriched in OPCs vs OLs and POLs

3.3.4 Comparing adult and neonatal OL lineage transcriptomics

In order to validate my database, I compared it to a well established neonatal OL lineage database published by Zhang et al., (Zhang et al., 2014). For comparing the two datasets, I selected the top 100 upregulated genes (p-value < 0.05) for each two cell type comparison (i.e. OL vs OPC, OL vs POL, POL vs OL, POL vs OPC, OPC vs OL and OPC vs POL). The results are summarised in Venn diagrams in figure 3.7, where in every figure, a list of common genes are shown.

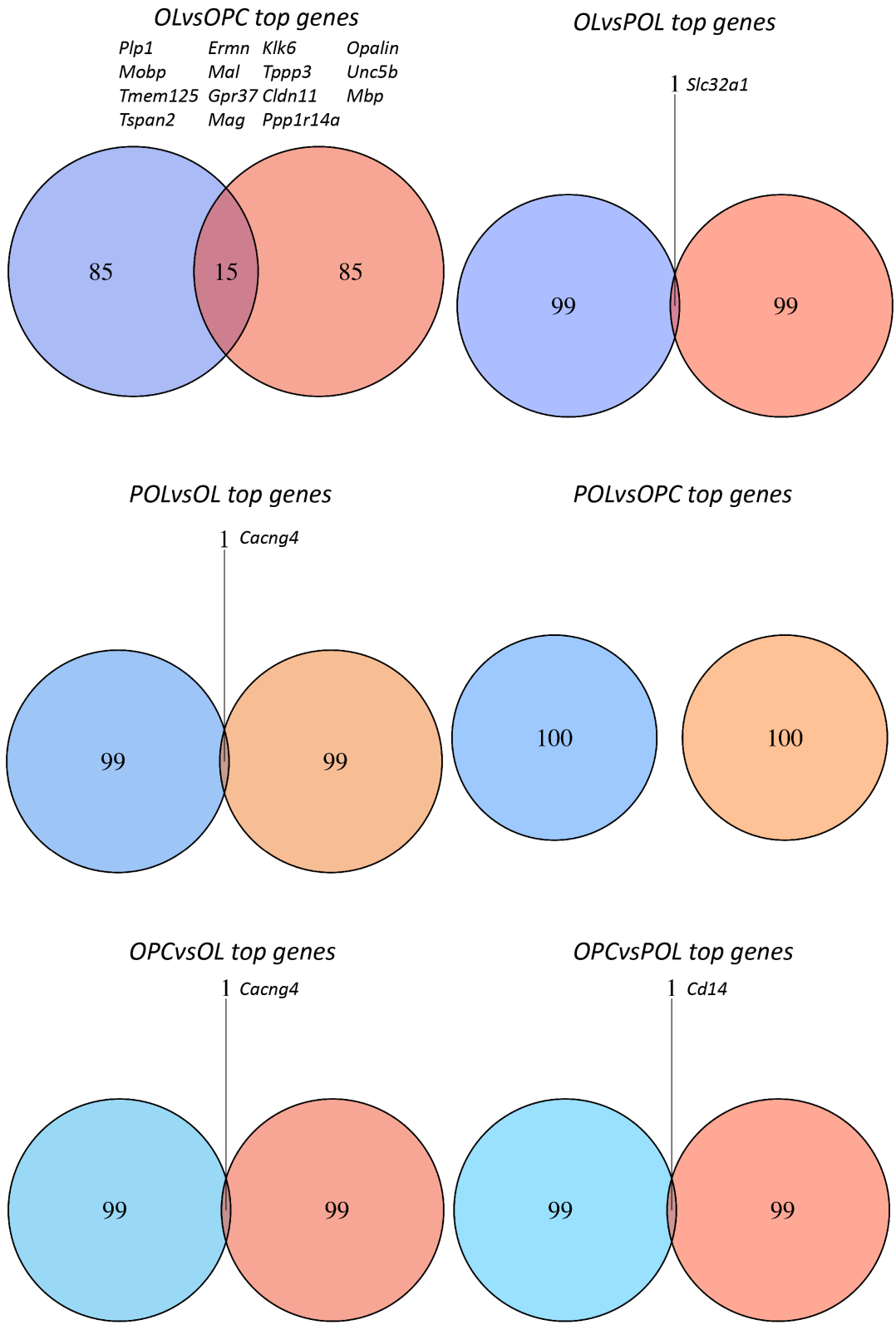

Fig. 3.7 **Comparison of neonatal and adult OL lineage cells** Top 100 significantly expressed genes in each cell type when compared to to other cell types were compared to top 100 enriched genes as was shown by Zhang et al., (Zhang et al., 2014). Genes from adult cell comparison are marked in orange circles, wheareas neonatal genes are marked by blue circles. For each comparison a venn diagram was created to show how many genes are similar between the adult database generated by this study to the database published by Zhang et al. For example, comparing the top 100 enriched genes in OLs vs OPCs as described in both databases revealed 15 shared genes only.

Comparing enriched genes in OLs versus OPCs yielded 15 common genes, including mainly known myelin genes such as *Mbp, Mobp* and *Plp1*. A similar comparison of genes upregulated in OLs in comparison to POLs revealed only a single gene in common *Slc32a1*, similar to the other comparisons which also yielded a single common gene, with the exception of upregulated genes in OPCs versus POLs which yielded no common genes.

Expression of quiescence markers in adult OPCs

Since the adult OPCs I isolated did not distinguish themselves by expressing 'classical' OPC markers (i.e. *Pdgfra, Cspg4, Sox2*) known from the literature (Zhang et al., 2014), I decided to explore in depth which genes are differentially expressed between OPCs and OLs. Since adult OPCs reside in quiescent like state, and exhibit low rate of myelination (Young et al., 2013), I hypothesised that adult OPCs express quiescence genes similar to quiescence stem cells from other tissues. Together with colleagues in the lab (Neumann and Segel, personal correspondence) and recent literature (Barker, Tan, & Clevers, 2013; Basak et al., 2017; Klempin, Marr, & Peterson, 2012; Cheung & Rando, 2013), I identified multiple genes that are associated with quiescence stem cells from various tissues and compiled a list of candidate genes that represent quiescence cells in adult known stem cell niches.

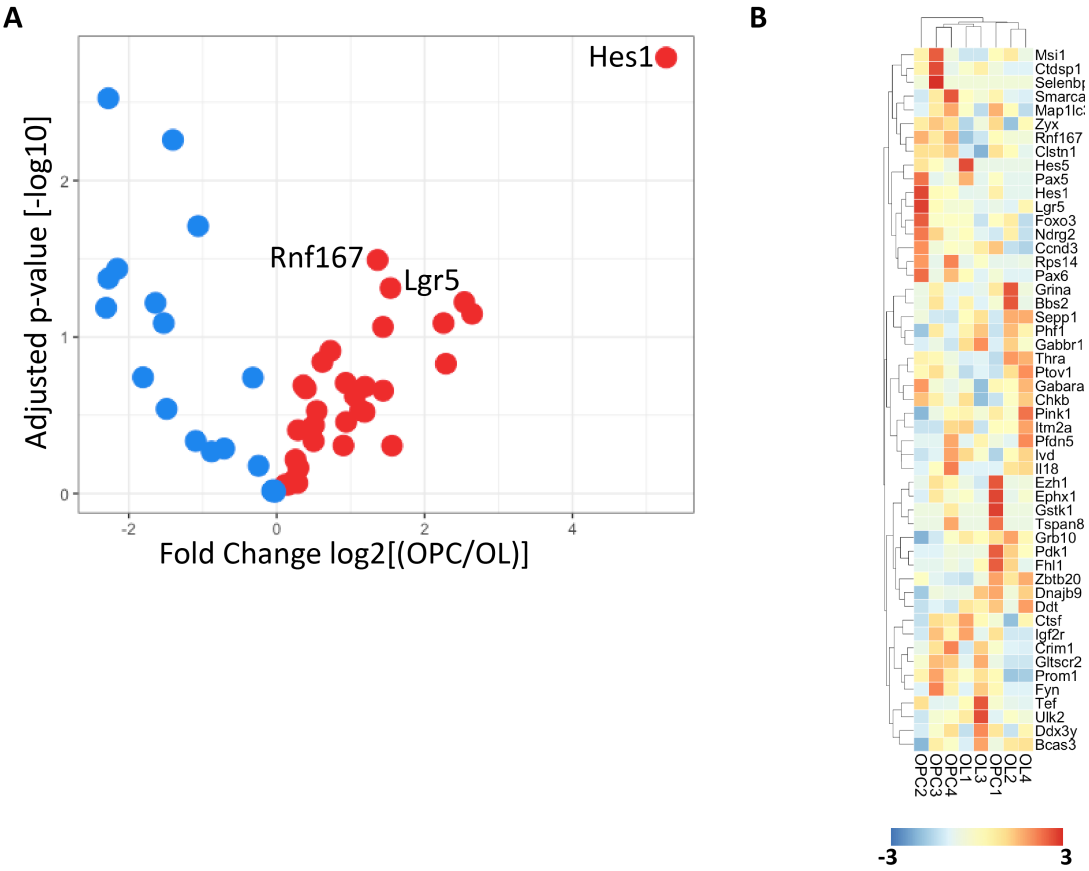

Fig. 3.8 **Expression of quiescence genes in adult OPCs** Comparison of quiescence related genes (gene list was based on multiple publications, mainly (Barker et al., 2013; Basak et al., 2017; Klempin et al., 2012; Cheung & Rando, 2013)) in OPCs versus OLs. **(A)** Volcano plot describing the RNA expression (log2 of fold changes in FPKM, x-axis) and p-value (-log10) of selected quiescence genes. Overexpressed genes in OPCs are marked in red, while higher expressed genes in OLs are marked in blue. For each group, significantly upregulated (adjusted p-value < 0.05) are labeled with the corresponding gene name. **(B)** A heatmap listing all the genes chosen for this analysis, and relative expression over all samples. Red colours represent higher expression values, while blue colours represent lower expression.

Figure 3.8 illustrates the results of the analysis of pre-chosen quiescence related genes. Although only three genes were significantly upregulated in OPCs in comparison to OLs, it is clear that the overall trend is of more genes upregulated in OPCs, rather in OLs. Out of 51 genes in total, 33 (64.7%) were upregulated in OPCs, with only 3 genes (*Hes1, Rnf167* and *Lgr5*) were significantly upregulated. Similar analysis comparing POLs and OPCs revealed only a single gene which was significantly different, *Grb10*, which was upregulated in POLs in comparison to OPCs, similar to its status in OLs vs OPCs (data not shown).

I further explored specific pathways that might be upregulated in adult OPCs

using Ingenuity Pathway Analysis (IPA, Qiagen). Figures 3.9 and 3.10 show the top pathways selected as enhanced in OPCs in comparison to OLs and POLs, respectively. Both pathways include genes which are mostly known for their role in immune cells activity. This corresponds with the immune related GO terms that were identified by the GOrrila analysis, described in table 3.3

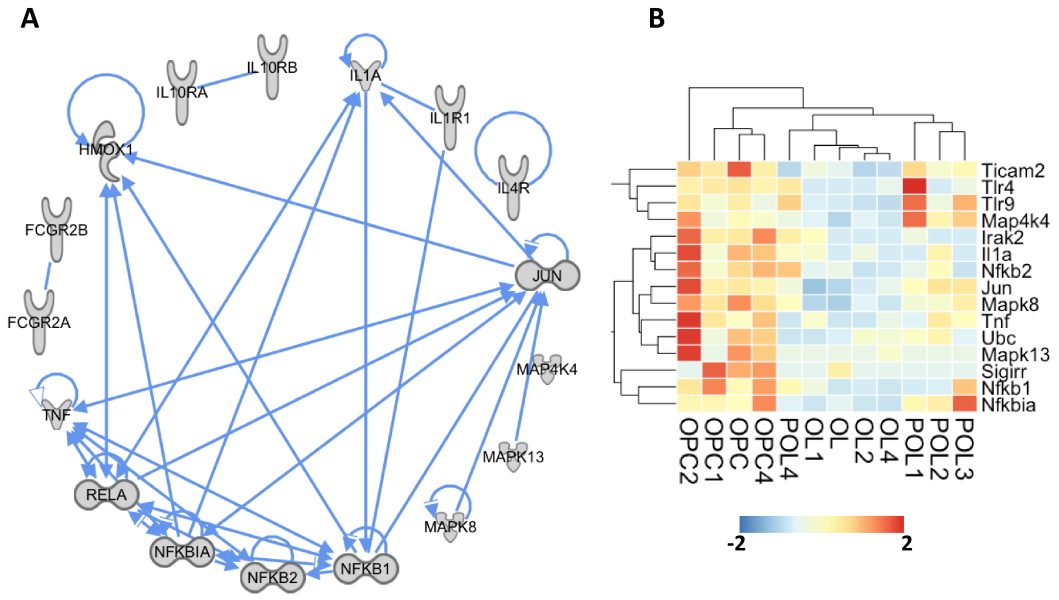

Fig. 3.9 **Expression of NfκB related pathway in adult OPCs vs OLs** Genes significantly upregulated in OPCs vs OLs (adjusted p-value < 0.05) were submitted to IPA. Top pathway predicted was IL10 pathway (enrichment p-value = $3.63*10^{(-9)}$). **(A)** Illustration of the relationship of the different molecules in the pathway. **(B)** Heatmap illustrating the changes in expression in the genes composing the pathway between the different cell types. The heatmap shows a clear separation between OLs and OPCs, whereas, POLs show a mixture of expression between the two cell types. Red colours represent higher expression levels, while blue colours represent lower expression levels.

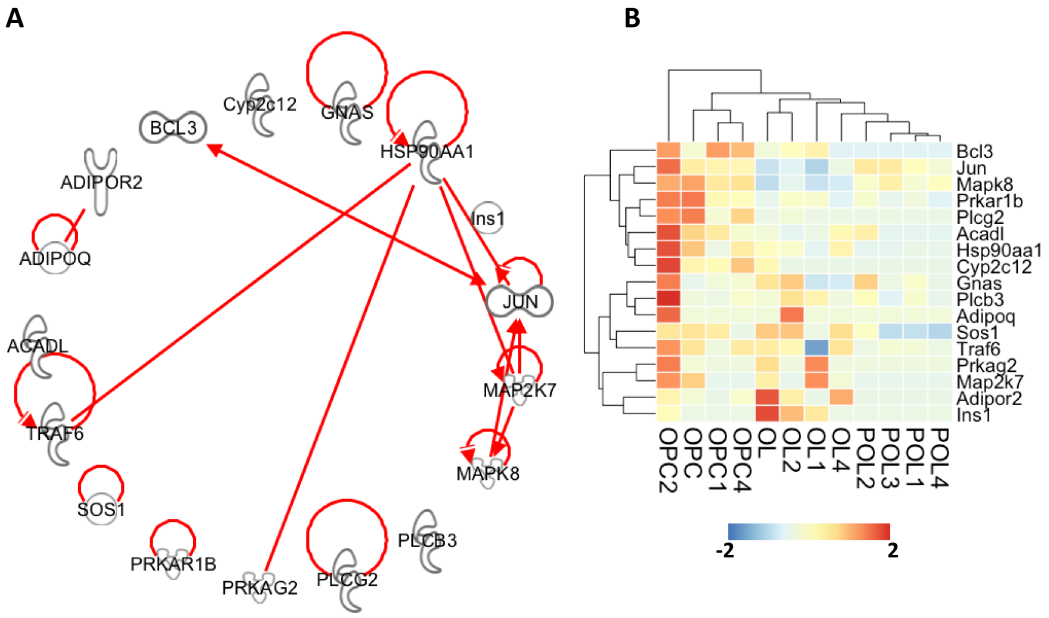

Fig. 3.10 **Expression of PPARα/RXRα related pathway in adult OPCs vs POLs** Genes significantly upregulated in OPCs vs POLs (adjusted p-value < 0.05) were submitted to IPA. Top pathway predicted was PPARα/RXRα pathway (enrichment p-value = $3.22*10^{(-5)}$). **(A)** Pathway illustration of the connections between the various molecules detected in the analysis. **(B)** Heatmap illustrating the changes in expression of the pathway genes. Gene expression reveals clear separation between cells types as expected. As this pathway was detected as upregulated in OPCs vs POLs, but not vs OLs, it is not surprising that expression pattern shows upregulation in OPCs and OLs, while being downregulated in POLs. Red colours represent higher expression levels, while blue colours represent lower expression levels.

3.3.5 Methylation changes across the adult OL lineage

The role of DNA methylation in OL lineage cells was studied recently (Moyon, Huynh, et al., 2016; Moyon et al., 2017), but a full methylome was only constructed for neonatal OL lineage cells (Moyon, Huynh, et al., 2016). The ablation of *Dnmt1* in OL lineage cells (Using an *Olig1* promoter) resulted in inefficient OPC differentiation and reduced OPC proliferation. In contrast, in adult animals, the ablation of DNMT3A in differentiating OPCs (*Plp1* promoter) resulted in reduced remyelination. This suggests that DNA methylation plays an important role in OPC biology, both in developmental and adult cells. I therefore set out to explore the methylome of adult OL lineage cells using WGBS.

Promoter DNA methylation is considered to be an inhibitory mark for transcription, so as a first step I wanted to test if that is correct in my dataset. Similar to the results shown in chapter 5, I could not find this kind of correlation. Figure 3.11 illustrates

this: highly methylated genes in OLs (red and yellow dots) show both increased and decreased expression rates. This pattern is repeated for other cell type comparison, i.e. OL vs POL and POL vs OPC (data not shown).

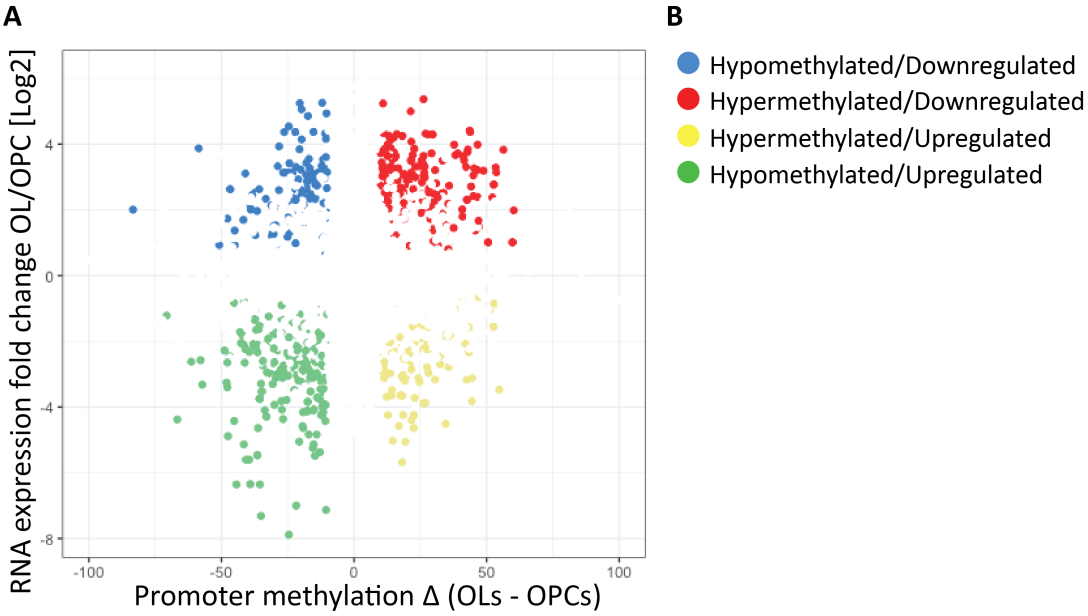

Fig. 3.11 **The effect of changes in promoter DNA methylation between OPCs and OLs on RNA transcript expression** For each gene, promoter DNA methylation was calculated as the change in methylation between OLs and OPCs and was plotted against log2 of the fold change in transcript expression. Coloured dots represent genes of which their promoters had 5X coverage at least for methylation analysis, and fold change was significantly different (adjusted p-value < 0.05).

Even though I could not point out a specific correlation between DNA methylation and transcription rates, I decided to analyse the GO terms of differentially methylated promoters between each cell type. The results are summarised in the tables below. The GO terms are different than the ones detected by the analysis of the transcriptional changes between the cell types and some of the GO terms are common for both hypomethylated and hypermethylated changes. This is in line with the lack of direct correlation between transcript expression and promoter DNA methylation shown in figure 3.11, and suggests that DNA methylation play a more complex role in the control of transcription. Nevertheless, it is clear that the methylation changes are focused in components of the cells which are most likely to be monitored during OPC differentiation and OL maturation. For example, OLs show changes in methylation (hypo and hyper) mainly in membranous proteins. This is true when comparing to POLs and OPCs.

OPCs on the other hand show hypermethylation in processes that are related to stimulus

detection. A more in depth view revealed that many of the genes clustered to these processes are olfactory related genes. This could be related to the inhibition of neuronal fates in OPCs when transitioning to POLs.

3.3.6 Sex based differences in OL lineage cells

Using both males and females for the isolations (n = 2 for each sex) allowed me further analysis in the potential effects of sex on OL lineage cells. Past published work has shown that aged females show enhanced remyelination in comparison to aged males (W. W. Li, Penderis, Zhao, Schumacher, & Franklin, 2006). Analysing the data divided according to sex, could reveal whether this difference in aged OPCs are already evident in the young adult OPCs.
As was shown previously in figure3.6A, all cells were clustered according to the cell type using t-SNE analysis, and there was no evidence for clustering according to sex. Figure 3.12A show the same t-SNE analysis, with labelling to distinguish the different samples. Sample 1 and 2 were derived from male rats, while 3 and 4 were from female rats. Samples did not seem to cluster according to the sex of the animal. In order to study each cell type separately, I used PCA, but this did not reveal any clustering according to sex (3.12B-D). I further tried to cluster the cells according to list of nuclear receptor genes (Di Canio et al., unpublished). This list includes multiple genes which encode to sex hormones receptors which I expected would be affected by the sex of the animal. Using K-means clustering did not show clustering according to sex either (fig3.13). This suggests that the sex of the animal has very little if no effect on the transcriptome of OL lineage cells (in this age range of 2-3 months).

OL vs OPC hypermethylation	
GO component	**Enrichment p-value**
Membrane part	1.58489E-16
Intrinsic component of membrane	2.24999E-14
Plasma membrane part	4.39997E-12
Plasma membrane	1.12001E-11
External side of plasma membrane	1.56E-09
OL vs OPC hypomethylation	
GO component	**Enrichment p-value**
plasma membrane bounded cell projection part	2.34E-06
Cell projection part	2.34E-06
leading edge membrane	1.76E-05
cell junction	5.62E-05
cell projection membrane	7.81E-05
OL vs OPC hypermethylation	
GO process	**Enrichment p-value**
Signal transduction	2.38E-15
G-protein coupled receptor signalling pathway	2.03E-13
Immune system process	3.17E-13
Immune response	3.50E-13
Positive regulation of immune system process	5.47E-13
OL vs OPC hypomethylation	
GO process	**Enrichment p-value**
Negative regulation of supramolecular fibre organisation	7.77E-05
Regulation of transport	8.14E-05
Negative regulation of chemokine production	1.24E-04
Regulation of cell projection organisation	1.71E-04
Cellular hyperosmotic response	1.81E-04

Table 3.4 Top GO term in differentially methylated promoters between OPCs and OLs

OL vs POL hypermethylation	
GO component	**Enrichment p-value**
Membrane part	1.82E-16
Intrinsic component of membrane	1.14E-15
integral component of membrane	4.85E-15
Plasma membrane	1.32E-11
Plasma membrane part	4.27E-11
OL vs POL hypomethylation	
GO component	**Enrichment p-value**
Cytoplasmic region	1.32E-05
Neuron part	3.03E-05
Synapse part	3.04E-05
Cell projection	3.48E-05
Perikaryon	3.80E-05
OL vs POL hypermethylation	
GO process	**Enrichment p-value**
Immune system process	1.05E-12
Positive regulation of immune system process	2.82E-12
Regulation of immune response	4.84E-12
Signal transduction	3.83E-11
G-protein coupled receptor signalling pathway	4.99E-11
OL vs POL hypomethylation	
GO process	**Enrichment p-value**
Hyperosmotic response	1.83E-04
Cellular hyperosmotic response	4.40E-04
Response to osmotic stress	7.75E-04

Table 3.5 Top GO term in differentially methylated promoters between POLs and OLs

POL vs OPC hypermethylation	
GO component	**Enrichment p-value**
Membrane part	2.63E-13
Intrinsic component of membrane	4.69E-13
Integral component of membrane	8.12E-13
Plasma membrane	2.20E-08
External side of plasma membrane	1.39E-06
POL vs OPC hypomethylation	
GO component	**Enrichment p-value**
membrane part	3.81E-07
Intrinsic component of membrane	3.19E-06
Integral component of membrane	5.22E-06
Proton-transporting two-sector ATPase complex, catalytic domain	4.77E-04
Cell projection membrane	5.86E-04
POL vs OPC hypermethylation	
GO process	**Enrichment p-value**
G-protein coupled receptor signalling pathway	3.39E-14
Detection of chemical stimulus	1.84E-11
Detection of chemical stimulus involved in sensory perception	2.73E-11
Detection of stimulus involved in sensory perception	8.99E-11
Detection of stimulus	1.04E-10
POL vs OPC hypomethylation	
GO process	**Enrichment p-value**
hyperosmotic response	1.83E-04
cellular hyperosmotic response	4.40E-04
response to osmotic stress	7.75E-04

Table 3.6 Top GO term in differentially methylated promoters between OPCs and POLs

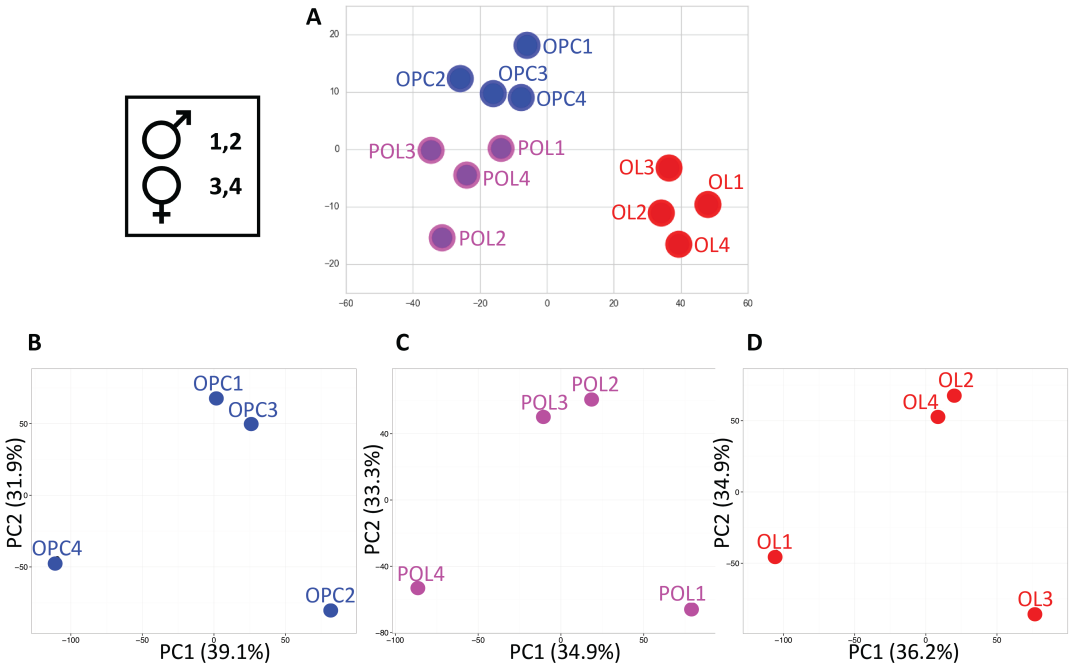

Fig. 3.12 **Transcriptome analysis according to sex in OL lineage cells (A)** t-SNE analysis of all samples. Male samples are labeled 1 and 2, while the female samples are 3 and 4. No clustering according to sex is visible. **(B-D)** PCA analysis for each cell type separately. None of the samples show clustering according to the sex of the animal.

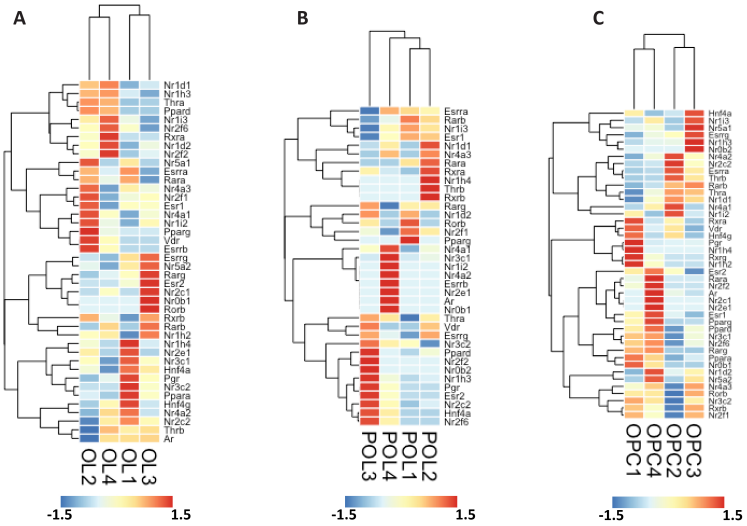

Fig. 3.13 **Expression of nuclear receptors in OL lineage cells (A-C)** Heatmaps showing the clustering of each cell type using only nuclear receptor genes. In this case cells were again clustered differently for each cell type, regardless of the sex of the animal. This is despite using a smaller list of genes which includes sex hormones receptors

I next asked whether the sex of the animal affects the alternative splicing. As shown in fig3.14B the total number of significant (adjusted p-value < 0.05) differentially expressed exons between males and females is dramatically lower than the when comparing cell types (53 vs 3218). This is also true when testing the changes between each two cell types (fig3.14B, right hand graph). This is visualised in the volcano plots shown in figure 3.14. When comparing the number of exons differentially expressed between each two cell types, the most distant cell types (OPCs and OLs) have the fewest significant differentially expressed exons (343), in comparison to the changes between OLs and POLs (2411) or OPC and POLs (2912). This leads to the conclusion that the main differences between distant cell types on the lineage are in expression of specific full transcripts (genes turned on and off), while the subtle changes of alternative splicing mainly differ between cells that are closer on the lineage.

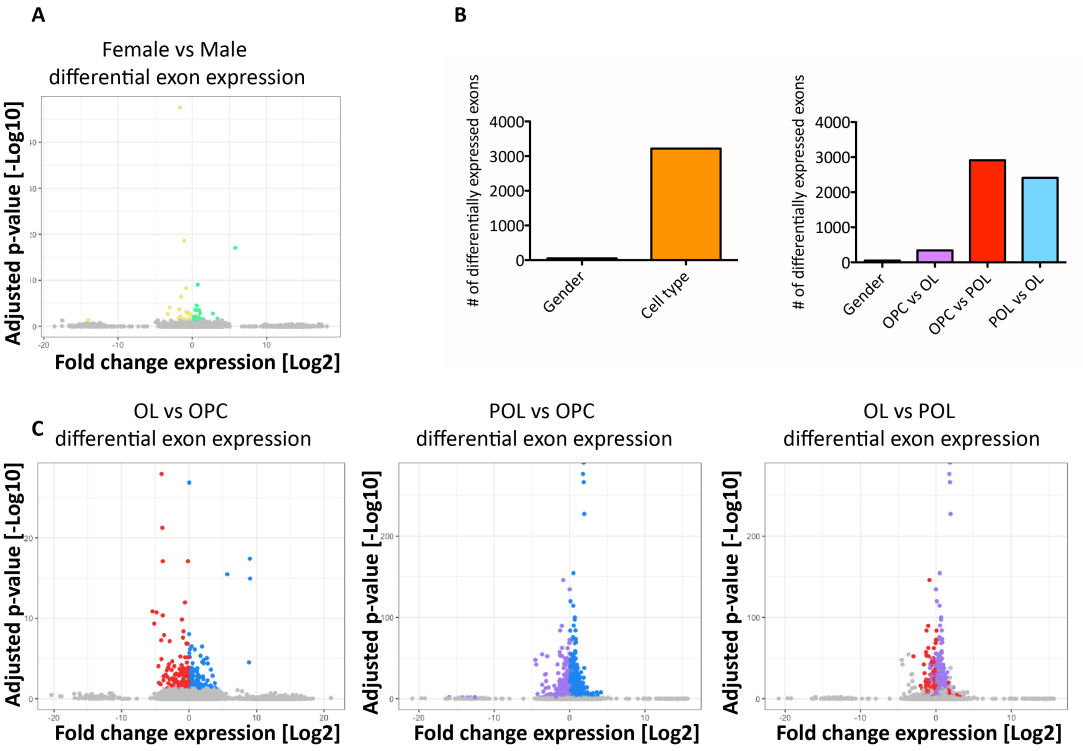

Fig. 3.14 **Differential exon expression in OL lineage cells (A)** Volcano plot showing differentially expressed exons between males and females. Coloured dots represent exons which were significantly differentially expressed (adjusted p-value < 0.05). **(B)** Bar plots summarising the difference in number of significantly expressed exons according to gender and cell type (53 and 3218 respectively. The right hand plot shows the number of differentially expressed exons within each comparison: OPC vs OL (343 exons), OPC vs POL (2912) and OL vs POL (2411). **(C)** Volcano plots visualising the exons differentially expressed between the different cell types. Coloured dots represent significantly (adjusted p-value < 0.05) differences.

3.3.7 Differential exon usage in OL lineage

After analysing changes in full transcript expression between the the different cell types in the adult OL lineage cells, I investigated the possible alternative splicing events between the different cell types, that would lead to differential exon usage between the various cell types. An overview of this analysis was presented in the previous section, in figure 3.14.

I ranked the top genes that exhibit significantly differential exon usage between each two cell types. The top 50 genes in each comparison are listed in figure 3.15A-B. A further GO analysis of all of the genes is then listed in tables 3.7 and 3.8. Most processes enriched between POLs and OLs are related to cell adhesion and cell surface genes. This is not surprising as the maturation of oligodendrocytes involves connection between the OL and axons. Thus, most of the genes were also located in the projection components of the cell. This was true also for the genes which show differential exon usage between OPCs and POLs. Although, the main enriched processes in these genes were not as specific to cell adhesion as the ones mentioned before.

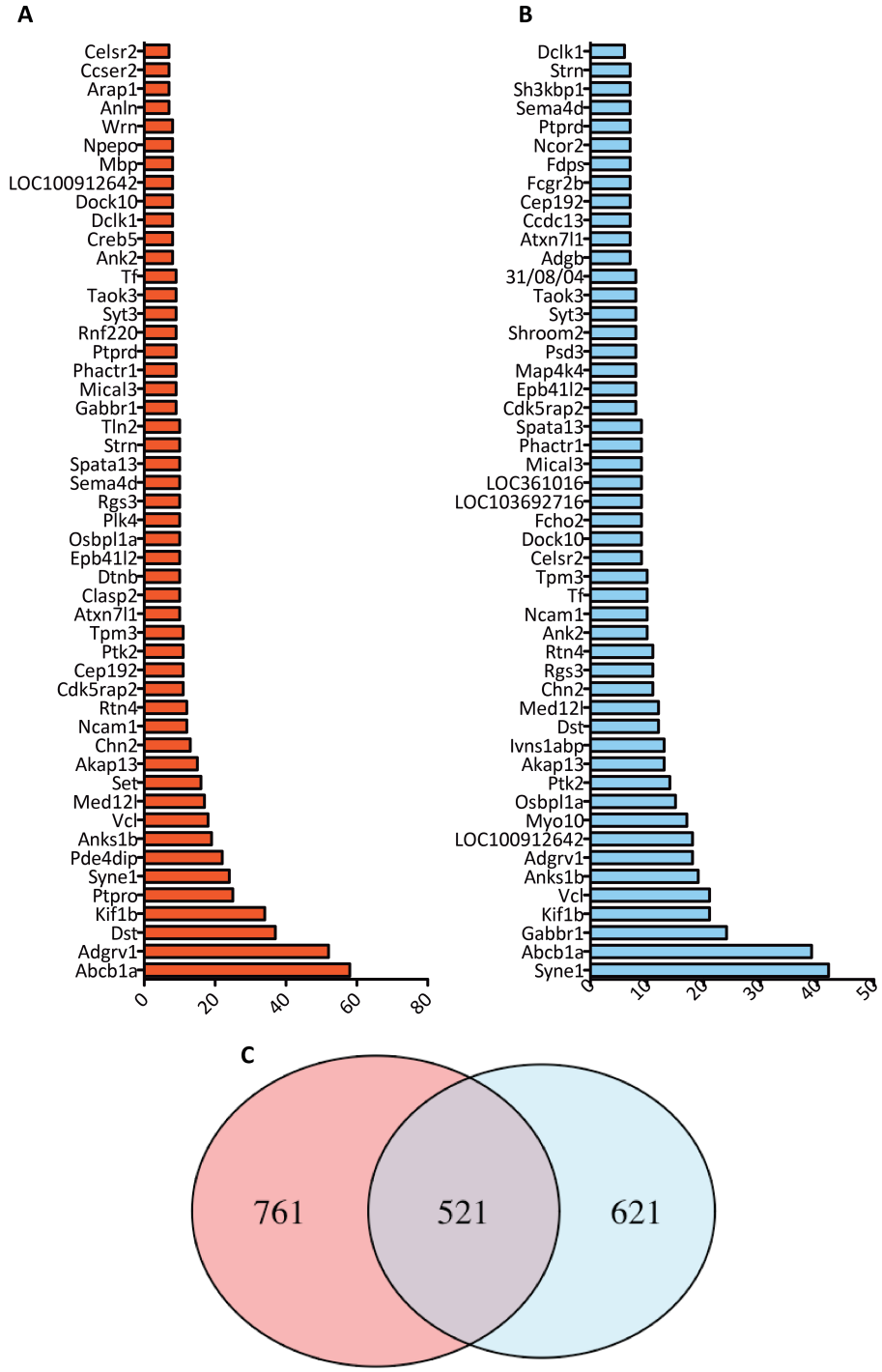

Fig. 3.15 **Top differential exon expression in OL lineage cells** **(A)** Top 50 genes which show highest number of significantly differential exon usage between OPCs and POLs. **(B)** Top 50 genes which show highest number of significantly differential exon usage between OLs and POLs. **(C)** Venn diagram of both comparisons described above. It shows that approximately half of the genes that have differential exon usage are similar in both comparisons.

OL vs POL	
GO process	**Enrichment p-value**
Cell-cell adhesion	1.95E-05
Cell-cell adhesion via plasma-membrane adhesion molecules	3.89E-05
Cell projection organisation	1.89E-04
Single-organism cellular process	2.27E-04
Cell adhesion	3.30E-04
GO component	**Enrichment p-value**
Cytoskeletal part	1.27E-06
Neuron part	6.21E-06
Neuron projection	1.00E-05
Lamellipodium	1.53E-05
Cell projection	1.98E-05

Table 3.7 GO analysis genes showing significant exon differential usage between OLs and POLs

OPC vs POL	
GO process	**Enrichment p-value**
Negative regulation of axonogenesis	3.35E-05
Single-organism cellular process	5.31E-05
Positive regulation of ion transport	8.62E-05
Regulation of cellular component movement	8.99E-05
Positive regulation of endocytosis	1.13E-04
GO component	**Enrichment p-value**
Cell junction	1.79E-08
Neuron projection	6.17E-07
Cell projection	3.78E-06
Plasma membrane bounded cell projection	6.65E-06
Neuron part	8.09E-06

Table 3.8 GO analysis genes showing significant exon differential usage between OPCs and POLs

3.3.8 Transposable elements in OL lineage cells

Using the deep RNA sequencing of multiple samples, I was able to research the changes in transposable elements (TEs) in the OL lineage. Figures 3.16 and 3.17 show the top 25 TE classes which are upregulated between each two cells types (OPC vs POLs and OLs vs POLs, respectively). Reviewing these lists will reveal that many of the classes are mutual in all comparisons. This is further illustrated by the Venn diagram displayed in figure 3.18. This suggests that these TE classes do not play a specific role in the differentiation along the lineage. In order to verify that no specific single TEs are involved in OL lineage differentiation, I plotted the top 500 TEs for each cell type. This time I have used each TE chromosomal address in order to identify unique TEs for each cell type (TE signature). This is illustrated by the Venn diagram in figure 3.19. This diagram illustrates that the number of unique TEs is the highest in OLs, suggesting that TEs that were activated in previous differentiation stages remained active throughout the lineage.

DNA methylation is usually considered one of the mechanisms used by cells to suppress the unwanted activation of TEs. I therefore investigated whether that is the

case in my dataset. Similar to the results shown in chapter 4 I could not establish a correlation between TE expression and methylation levels. This was true for all cell types (fig3.20A-C). I could not find any correlation, even when observing specific TE families such Alu or L1 (fig3.20D and E respectively).

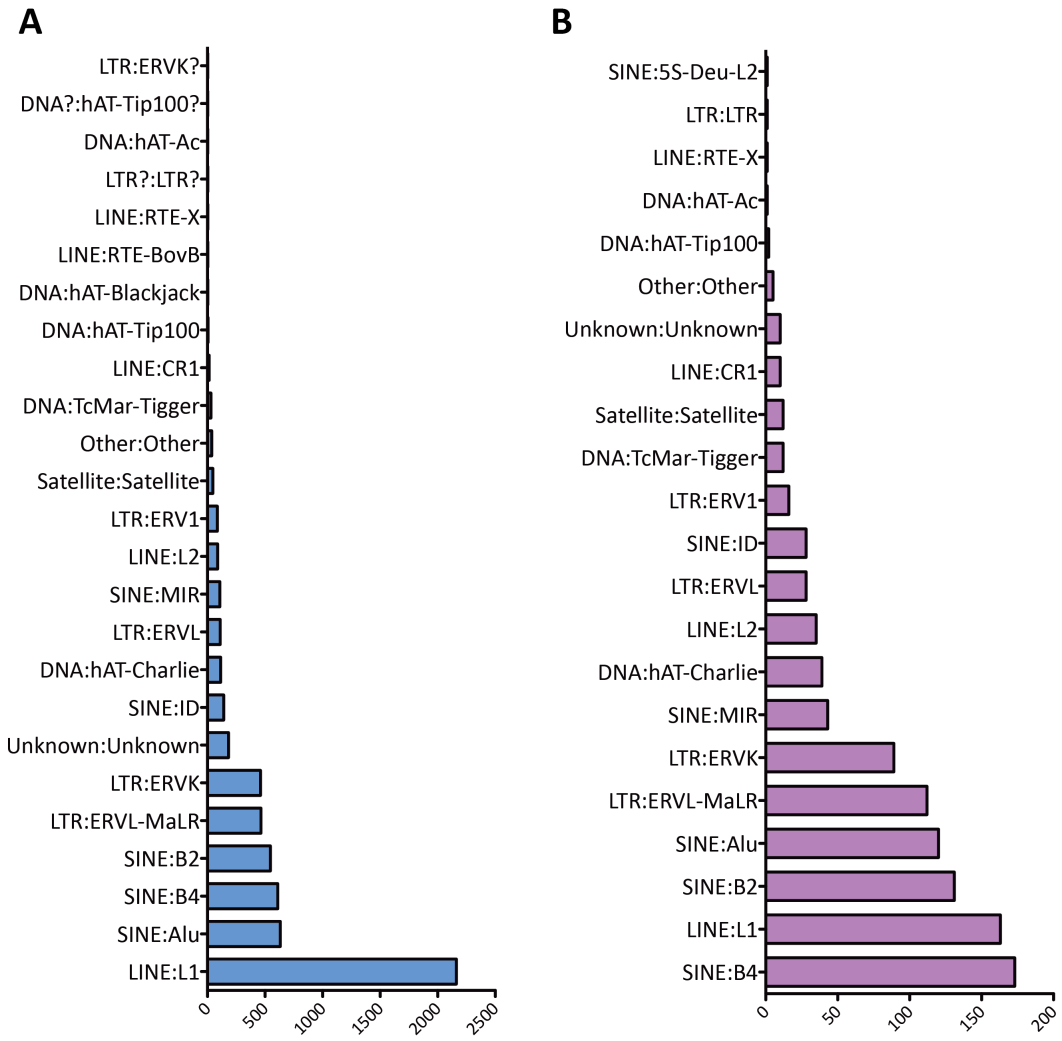

Fig. 3.16 **Top 25 transposable elements classes expressed in OPCs and POLs (A)** Top 25 TE classes which are significantly upregulated in OPCs vs POLs. **(B)** Top 25 TE classes which are significantly upregulated in POLs vs OPCs.

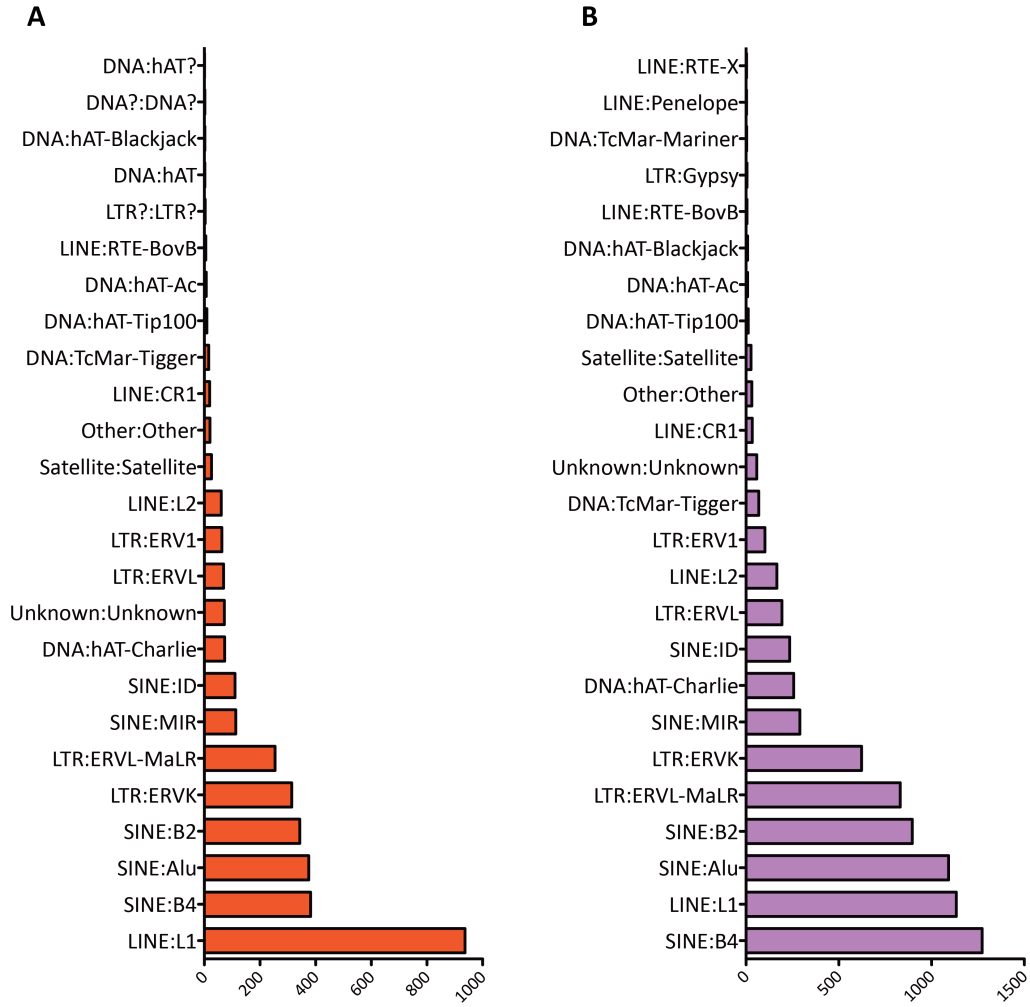

Fig. 3.17 **Top 25 transposable elements classes expressed in OLs and POLs (A)** Top 25 TE classes which are significantly upregulated in OLs vs POLs. **(B)** Top 25 TE classes which are significantly upregulated in POLs vs OLs.

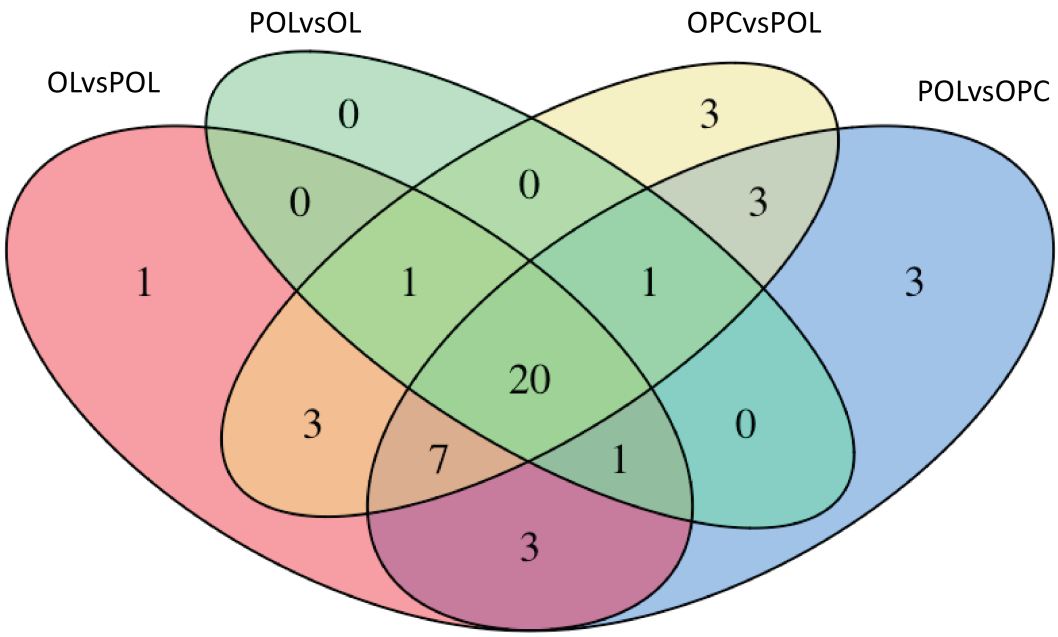

Fig. 3.18 **Venn diagram of TE classes significantly expressed between OL lineage cell types**

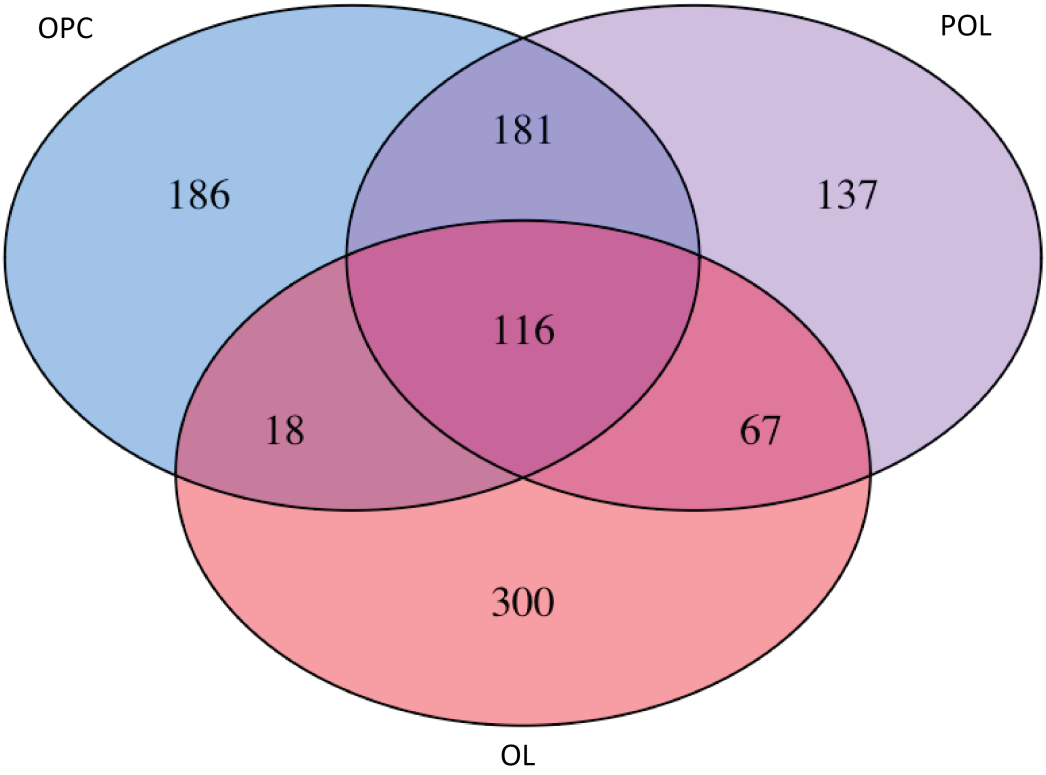

Fig. 3.19 **Venn diagram of 500 top expressed TEs in the different OL lineage cell types** As expected the POLs have the least mount of unique TEs (27%), followed by OPCs (37%) and finally OLs (60% unique TEs).

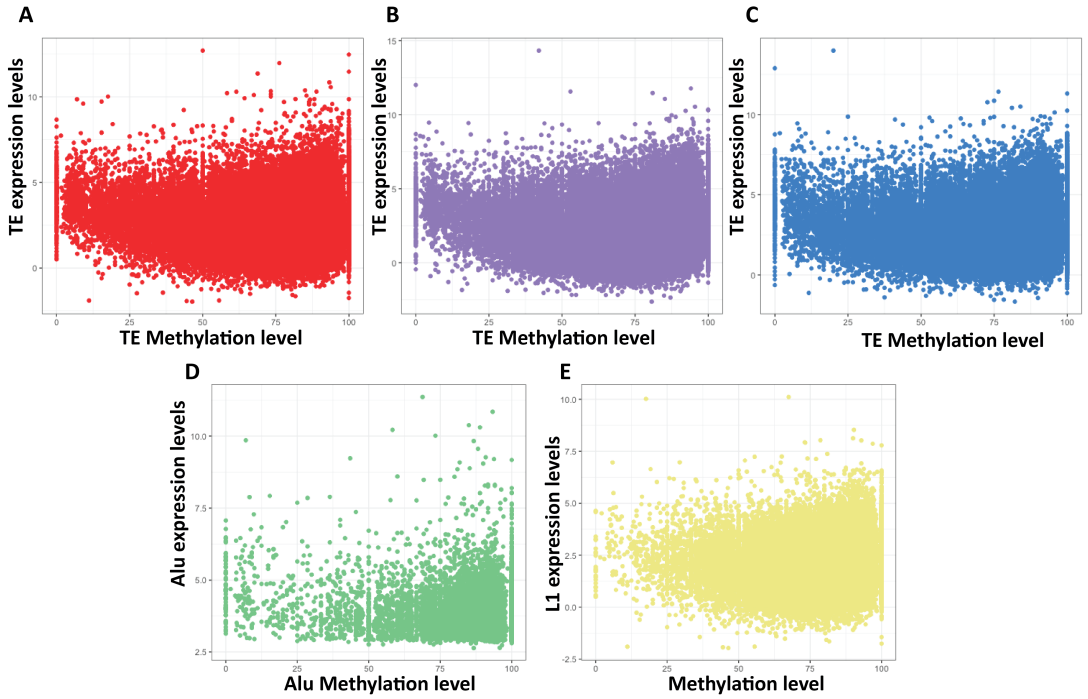

Fig. 3.20 **Expression and methylation levels of TEs in OL lineage cells** All TEs in this analysis were covered by at least 5X coverage for methylation analysis. **(A-C)** Scatterplots illustrating the levels of of expression and methylation of specific TEs in each cell type (OL, POL and OPC - from left to right) It is clear that no correlation can be found between the expression levels of specific TEs and their methylation levels. **(D-E)** Scatterplots showing the expression levels and methylation of Alu (D) and L1 (E) elements in OLs. Again, no correlation can be found between expression levels and methylation levels.

3.4 Discussion

3.4.1 Adult OL lineage isolation

In this part of my project I successfully isolated three different cell type groups within the OL lineage from adult rats. Even though my *in vitro* experiments show predictable results regarding the function and activity of these cells as would have expected from similar studies done in neonatal OL lineage cells, comparing the transcriptomes of adult OL lineage cells yielded different changes between the cell populations compared to the differences detected by previous studies,

For my experiments I decided to begin with MOG positive cells (will be termed OL from here on) and not with A2B5 positive cells (will be termed OPC in this section from now on). This is in oppose to the method used by Zhang et al (Zhang et al., 2014) where OPCs were isolated prior to OLs. I chose to isolate OLs first as I believe there

has to be an overlap between the population so there are cells in transition between OPCs and OLs, which must express both markers. But, it is likely that cells that express MOG, will not be able top revert back to progenitor state, and are already destined to be in the OL fate. Thus, isolating OLs first, allowed me to isolate pure uncommitted OPC population.

Figure 3.5 also support the method I chose, as it shows that proportions of the different sub-populations of within the OL lineage (as represented by the numbers of cells I isolated from each animal) show the developmental trend expected from these cells. Thus, the proportion of mature OL rises with development, whereas the proportions of OPCs declines. This is in line with other reports regarding developmental myelination in rodents, which is known to take place during the first couple of months postnatally (A. H. Crawford, Tripathi, Richardson, & Franklin, 2016).

My results show a distinction between the differences stages in the OL lineage, as seen in figure 3.6. My data also allows a more in depth look into the differences between adult OLs and adult OPCs than were observed before. For example, Moyon et al., (Moyon et al., 2015) compares adult OLs to adult OPCs and reveal changes in only 37 genes. This can stem from multiple differences between the two datasets. Firstly, Moyon et al., used a microarray analysis in comparison to my database which is based on full RNA sequencing. A second, and maybe a more important difference is the method used to isolate the different cells. Moyon used FACS sorting to isolate their cells, using PDGFαR::GFP mice to isolate OPCs, and PLP-GFP mice for OLs. In the PDGFαR::GFP mice, the GFP construct is attached to the histone H2B. This can create a state in which, even though *Pdgfra* expression has ceased, the GFP will linger on. This way, cells which are supposedly PDGFRA positive, are not anymore in reality, thus creating a mix population between OPCs and OLs. This is shown by the single cell sequencing done by Marques et al., (Marques et al., 2016) which used the PDGFRA-H2B-GFP mice to isolate cells from the full OL lineage. Moreover, according to my dataset, unlike neonatal OPCs, which are in constant activation mode and therefore express high levels of *Pdgfra*, adult OPCs are in a resting quiescence state and their expression levels of *Pdgfra* are similar to those expressed by OLs. This again support my original decision to begin with MOG isolation, as this included both mature OLs as well as OPCs which are en route of differentiation. Another observation made by Moyon et al., was that activated adult OPCs upregulate inflammatory mediators as part of their response to demyelination, and specifically indicate the role of *Il1b* and *Ccl2*. In similar fashion, I also found that OPCs and POLs upregulate the expression of both genes, and mainly *Il1b* compared to OLs. This could also be due to the presence of contaminating microglia, but I found this unlikely due to the high percentages of O4+

and A2B5+ cells identified in flow cytometry (fig3.4).
The *in vitro* culturing experiments shown here only compared the cells differentiation capacities. In the future, I would like to perform additional experiments using these cells in order to define their proliferation potential and multipotency. For that I will test what proportions of the cells in each group are proliferative as well as the proportions of cells that can become astrocytes. According to the results I have obtained so far, I would expect that OLs will show the lowest proliferation capacity as well as the lowest astrocyte formation. POLs will show high proliferation capacity, but low number of astrocytes and OPCs will show low proliferation (see quiescence cells in section below) but high capacity for astrocyte formation as these cells are the only ones which are not committed to the OL fate.
The method of isolation I present here can also be used to study the effects of ageing on OLs biology. Using MOG isolation, aged and young OLs can be isolated and their transcriptomes can be compared and analysed. It will interesting to investigate whether there are changes in expression of key genes in aged OLs. I would expect to see changes in the expression of genes related to myelin compaction, as myelin integrity has been shown to deteriorate in normal ageing brains using MRI imaging (Bartzokis et al., 2003). Another feature of OLs that could interesting to explore would be changes in genes related to the tropic support provided by OLs to axons. These changes could be due to changes in expression of full transcripts, or as shown in chapter 5, can be due to changes alternative splicing.

3.4.2 Differences between adult and neonatal OL lineage cells

Comparing my results with those obtained by previous studies (figure 3.7, (Zhang et al., 2014)) revealed major differences in the gene expression signature between cells that are suppose to represent similar stages in the OL lineage. There are several possible explanations for this result:

1. Using different animals for cell isolation -
   Even though mice and rats are both rodents and share many similar traits, it is only reasonable that the main changes will be revealed when observing specific changes in gene expression. Moreover, several studies conducted by various members of my lab (Neumann, personal communication) have shown that rat and mouse OPCs specifically differ considerably in their *in vitro* behaviour and therefore they will harbour transcriptional differences as well.

2. Different isolation sequence -

Zhang et al, used the following sequence of isolations: OPCs, OLs and finally POLs. Whereas I have isolated OLs first and only then proceeded to isolate OPCs and POLs from the remaining pool of cells. As discussed above, using this method ensured that my OPCs do not contain any cells that are already committed to the OL lineage, thus the population is composed mainly of true progenitor/stem cells.

3. Inherent differences between neonatal and adult OL lineage cells -
   Zhang et al., have used neonate mice (P7) for the isolation of cells from the OL lineage. At this stage, most OPCs are highly activated and intense myelination is carried out throughout the rodent CNS. This is in a striking contrast to the CNS state in the adult CNS brain, from which I have isolated the cells. At the age of 2-3 months, most developmental myelination in rats are completed, and OPCs which reside in the brain are in a quiescence state (see next section) and are definitely not in an activated state as their neonatal counterparts.

3.4.3 Adult OPCs are quiescent stem cells

Are OPCs adult stem cells? This has been questioned and debated before (A. Crawford et al., 2014; Franklin & Ffrench-Constant, 2008). The main criteria for defining a cell as a stem cell are their multipotency, ability to self-renewal, asymmetric division and quiescence state, common to many other tissue specific stem cells. Even though majority of OPCs usually differentiate to OLs both in normal conditions as well as following lesions (Zawadzka et al., 2010), they posses multipotent abilities and can differentiate into multiple other cell types including astrocytes (Raff et al., 1983), neurons (Belachew et al., 2003) and even non-CNS cells, such as Schwann cells (Zawadzka et al., 2010). Adult OPCs also show proliferation capabilities (Dawson et al., 2003) and there is evidence that they can undergo asymmetrical division (Sugiarto et al., 2011). But it has not been known whether or not adult OPCs can actually be described as quiescence stem cells, similar to other tissue specific stem cells? (Cheung & Rando, 2013)

The overexpression of known quiescence markers in my OPC isolated population when compared to the OL population, suggests so. The list of genes I used to define quiescence was according to genes expressed in other stem cells, mainly haematopoietic stem cells (HSCs), muscle stem cells (MuSCs) and hair follicle stem cells (HFSCs) (Cheung & Rando, 2013). To that list I added some known quiescence genes described regarding other stem cell niches, such as *Hes1/5* , *Lgr5* and *Pax5/6* (Barker et al.,

2013). Even though most of the genes on the list were upregulated in OPCs, only a few were significantly upregulated. From observing the individual sample data (figure 3.8B) I could infer that there was high variability within samples of each group in the expression levels of these genes. This combined with low amount of mRNA in OPCs in general (especially when compared to the amount in OLs) can explain the difficulty in obtaining significant results. Moreover, many of the genes I used for comparison were detected in other tissues, and are not widely expressed in CNS resident cells. Therefore it is not surprising that the genes that are significantly expressed in OPCs included genes that were detected in quiescence NSCs.
In this section I intend to explore some of the factors upregulated in OPCs and how these factors promote quiescence as been described in other cell types.

- **Lgr5** -
  The *Lgr5* gene encodes an orphan G-protein-coupled receptor. It is a downstream target of the Wnt pathway, and was observed in several types of carcinomas. In the colon and the small intestine, *Lgr5* is expressed by cycling cells, as it is expressed by the crypt base columnar cells. These cells are based in the bottom of the crypt (as their name suggests) and are interspersed between differentiated Paneth cells (Barker et al., 2007).
  Similarly, *Lgr5* is also expressed by cycling hair follicle stem cells (Jaks et al., 2008). Interestingly, these are also the first cells to respond to anagen (formation of hair follicle)-initiating signals. This is similar to the role of OPCs which also responds to external stimuli. This is also in line with some of the GO terms that are enriched in OPCs in comparison to OLs and POLs, including detection of chemical stimulus and cell activation.
  Moreover, as a downstream target of Wnt signalling, it is not surprising that OPCs overexpress *Lgr5* as Wnt inhibition is required for OPC differentiation.

- **Hes1** -
  Hes1 (mammalian hairy and Enhancer-of-split homologues) is a bHLH type transcription factor which is a downstream of the NOTCH signalling pathway. NOTCH signalling is required for the maintenance of of NSCs (Hitoshi et al., 2002). More specifically, overexpression of *Hes1* inhibits the differentiation of neural stem cells (Ishibashi et al., 1994) and has been shown to control neuronal differentiation (Nakamura et al., 2000).
  In OPCs, HES1 expression is downregulated following cuprizone administration, and when not inhibited, it blocks differentiation (Shen, Sandoval, et al., 2008).

In summary, these two examples present genes which are known to be part of the quiescence states in multiple stem cells resident cells, and therefore their upregulation in adult OPCs further supports the notion that these are quiescence stem cells.

Ingenuity Pathway Analysis (IPA) further revealed interesting pathways which are activated in adult OPCs when compared to POLs and OLs. These were NfκB and PPARα pathways (figures 3.9 and 3.10). NfκB pathway was related to the maintenance of pluripotency in both human embryonic stem cells (hES, (Armstrong et al., 2006)) as well as human induced pluripotent cells (IPS, (Takase et al., 2013)). In both cases, NfκB expression was downregulated with the differentiation of the cells. Since NfkB and its dimers have multiple targets in in the genome, only through ChiP-sequencing experiments we will be able to elucidate its specific role in OPCs in particular and stem cells in general. Therefore, I assume that NfκB plays a similar role in the maintenance of the progenitor state in adult OPCs.

This observation regarding the 'stemness' of adult OPCs, raises the question whether the cells I isolated weren't actually neural stem cells (NSCs) and not OPCs? This notion can be supported by the reports of several groups regarding the role of adult NSCs in remyelination. For example, Xing et al., show that in the cuprizone model, NSCs originating in the SVZ were responsible for a substantial amount of remyelination and regeneration (Xing et al., 2014). The generation of OLs by NSCs and OPCs was spatially segregated, and morphological differences were noticed as well. More recently, Samanta and colleagues introduced the mechanism underlying the recruitment of NSCs to remyelination, which included the inhibition of *Gli1* (Samanta et al., 2015). This is similar to the expression pattern of *Gli1* shown in my dataset as *Gli1* is significantly upregulated in OPCs in comparison to OLs (fold change = 3.28, adjusted p-value < 0.001). Thus, NSCs can participate in remyelination and can therefore 'contaminate' the OPC group presented here. I find this unlikely, due to the expression pattern of *Nes* in my database. NSCs are characterised by high expression of Nestin (). This was not evident in my database as OPCs did not display upregulation of Nestin in comparison to OLs or POLs (adjusted p-value = 0.86 and 0.44 respectively). Therefore, I do not believe that my results are due to contamination of the OPC pool by NSCs. The lack of higher expression of *Nes* together with lack of neurons developing in any of my *in vitro* cell culture experiments (data not shown) both support the notion that adult OPCs and NSCs are in fact two distinct and separate cell populations.

In conclusion, using a novel technique to isolate and distinguish between the various cells in the adult OL lineage allowed me to identify a unique signature of the OPCs in the adult CNS, which was cannot be observed when studying neonatal OPCs. This is due to extreme differences in the environments and roles OPCs between

the neonatal and adulthood phases. The data I present here supports the notion that similarly to other tissues in the mammalian body, the CNS also harbours a resident quiescent stem cell population. Further experiments will be needed in order to characterise more clearly the unique state of OPCs in adulthood. Open questions include the spatial organisation of adult OPCs (do they have a niche much like other stem cells?) and their role in normal conditions.

3.4.4 Transposable elements in OL lineage

Transposable elements (TEs) are usually mentioned in relation to DNA damage and ageing, in which they have been shown to increase in expression (this topic is covered extensively in chapter 4 in relation to ageing OPCs). The potential role of TEs in cell differentiation and stem cell maintenance is currently unknown. In this chapter I showed evidence for upregulation of multiple families of TEs in the different OL lineage cell types. At a first glance this can support the notion of a novel role for TEs in OL maturation (and maybe even in other cell types). Unfortunately, I do not think this is the case here. The fact that same families of TEs show upregulation in all cell types suggest that there is no specific role for a TE family in a specific cell type. Using a Venn diagram I show that each cell type only has very few cell type specific TEs uniquely expressed (Figure 3.19). Moreover, as the role of TEs in cell differentiation is currently unknown, it is difficult to verify that these expression patterns are not solely due to correlation with other genes, i.e. - TE which are adjacent to genes which are upregulated can be upregulated as well just by proximity.
Figure 3.19 shows the common specific TEs between cells types. As expected, POLs exhibit the least number of uniquely expressed TEs. I expected that since these cells share most the most active TEs both OPCs (TEs that were activated did not get shut down) and whatever new TEs have been activated in this state, are now shared with the next phase of maturation - the OLs. Surprisingly though, OLs show the highest number of uniquely expressed TEs (60% of the top 500 expressed TEs), which contradict somewhat the theory I have just proposed. I would have expected that OPCs would have higher ratio of expression of TEs as they are progenitor cells and it has been shown that at least neural progenitor cells express higher ratios of TEs (Coufal et al., 2009).

To further investigate the potential role of TEs in OL lineage cells, more specific genomic studies and experiments must be conducted. For example, each significantly upregulated TE should be verified not to be adjacent to other upregulation genes. Furthermore, functional essays must be conducted in order to discover functional roles

of TEs.
Figure 3.20 shows the relationship between TE expression levels and their methylation levels. I could not find such correlation, in contrast to previous studies (Slotkin & Martienssen, 2007; Woodcock, Lawler, Linsenmeyer, Doherty, & Warren, 1997). It is possible that DNA methylation is not the main mechanism in which OL lineage cells silence TE expression, and they employ other epigenetic mechanisms, such histone modification and RNA silencing (Slotkin & Martienssen, 2007).

3.4.5 Differential exon usage in OL lineage cells

Alternative splicing is an elaborate mechanism that allows eukaryotic cells to enhance the number of proteins they can produce from a smaller amount genes. In the OL lineage, Moyon et al., have recently reported that knock out of *Dnmt1* in OPCs led to dysfunctional alternative splicing (Moyon, Huynh, et al., 2016). Therefore I set fourth to examine which genes in the adult OL lineage could be affected by alternative splicing. To examine this I isolated the genes which exhibited significant changes in differential exon usage (adjusted p-value < 0.05) and submitted them to GO analysis using the GOrrila web tool, in the same way I used it previously for analysing changes in full transcripts (tables 3.7 and 3.8 and tables 3.1, 3.2 and 3.3 respectively). In both comparisons (OLs vs POLs and POLs vs OPCs) most genes were identified to be part of the membranous part of the cell, and were even identified into the same categories exactly. This suggests that genes that exhibit alternative splicing in OPCs vs POLs, also show significant changes in exon usage when POLs transition into OLs. This is also evident when comparing the top 50 genes that show significant exon usage - many of the genes are mutual in both comparison as illustrated by the Venn diagram (fig3.15C).
It is therefore clear that these changes in alternative splicing are relevant to the correct OPC differentiation. It yet unknown what are the specific mechanisms that control these splicing events, as my attempts to correlate changes in DNA methylation to differential exon did not yield a correlation (see chapter 5 for specific illustrations). It is possible that further research will uncover the mechanisms that control alternative splicing in OL lineage cells.

3.4.6 Sex based differences in OL lineage cells

One of the main advantages of the isolation method used in this chapter is the ability to isolate all three OL lineage cell types from the same rat. This allowed me to retrieve

multiple samples form both male and female young adult rats. Sex hormones receptors, such as Estrogen receptors have been implicated to have a role in OPC differentiation and remyelination (Gonzalez et al., 2016) as well as progesterone receptors (Hussain et al., 2011). Moreover, pregnant females show decreased symptoms of EAE and MS (Voskuhl & Palaszynski, 2001). This led me to compare my female and male samples. Surprisingly, clustering of the samples was not according to the sex of the animal, but seemed to be random (figure3.12). The lack of clustering according to sex was evident when using different clustering methods, including t-SNE (figure3.12A) , PCA (figure3.12 B-D) and K-means (data not shown). This was also true for when samples were clustered according to nuclear receptors which include multiple sex hormone receptors (figure3.13). Males and females also exhibited a very small changes in differential exon usage in comparison to the striking differences exhibited by the different cell types (figure3.14). These results fall in line with evidences from toxin induced demyelination study performed by Li and colleagues (W. W. Li et al., 2006). In this study male and female rats showed differences in remyelination rates only in advanced ages. Thus, sex based differences in OL lineage cells (and OPC specifically) arise only in later ages than the ones I studied in this chapter. Clearly in order to study these potential changes, the study described in this chapter is needed to be replicated by using aged female and male rats. I expect that this will provide us with knowledge regarding the changes in aged male and female OPCs. As to the studies exhibiting differences in MS and EAE between males and females - one must remember that these are autoimmune based diseases, and as such the adaptive immune system plays a pivotal role in the advancement of the disease. Therefore, according to the results I provide in this chapter, together with the conclusions from toxin induced demyelinating studies, I assume that the underlying reasons for male/female differences in EAE/MS are due to the changes in the immune system.

Chapter 4

Transcriptome and methylome of aged OPCs

4.1 Introduction

One of the main obstacles for successful remyelination is the failure of OPCs to differentiate into mature myelinating OLs following demyelination lesions(Kuhlmann et al., 2008; Woodruff et al., 2004). This phenomenon increases with ageing and is a feature of MS in the chronic stages. As patients usually reach this stage only in older ages, it is sensible to assume that changes that occur with ageing are related to this decline in remyelination and chronic deterioration. Although previous studies have described the changes in remyelination with ageing in general (Shields et al., 1999; Sim et al., 2002), and some have focused on the changes in the macrophage/microglia population (Zhao et al., 2006), only few studies so far have described the intrinsic changes that occur in the OPCs (Shen, Sandoval, et al., 2008).

In a study from 2015, Moyon et al., (Moyon et al., 2015) have described the changes between neonatal and young adult OPCs. The authors specify that adult OPCs exhibit a more differentiated phenotype in comparison to the neonatal counterparts. They further report that during remyelination, activated OPCs are reverted back into neonatal stage before fully differentiating into OLs. Since this study was limited to neonatal and young adult OPCs, and did not include aged OPCs, it does not explain the changes in aged OPCs which limit their differentiation capabilities. Previous studies by Shen and colleagues (Shen, Liu, & Li, 2008; Shen, Sandoval, et al., 2008) show that changes in HDAC activity in aged OPCs can explain at least partially the lack of differentiation in aged OPCs. Therefore, there is reason to believe that aged OPCs are internally different and exhibit transcriptional and epigenetic changes in comparison

to young adult OPCs.

Using newly developed isolation methods recently established in the Franklin lab (B. Neumann et al., n.d.) I was able to isolate OPCs from both young and aged adult rats in order to study the changes in their transcriptome as well as methylome and therefore establish a database that will enable research into potential new targets in aged OPCs

4.2 Experimental strategy

OPCs were isolated using A2B5 magnetic sorting (MACS) (fig4.2) or fluorescent activated (FACS) (fig4.1) from both young and aged male rats. Young adult OPCs were isolated from rats who were 2-3 months old, as this is considered to be an age where remyelination is highly efficient, while developmental myelination is considered to be complete. This is similar to the age chosen by previous studies in order to explore young adult OPCs (Moyon et al., 2015). Aged OPCs were isolated from 18-22 months old rats. Rats older than 24 months began developing tumours and other health problems, which required them to be culled before use. OPCs used for DNA isolation were isolated using FACS sorting, while RNA was taken from MACS sorted cells. DNA / RNA were isolated immediately after cells isolation, using Qiagen DNeasy and RNeasy kits respectively. FACS sorted OPCs were only used for DNA isolation, as attempts to isolate high quality RNA from these samples were not successful. Therefore, MACS isolated cells were used for RNA extraction.

A2B5 monoclonal antibody was used to isolate the OPCs, as this antigen has been associated with OPCs for many years, and has been used previously used to label adult OPCs (Shi, Marinovich, & Barres, 1998; Wolswijk, Riddle, & Noble, 1991). Cells isolated in this method in my lab, stained 2 hours post plating show high percentages of OLIG2 and PDGFRA expression (Neumann et al., 2017). Isolations for methylation sequencing were done using FACS using A2B5-488 conjugated antibodies and CD11B-APC conjugated antibodies to remove and isolate microglia (4.1A). Percentages of OPCs between aged and young rats did not change significantly, in contrast to CD11B numbers which have significantly increased in aged rats (fig4.1B-C). The increase in CD11B in aged rats could be attributed to both increase in microglia numbers with ageing, or an increase in infiltration of peripheral immune cells, which are also CD11B positive (e.g. circulating monocytes, neutrophils, natural killer cells, granulocytes and macrophages).

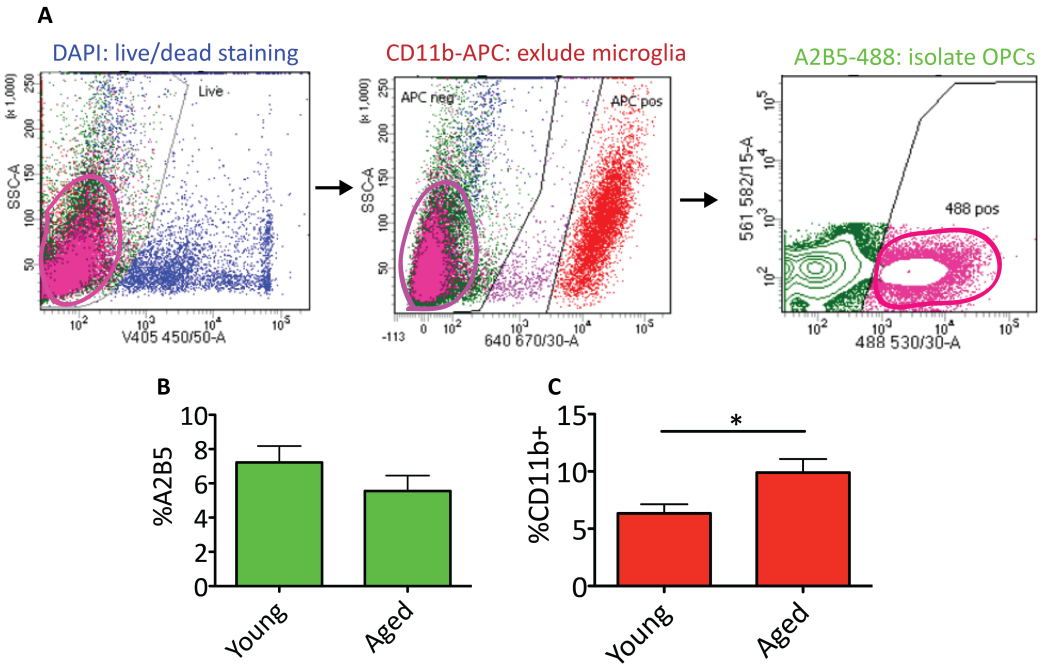

Fig. 4.1 **FACS isolation of A2B5 and CD11B positive cells from aged and young rats (A)** Illustration of the FACS strategy used to isolate both OPCs and microglia from young and aged rats. Desired population is circled by magenta circle in all FACS plots. Prior to FACS, single cell suspension was generated as previously described (section 2.3.1). Cells were divided to live and dead by DAPI staining, as only dead cells incorporate DAPI into their genome. Subsequently CD11B+ cells were removed, before A2B5 cells were isolated. **(B)** Percentage of A2B5+ cells did not significantly differ between aged and young rats (t-test, n=5, p-value = 0.2423). **(C)** CD11B+ percentages increased significantly with ageing (t-test, n = 5, p-value < 0.05)

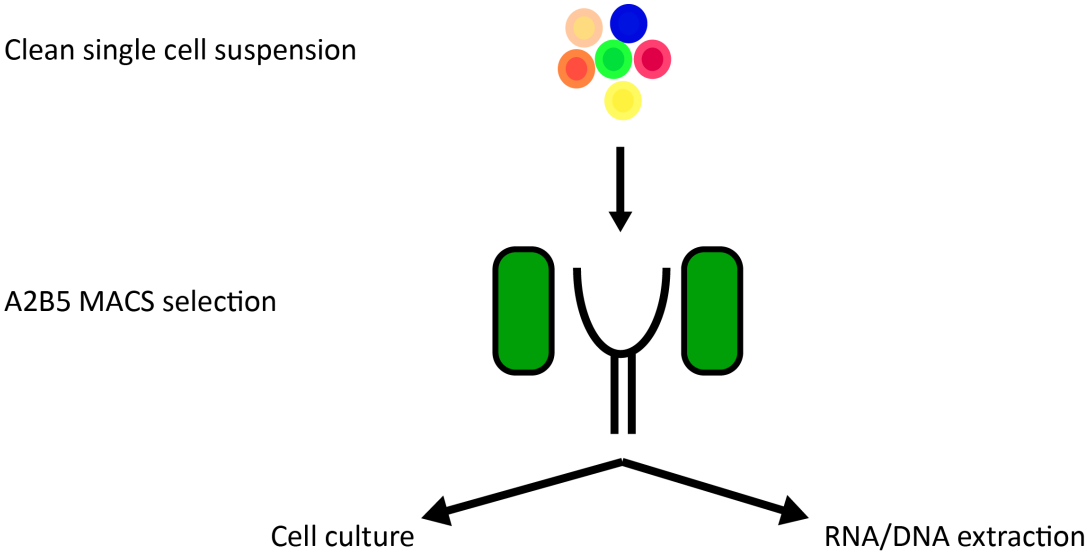

Fig. 4.2 **MACS isolation of A2B5 positive cells from aged and young rats** Schematic description of MACS isolation used to isolate OPCs from young and aged rats. Single cell suspension was generated in similar method as previously described (see section 2.3.1). Cells were labelled using A2B5 antibody, and were subjected to magentic isolation as previously described. Average isolation yielded 10^6 cells.

4.3 Results

4.3.1 Age related changes in OPC transcriptome

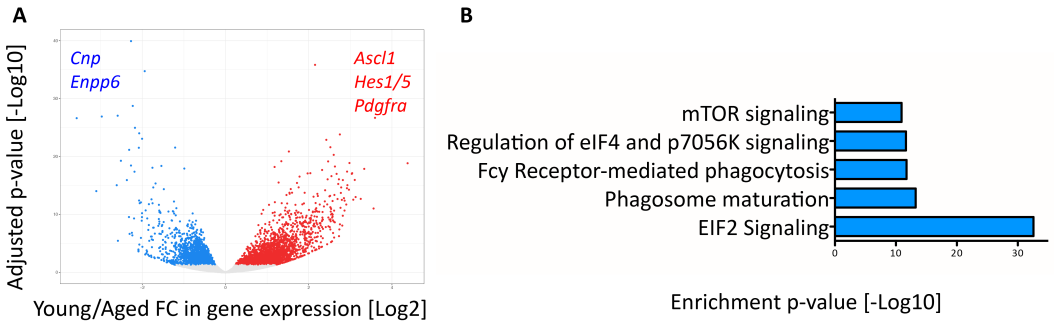

Fig. 4.3 **Changes in gene expression between aged and young OPCs** Following the acute isolation of aged and young OPCs (n = 3) using A2B5 MACS, total RNA was isolated and RNA sequencing was performed. **(A)** Volcano plot visualising expression data for genes sequenced. Coloured dots represent genes that were significantly different between the two conditions (adjusted p-value < 0.05). Red dots represent genes that are upregulated in young OPCs, while blue dots mark genes that are upregulated in aged OPCs. Young OPCs show relative upregulation of progenitor genes such as *Ascl1*, *Hes1/5* and *Pdgfra*. Whereas, aged OPCs show the upregulation of early differentiation genes such as *Cnp* and *Enpp6* (adjusted p-value < 0.05 for all genes). **(B)** IPA results of genes upregulated in aged vs young OPCs. Top five significant pathways are shown, with their respective enrichment p-value (-log10).

As a first step to describe the intrinsic changes between aged and young OPCs, I used the MACS A2B5 isolation method (see Materials and Methods, as well as Nuemann et al., 2017) in order to isolate RNA from both young (2-3 months old) and aged (20-24 months old) OPCs and perform a full RNA sequencing. The volcano plot view (fig4.3A) illustrates that there are multiple genes which are significantly differentially expressed between aged and young OPCs. Young OPCs showed increased expression of progenitor and self renewal genes, such as *Ptprz1, Pdgfra, Hes1, Hes5* and *Ascl1*. This was in contrast to the aged OPC population which exhibited higher levels of early differentiation markers, including *Enpp6, Sirt2* and *Cnp1*, but no myelin genes such as *Mog, Mbp, Mag, Mobp*. Aged OPCs also did not show a higher expression of Wnt pathway inhibitors, such as *Apc* or *Axin2*, which are usually upregulated in differentiating OPCs. Selected genes from this list were then confirmed using qRT-PCR (Neumann et al., 2017). Using a recent single cell sequencing study (Marques et al., 2016) I compiled list of genes which identify OPCs and OL lineage cells. Plotting the expression of the 50 OPC signature genes using a heatmap presentation (fig4.4A), shows that most genes are overexpressed in young OPCs, and not in aged

ones. More detailed examination revealed that 56.25% of the genes were significantly overexpressed in young OPCs in comparison to only 8.33% overexpressed in aged OPCs (adjusted p-value < 0.05) (Neumann et al., 2017). This is illustrated in a pie chart (fig4.4B). Moreover, using k-means clustering according to OL lineage signature genes showed that aged OPCs cluster more closely to mature OLs in comparison to young OPCs (fig4.4C), while still clustering closely to young OPCs.

Figure 4.3B shows the top pathways as predicted by Ingenuity Pathway Analysis (IPA), when analysing the preferentially expressed genes in both aged OPCs. These pathways include both metabolism related processes (such as mTOR) as well as protein translation initiation pathways (EIF2 signalling and eIF4 signalling).
Genes involved in mTOR pathway and were found to be significantly upregulated in aged OPCs in comparison to young ones are illustrated in their cellular compartments in figure 4.5A. A visualisation of this can be observed using a heatmap (fig4.5B).

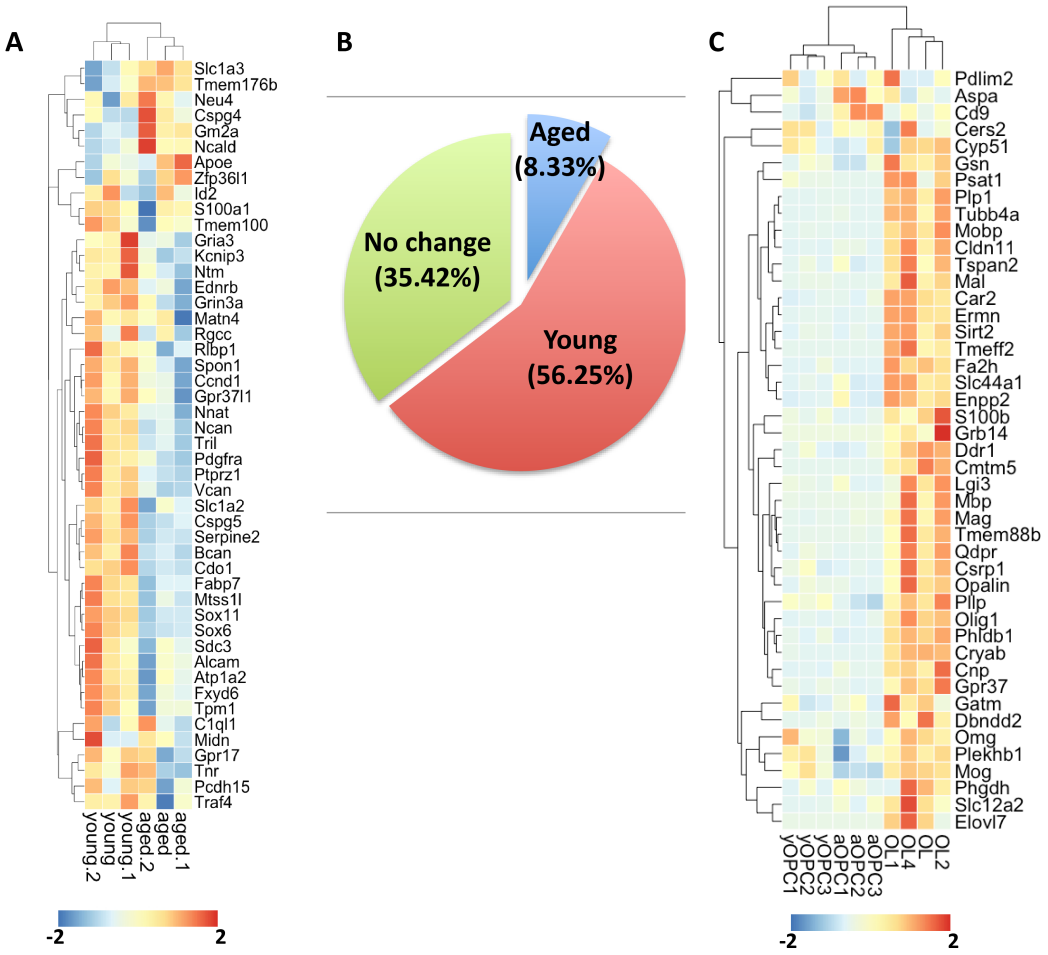

Fig. 4.4 **Upregulation of OL genes in aged OPCs (A)** Heatmap showing the expression of OPC genes (according to recently published single cell sequencing database (Marques et al., 2016)). Red colours represent high expression, whereas blue colours represent low expression. It is clear that most genes show higher expression in young OPCs in comparison to aged OPCs. **(B)** Pie chart illustrating the relative percentages of significantly (adjusted p-value < 0.05) upregulated OPC signature genes in aged and young OPCs. Whereas only 8.33% of the genes are upregulated in aged OPCs (marked in blue), more than half of the genes are upregulated in young OPCs (56.25%, marked in red), including key stem cell genes. 35.42% of the genes showed no significant change in expression between aged and young OPCs. **(C)** Heatmap representing clustering of young OPCs, aged OPCs and young OLs using OL lineage genes (Marques et al., 2016). The heatmap shows that OPCs (regardless of their age) cluster tightly together, but also that aged OPCs cluster closer to OLs than young OPCs, supporting the claim that aged OPCs are more progressed in the OL lineage and lose some of their progenitor markers. Red colours represent higher expression, whereas blue colours represent lower expression levels.

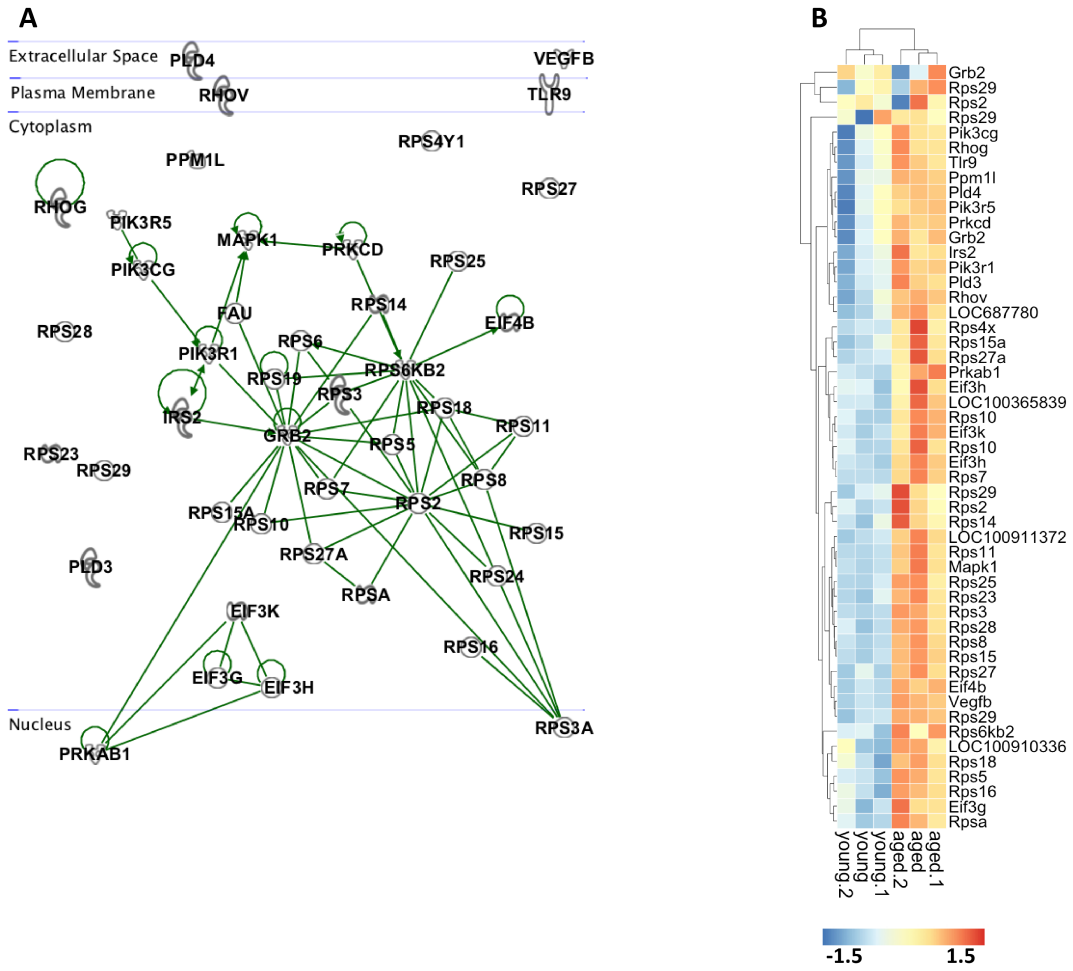

Fig. 4.5 **Upregulation of mTOR pathway in aged OPCs** Using Ingenuity Pathway Analysis (IPA, by Qiagen) multiple pathways were shown to be upregulated in aged OPCs in comparison to young ones (see fig4.3C for the full list). **(A)** mTOR pathway related genes which show significant upregulation in aged vs young OPCs. The illustration presents the relationship between the molecules and their cellular compartments. **(B)** Heatmap showing the mTOR related genes relative expression in young and aged OPCs. Red colours represent higher expression, whereas blue colours represent lower expression levels.

4.3.2 Changes in expression of nuclear receptors in ageing OPCs

Nuclear receptors, even though usually expressed in low copy number, play a pivotal role in intracellular signalling pathways. In OPCs, it has been shown that the Retinoid Acid (RA) receptor families are crucial for OPC differentiation, with emphasis on the RXR-γ receptor (Huang et al., 2011) and stimulation of of Vitamin D receptors (VDR; known binding partner of RXR-γ) can promote OPC differentiation (de la Fuente et al., 2015). Recent work in my lab (Neumann 2017, unpublished) has shown that RXR

agonists, as well as T3 do not enhance the differentiation of aged OPCs *in vitro*. I therefore asked if there are changes in the expression of various nuclear receptors in ageing OPCs in comparison to young ones, as this might serve as an explanation to the recent results I have just mentioned.

To test the changes in nuclear receptor expression, I compiled a list according to known literature that included 47 genes (Di Canio et al., unpublished), which many of are known to play a role in OPC differentiation. Figure 4.6A summarises the differences in gene expression, where coloured dots represent genes which are significantly changed with age (adjusted p-value < 0.05). Red dots signify genes that are upregulated in young OPCs, whereas blue dots represent genes upregulated in aged OPCs. Figure 4.6B summarises the percentage of genes which changed expression in aged and young OPCs (22.92% of the genes are downregulated in aged OPCs, in comparison to a single gene which is upregulated in aged OPCs).

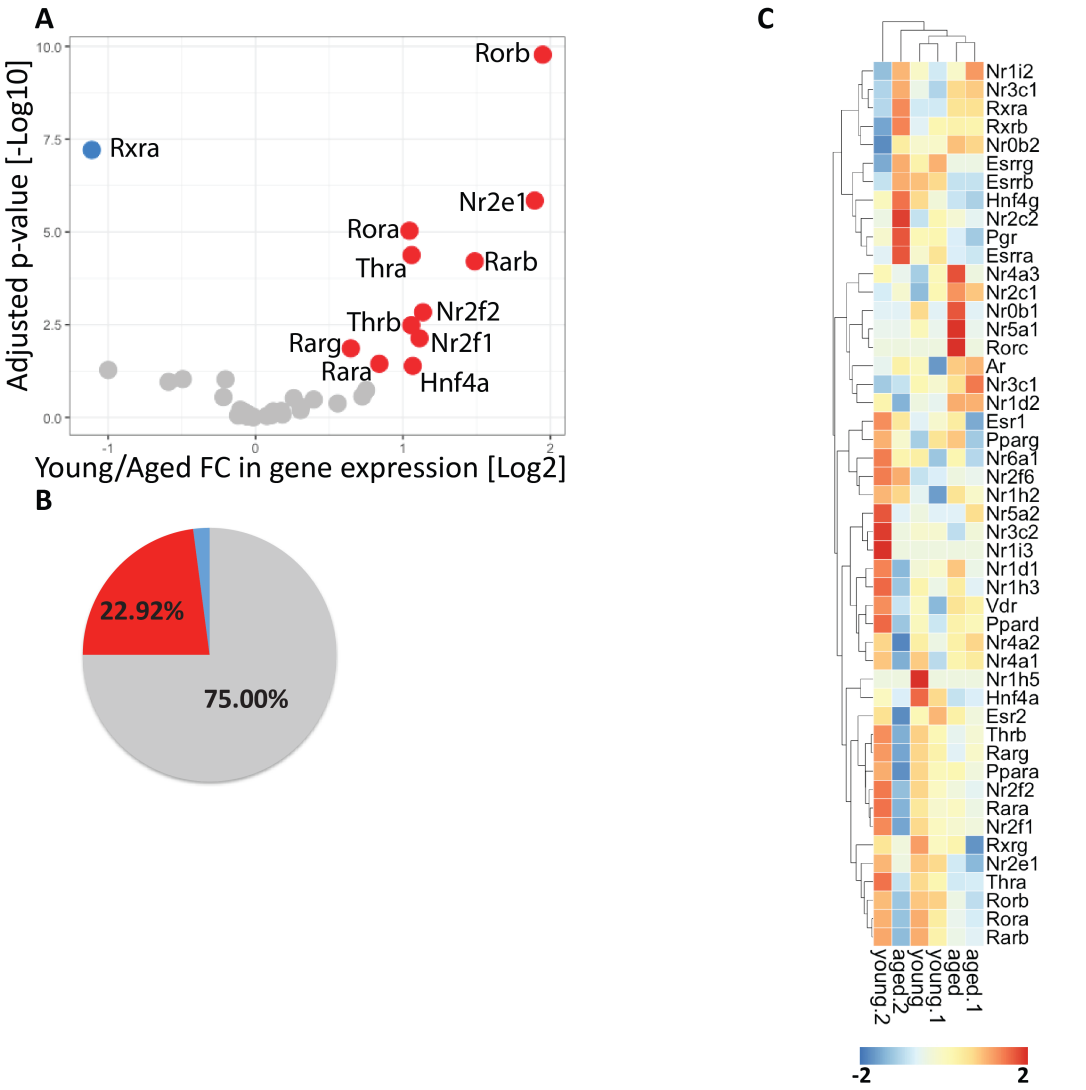

Fig. 4.6 **Changes in nuclear receptors expression in ageing OPCs (A)** Volcano plot visualising transcriptome data from young and aged OPCs of selected nuclear receptor genes. Significantly higher expressed genes in young OPCs are marked in red dots, and significant higher expressed genes in aged OPCs are marked in blue dots (adjusted p-value < 0.05). **(B)** Pie chart displaying the percentages of genes differentially expressed between young and aged OPCs. 75% of the genes were not significantly changed between young and aged OPCs (marked in grey in the doughnut chart). When examining the 25% of the genes which exhibit significant changes in expression, 96% are downregualted in aged OPCs in comparison to young OPCs (marked in red). **(C)** Heatmap illustrating the changes in gene expression of nuclear receptors genes in young and aged OPCs.

4.3.3 Changes in DNA methylation in ageing OPCs

After observing the changes in the transcriptome of aged and young OPCs, I continued to investigate more potential intrinsic changes between young and aged adult OPCs. Knowing that epigenetic changes play a significant role in ageing ((Issa, 2014) and chapter 1 for a full review), I decided to compare the methylome of aged and young OPCs. For this, OPCs were FACS sorted from aged and young OPCs (n = 2 for each age group), according to A2B5 positive expression and the lack of CD11B expression (see fig4.1). DAPI staining was used to distinguish between live and dead cells. Throughout the isolations, live cells accounted for more than 90% of the total cells identified (according to SSC and FSA analysis). Freshly isolated cells were lysed immediately, and total genomic DNA was extracted and Whole Genome Bisulfite Sequencing (WGBS) was performed (see Materials and Methods for further details).

Global changes in aged OPCs methylome

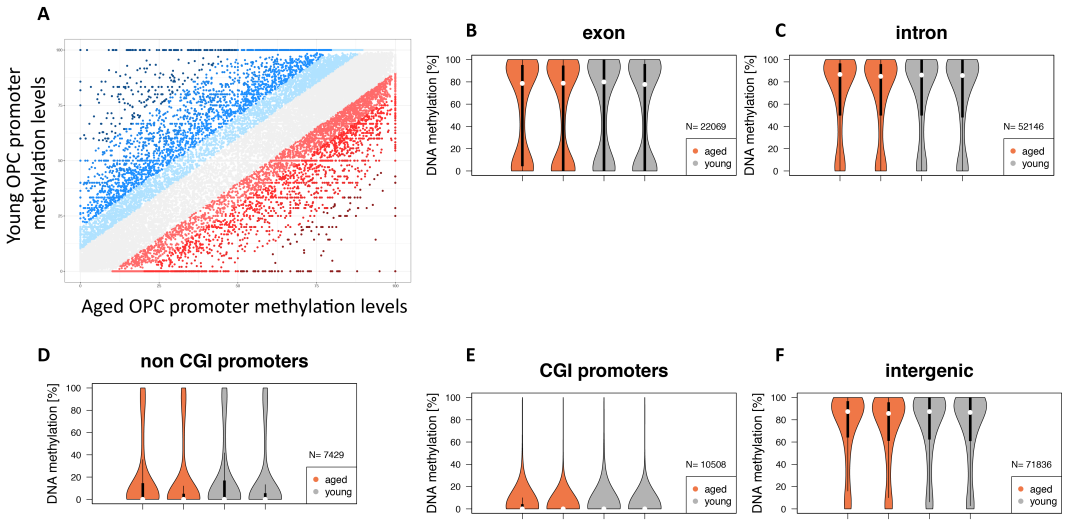

Fig. 4.7 **Global genome changes in methylation in ageing OPCs** Following A2B5 FACS sorting, total genomic DNA was purified from aged and young OPCs (n = 2) for WGBS. **(A)** Scatter plot including the methylation levels in CpGs in all promoters. Coloured dots represent CpGs in which methylation change was higher than 10%. Red dots represent CpGs which are hypermethylated in aged OPCs, while blue dots represent CpGs which are hypermethylated in young OPCs. The darker the colour, the greater the change in methylation between the samples. Only CpGs with a minimum of 5X coverage were used for the analysis. **(B-F)** Violin plots showing methylation rates in various genomic regions. As can be seen, there are no observable changes in any of the examined regions.

After obtaining WGBS results for both aged and young OPCs, I examined the data to identify global changes in the methylome of aged OPCs. As can be observed in figure 4.7, although there are many changes in CpGs in promoters (fig4.7A), there was no overall genomic trend when observing specific functional genomic regions (fig4.7B-F).

Specific changes in aged OPC promoter methylation

Since there were no significant global changes in the methylome of aged OPCs, I focused my analysis specifically on promoter methylation, as it thought be more closely related to gene expression (A. P. Bird, 1985). Comparing the methylomes of aged and young OPCs revealed multiple specific changes in methylation, but not global changes (figure 4.7 A), in contrast to previous studies (Issa, 2014). Figure 4.8A plots all the promoters which are differentially methylated between aged and young OPCs. Next, I examined the same genes I used for defining young OPCs. Unlike the transcriptomic results, which have shown a clear overexpression of OPC related genes in young OPCs, this did not replicate in their methylome. Thus, most differentially methylated genes were hypermethylated in young OPCs in comparison to aged ones (21 genes versus 7). Including promoters of genes which were overexpressed in young OPCs and verified by qRT-PCR (such as, *Ptprz1*). Similar results were observed when I performed the same analysis for nuclear receptor genes (fig4.8B).

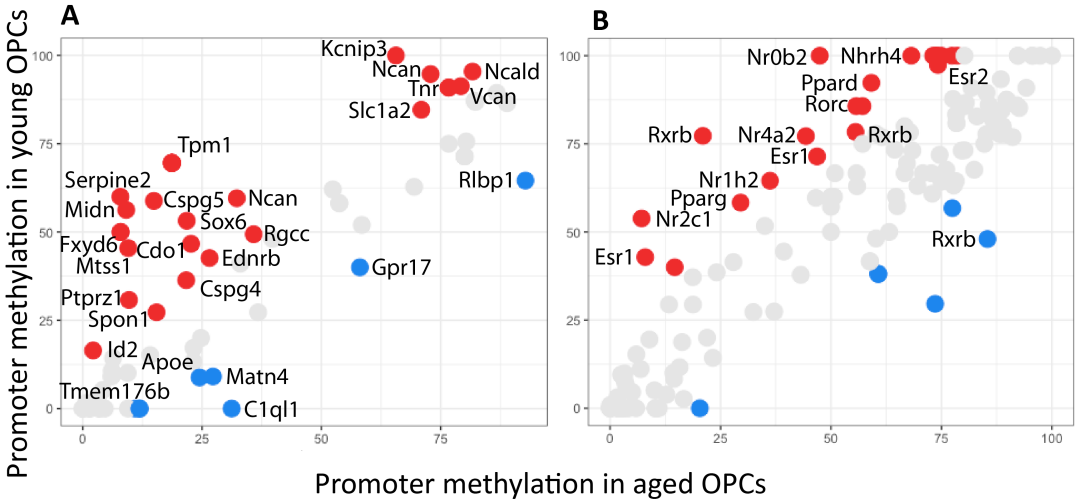

Fig. 4.8 **Changes in OPC promoter methylation (A)** Scatterplot shows the promoters of OPC associated genes and their level of methylation in aged and young OPCs. Blue dots represent promoters which are hypermethylated in aged OPC in comparison to young OPCs, and red dots represent the opposite. Change in methylation was analysed only for CpGs with at least 5X coverage, and methylation change of at least 10%. It clear that most promoters lose their methylation marks with ageing, suggesting loss of correct epigenetic landscape with ageing. **(B)** Another example for methylomic changes with ageing is shown by focusing on promoter methylation changes in nuclear receptors genes. This scatterplot shows loss of methylation mark in multiple promoters of key nuclear receptor indicating an epigenetic drift with ageing.

Since there was not correlation between the expression of OPC genes and their methylation (as well as for nuclear receptor genes), I then tested this correlation for all promoters. Figure 4.9 shows both changes in gene expression as well as DNA methylation for all genes. Hypo or hyper-methylation in the graph indicate the status in aged cells (i.e. hypomethylated promoter, is a promoter which exhibits loss of methylation markings in aged cells). Down or up-regulation of genes were also calculated as compared to status in aged cells (i.e. upregulated genes are genes with significantly increased expression in aged OPCs in comparison to young ones). In contrast to previous studies, I could not identify a strong correlation between promoter methylation levels and transcription levels. This is illustrated in fig4.9C pie chart which summarises percentages of each category.

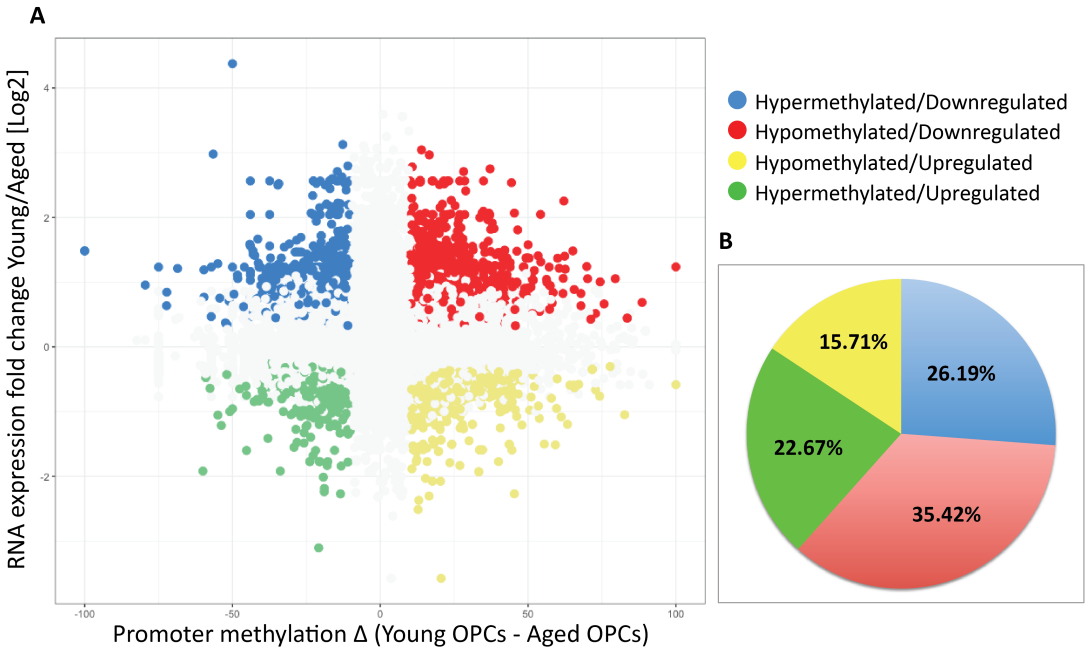

Fig. 4.9 **Correlation between DNA methylation and transcription levels in aged and young OPCs (A)** Scatterplot combining both methylation data and gene expression data in aged and young OPCs. Colourful dots represent genes which show significant change in methylation and gene expression between aged and young OPCs. Methylation and expression levels indicate aged OPC state. **(B)** Pie chart summarising the percentages of each category of genes. This shows that majority of hypomethylated genes in aged OPCs are also downregulated in aged OPCs, in contrast to previous reports regarding the relation between methylation and transcription

Changes in DNA methylation in intragenic regions and their effect on transcription

Since WGBS was used in my study, I was able to analyse the potential methylation changes in gene bodies as well as promoters. This in contrast to previous studies which examined methylation changes in OPCs and used more restricted methods such Reduced Representation Bisulfite Sequencing (RRBS) (Moyon, Huynh, et al., 2016).

For this analysis, Differentiated Methylated Regions (DMRs) were chosen as ones which include 3 CpGs at least, and a significant change was considered as one with more than 3 CpGs showing a change in methylation status. Only 869 intragenic regions answered these criteria. Since there are conflicting reports in the literature regarding the effects of intragenic DMRs, I tested the expression levels of transcripts regardless of the direction of change in methylation (hypo or hyper). The majority of genes exhibiting DMR between aged and young OPCs, also showed a decrease in expression in aged OPCs (fig4.10A). In fact 72.14% of these genes showed a significant

downregulation with ageing (fig4.10B). These proportions did not change significantly if only hypomethylated genes or hypermethylated genes were considered (fig4.10C-D respectively). These results suggest that regardless of the change in methylation at the gene bodies, the majority of these genes exhibit a decrease in expression in aged OPCs, in comparison to young ones.

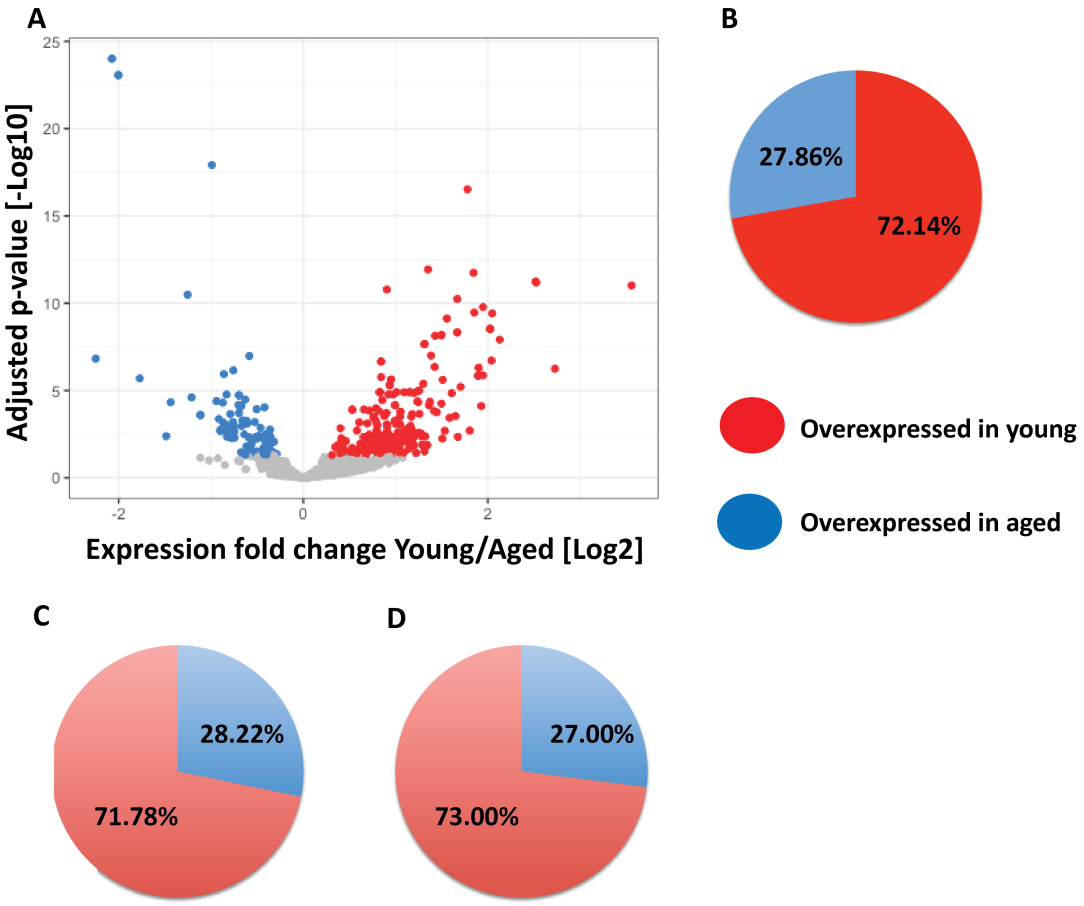

Fig. 4.10 **Age related changes in intragenic methylation** Intragenic regions were chosen as ones which contain a minimum of 3 CpGs, and at least 3 CpGs exhibited changes in methylation between aged and young OPCs. **(A)** Volcano plot showing only genes which exhibit significant (more than 3 CpGs changed) aged related changes in methylation in intragenic regions. Red coloured dots represent genes overexpressed in young OPCs in comparison to aged OPCs. Blue dots represent genes which exhibit overexpression in aged OPCs in comparison to young ones. **(B)** Pie chart summarising the changes in gene expression between aged and young OPCs in genes which exhibit significant changes in methylation in intragenic regions. A majority of these genes (72.14%) showed an increase in expression in young OPCs (coloured in red), in comparison to genes overexpressed in aged OPCs (coloured in blue). **(C)** Percentages of genes overexpressed in young and aged OPCs (coloured red and blue respectively), within genes which are hypomethylated in aged OPCs. **(D)** Percentages of genes overexpressed in young and aged OPCs (coloured red and blue respectively), within genes which are hypermethylated in aged OPCs.

4.3.4 Changes in alternative splicing in ageing OPCs

Alternative splicing has been studied in regards to ageing in general (Harries et al., 2011; Witten et al., 2011a) and neurodegeneration specifically (Witten et al., 2011b),

and so I asked if it can contribute to the ageing phenotype observed in aged OPCs. Differential Exon Expression (DES) analysis was performed using the DEXseq R package. Plotting genes which are overexpressed in young OPCs in comparison to aged ones and testing whether they also show upregulation in changes in exons expression (fig4.11A) revealed that there are almost no genes which are significantly changed in both the full expression as well as in individual exons. Thus, there is no connection between full transcript expression to changes in splicing. When examining which genes show significant changes in splicing (fig4.11B) it was clear that young OPCs exhibit higher rates specific exon expression. In order to focus on changes in alternative splicing, that will not be contaminated by changes in whole gene expression, I filtered out of the gene list all the genes that were differentially expressed between aged and young OPCs (changes in full transcripts). This allowed me to add another layer of observed changes between young and aged OPCs. GO analysis of the exons which are differentially expressed between young and aged OPCs reveal that the top processes which exhibit alternative splicing are involved in regulation of metabolic processes (fig4.11C).

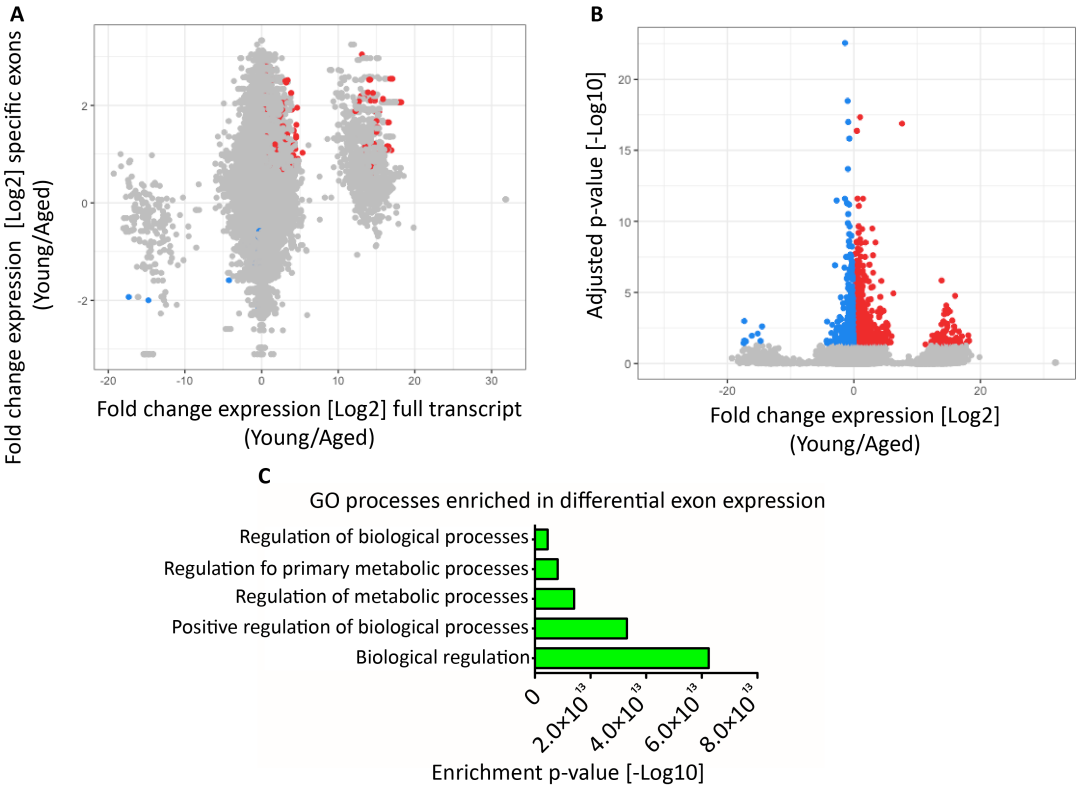

Fig. 4.11 **Differential exon expression in aged and young OPCs analysis (A)** Scatter plot showing Log2 fold changes in full transcript expression (X axis) plotted against Log2 fold changes in exons of the same gene (Y axis). Red dots represent genes which express significant (adjusted p-value < 0.05) upregulation full gene expression as well specific exon upregulation in young OPCs versus aged OPCs. Blue dots represent genes under the same criteria but only upregulated in aged OPCs versus young OPCs. Only a handful of genes show the same trend in transcript expression and differential exon expression. Thus, there is very little correlation between full transcript transcription and differential exon expression. **(B)** Volcano plot showing all the exons significant differential expression between young and aged OPCs. Blue dots represent exons overexpressed in aged OPCs, while red ones represent exons over expressed in young OPCs. Grey dots represent exons not significantly differentially expressed (adjusted p-value > 0.05). This plot illustrates that young OPCs show a higher number of unique spliced exons in comparison to aged OPCs. **(C)** Top 5 processes identified in GO analysis in significantly changed exons. Only genes of which full transcripts are not differentially expressed between young and aged OPCs were used for this analysis.

In order to investigate more specifically which genes show the most alternative splicing, I plotted the top 50 genes that show significant splicing events (figure 4.12). At the top of this list is *Kif1b* which exhibits 15 significant alternative splicing between aged and young OPCs. *Kif1b* has been shown before to play a pivotal role in the localisation of *Mbp* mRNA in OL processes during myelination (Lyons, Naylor, Scholze, & Talbot, 2009). The list includes several more genes which has been show to

be expressed in OPCs, such as *Myrf* (Emery et al., 2009) and *C3* (Hosokawa, Klegeris, Maguire, & McGeer, 2003). A visualisation of the differential exon usage of *Kif1b* and *Myrf* is shown in figure 4.13. I also plotted the differential exon usage of *Mbp*, as an example of a gene which expresses lower levels of significant change.

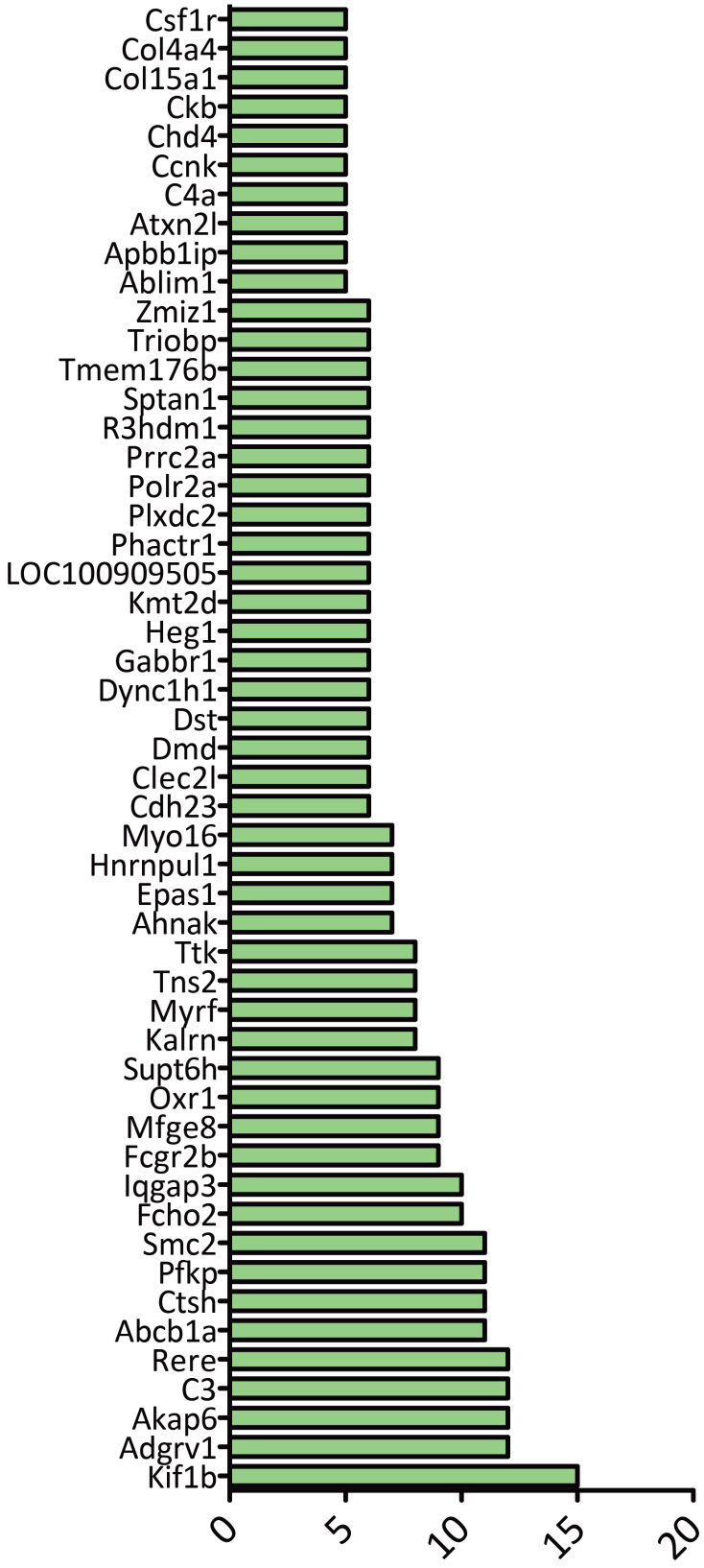

Fig. 4.12 **Top 50 genes exhibiting alternative splicing between aged and young OPCs** Top 50 genes which exhibit significant differential exons expression between aged and young OPCs (adjusted p-value < 0.05). Genes are ranked according to number of significantly differentiated exons expressed between young and aged OPCs.

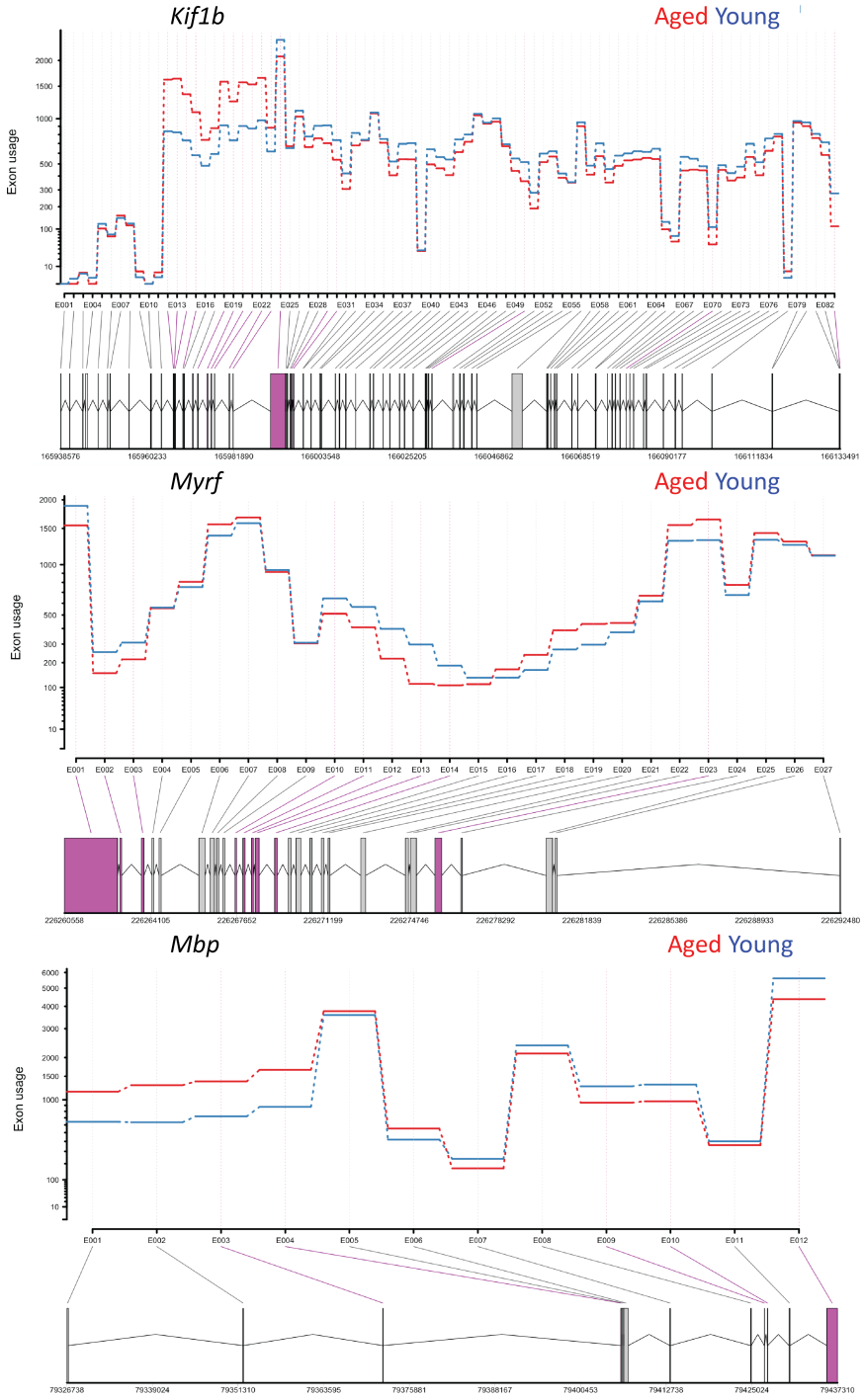

Fig. 4.13 **Visual representation of differential exon usage in *Kif1b*, *Myrf* and *Mbp***. This visual presentation allows to view the differences between genes in the amount of differentially expressed exons. Aged transcripts are marked in red, while young transcripts are marked in blue. Significantly different expressed exons (adjusted p-value < 0.05) are marked in magenta for each transcript. Note that *Mbp* exhibits a low amount of significantly differential expressed exons, in comparison to *Kif1b* or *Myrf*

Aged related alternative splicing in *Myrf*

Figure 4.13 illustrates the changes in exon expression in the *Myrf* transcript between aged and young OPCs. Since *Myrf* plays a crucial role in the regulation of many of the myelin genes I asked what could be the results of this alternative splicing. Out of 27 exons in *Myrf*, 9 show significantly changed expression between aged and young, and 8/9 are downregulated in aged OPCs (adjusted p-value < 0.05). Using the ensemble genome browser (fig4.14) I identified the protein domains that are potentially affected by the loss of specific exons in aged *Myrf* transcript. Exons 1-3 are are significantly downregulated in aged OPCs (log2 fold change = 0.28, 0.72, and 0.51, adjusted p-value < 0.05, respectively) and comprise the PF13888 domain, which is part of MYRF C-terminal domain. Exons 13-14 are also dowregulated in aged OPCs and are also part of MYRF C-terminal domain (PF13887). Exon 14 is also downregulated in aged OPCs and constitute a part of the ICA domain (PF13884) - Intramolecular chaperone auto-processing domain. This domains enables the auto-proteolysis of the protein. MYRF is translated as membrane bound protein, that in order to translocate to the nucleus is auto-processed into two domains. This is done by the ICA domain. This auto-processing is essential for the transcriptional activity of MYRF, and therefore is required for the activation of multiple myelin genes (Z. Li, Park, & Marcotte, 2013).

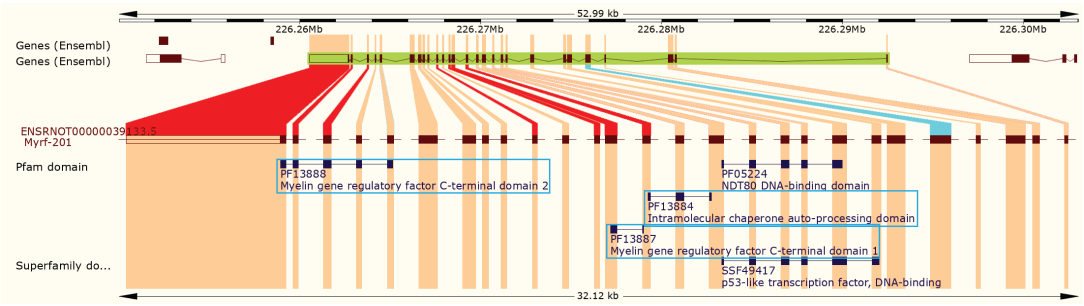

Fig. 4.14 **Genomic representation of *Myrf* exons** Using ensemble genome browser, protein domains in MYRF were identified according to the exons that constitute them. Exons are numbered from left to right according to the graph shown in figure 4.13. Exon marked in red are significantly downregulated in aged OPCs, while blue ones are upregulated in aged OPCs (adjusted p-value < 0.05). Full view of the genome browser is available in the following link: http://www.ensembl.org/Rattus$_n$*orvegicus*/*Gene*/*Splice*?*db* = *core*;*g* = *ENSRNOG*00000028274;*r* = 1 : 226260558 − 226292480;*t* = *ENSRNOT*00000039133

4.3.5 Transposable elements in ageing OPCs

Transposable elements (TE) are DNA sequences with the ability to change their location within the genome. TEs constitute a considerable fractions of the eukaryotic genome and are considered harmful to the host genome as their success in duplication can come at the expense of the host genome stability. Eukaryotic cells have developed epigenetic mechanisms in order to inhibit activation of TEs. One of the main mechanisms involved in TE suppression, is DNA methylation (Slotkin & Martienssen, 2007). Thus, I used the deep sequencing that was available to me from aged and young OPCs, to investigate the possible changes in TE expression in these cells. The results of this comparison are summarised in figure4.15. In total, 5877 TEs were significantly differentially expressed between aged and young OPCs (adjusted p-value < 0.05). Out of these TEs, 80.13% were upregulated in young OPCs in comparison to aged ones. Only 19.87% of the TEs were overexpressed in aged OPCs. Figure4.15A-B lists the the classes of TEs which are overexpressed in young and aged OPCs respectively. Venn diagram analysis showed that most of the families expressed in aged OPCs are also expressed in young OPCs (as expected), whereas young OPCs exhibit high expression of 13 classes of TEs, not expressed in aged OPCs. These include DNA TEs, LTRs, and SINE. This increased expression in TEs in young OPCs, included elements which have been previously reported to increase in senescent aged cells, such as Alu (De Cecco, Criscione, Peckham, et al., 2013).

In order to have a more detailed analysis of the different TEs expressed in aged and young OPCs, I have plotted the top 500 expressed TEs in aged and young OPCs, as well as in OLs. I could then test whether young and aged OPCs express different TEs, and also see if aged OPCs show a pattern of TE expression more similar to OLs than young OPCs do (similar to their gene expression profile described before). Venn diagram in figure 4.16 shows that between young and aged OPCs more than half of the top 500 expressed TEs are common. Moreover, it shows that young OPCs have more TEs in common with OLs than aged OPCs (32 vs 10). Since young OLs were used for this analysis this suggests that the age of the cells, has more effect on the expression of the TEs than how mature the OPC is.

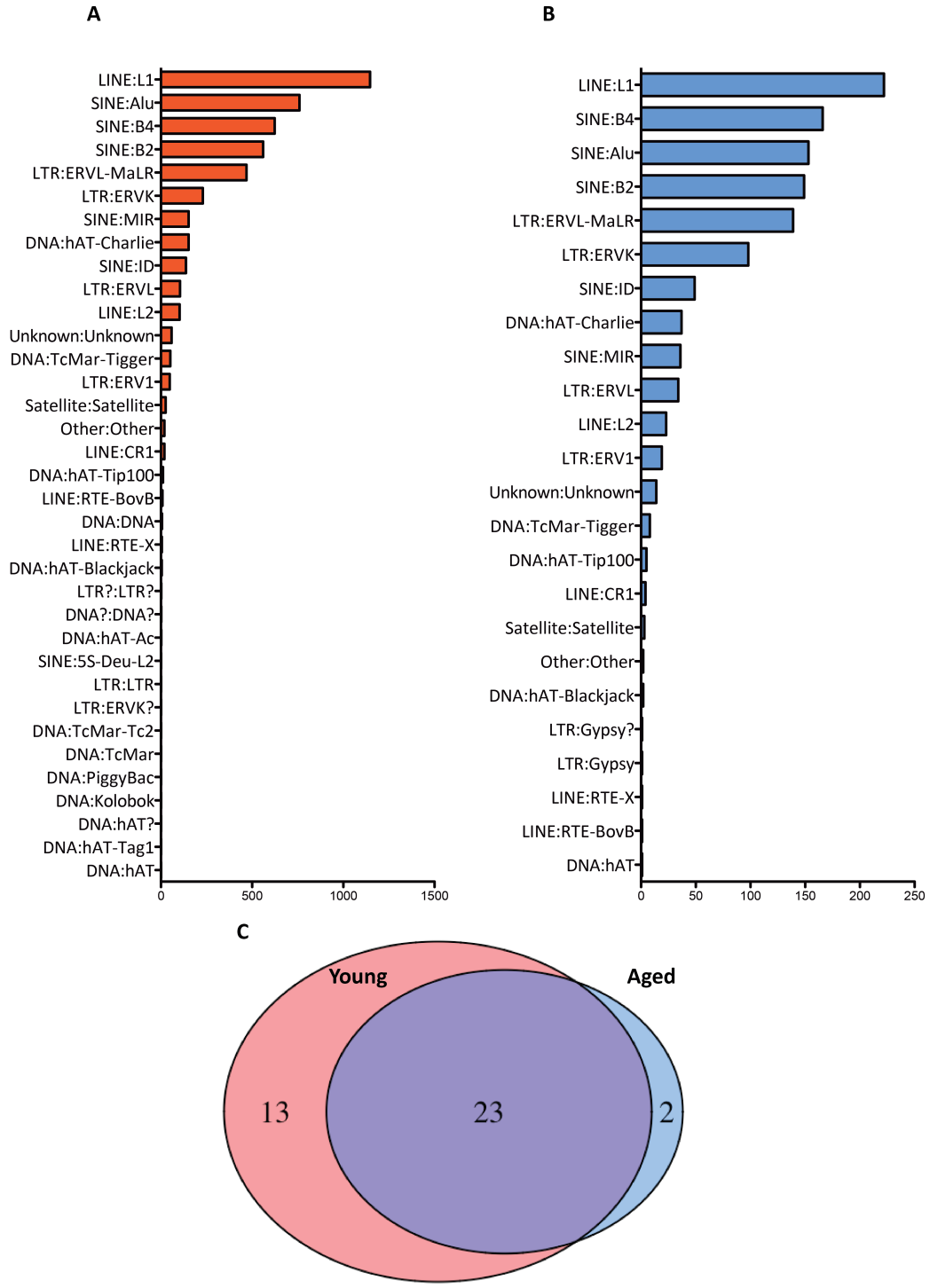

Fig. 4.15 **Differential expression of transposable elements in aged and young OPCs (A-B)** Classification of TEs significantly expressed in young and aged OPCs (respectively). **(C)** Venn diagram illustrating the common classes of TEs between aged and young OPCs.

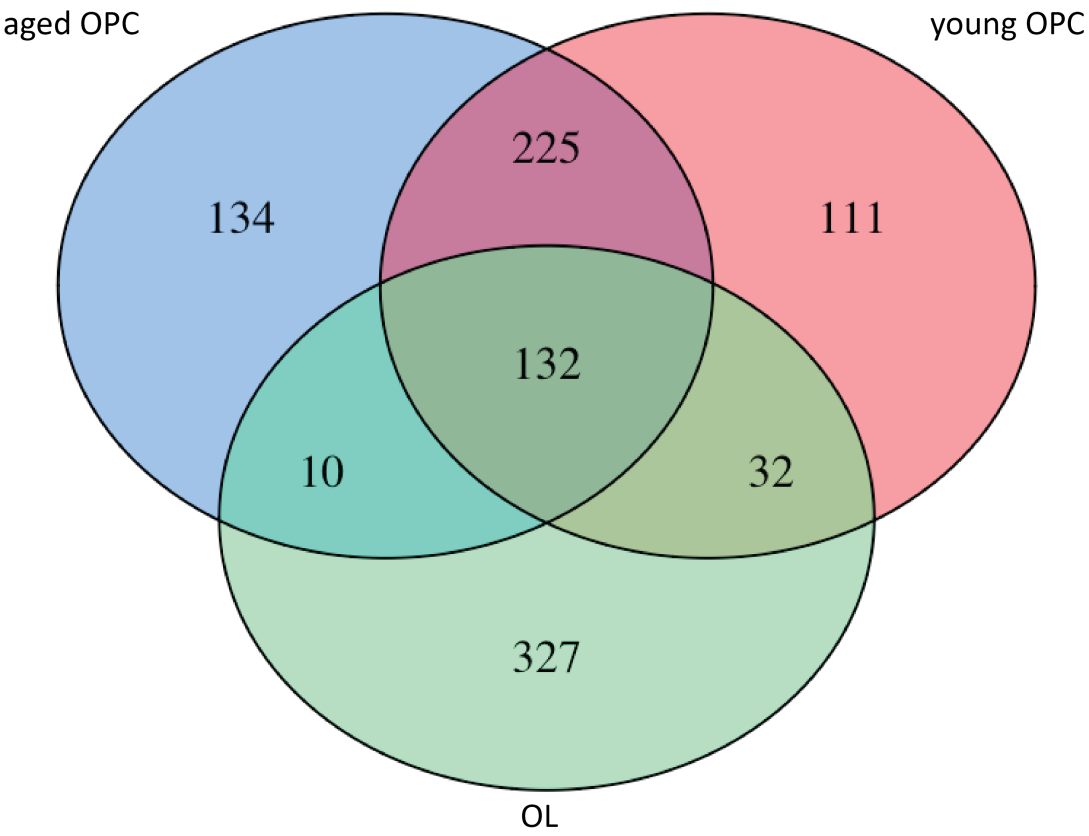

Fig. 4.16 **Top 500 expressed TEs in aged/young OPCs and OLs** Venn diagram showing top 500 expressed TEs in OLs (green), aged (blue) and young (red) OPCs. Out 500 TEs, 357 (71.4%) were common to both young and aged OPCs. OLs did not share higher number of TEs with aged OPCs, unlike gene expression results (see previous sections).

The changes in expression levels in TEs between aged and young OPCs did not correspond to similar changes in DNA methylation. Aged and young OPCs show similar numbers of hyper and hypo methylated TEs, and when plotting expression levels and methylation levels, no correlation was found in aged nor young OPCs (figure4.17). The scatterplots shown in fig4.17 A-B illustrates that there is no correlation between the methylation status of a specific TE to its expression levels.

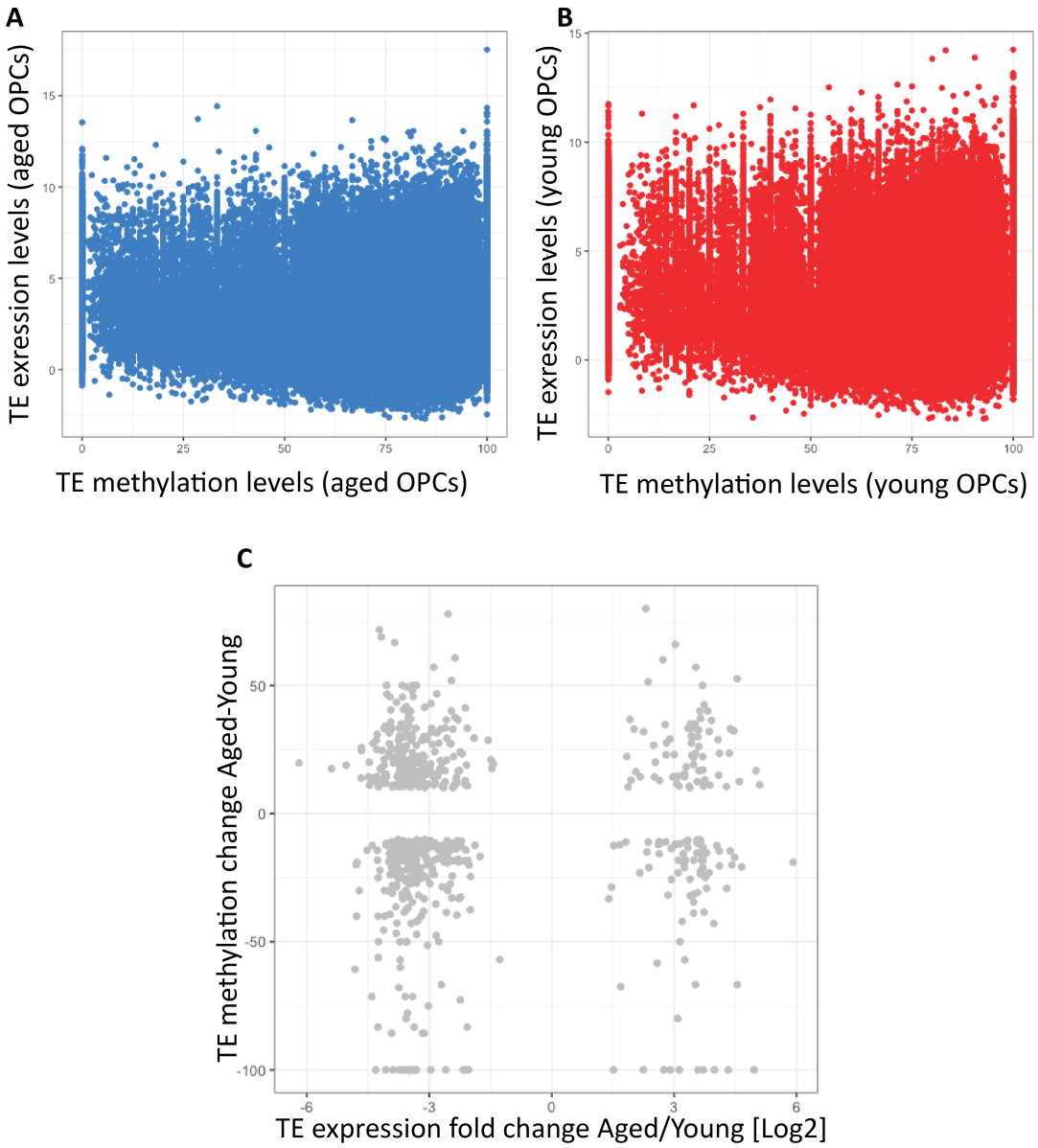

Fig. 4.17 **Age related changes in methylation of TEs (A-B)** Scatterplots of methylation levels and expression levels of TEs in aged and young OPCs (respectively). There is no correlation between the individual methylation levels of each TE, and its expression levels. **(C)** Scatter plot of TEs which exhibit significant change in expression (adjusted p-value < 0.05) and significant change in methylation (at least 5X coverage; methylation difference > 10). There is no clear correlation between aged related changes in expression and methylation.

Since I could not find global changes that support the notion that TE expression increases with ageing, I decided to focus on two main types of TEs widely studied Alu and L1. These elements were shown to increase in ageing and senescence cells (De Cecco, Criscione, Peckham, et al., 2013; De Cecco, Criscione, Peterson, et al.,

2013). I therefore filtered my data to include only these elements and examined both their expression and methylation. Figure 4.18 shows the number of elements from each family which are significantly increased in aged or young OPCs. It is clear that both elements are highly enriched in young OPCs, in comparison to aged ones. Figure 4.19 shows the changes in methylation of Alu and L1 elements. Similar to the global analysis shown above, there is no correlation in either of the TEs.

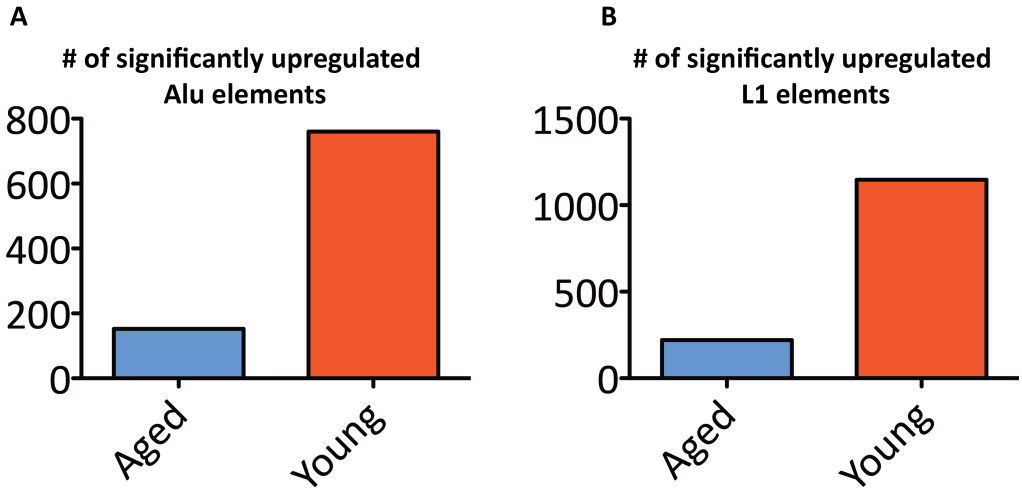

Fig. 4.18 **Age related changes in expression of Alu and L1 elements (A)** A bar plot summarising the number of significantly upregulated Alu elements in aged (blue) and young (red) OPCs. Whereas 760 Alu elements were significantly upregulated in young OPCs, only 152 were upregulated in aged OPCs (adjusted p-value < 0.05). **(B)** A bar plot summarising the number of significantly upregulated Alu elements in aged (blue) and young (red) OPCs. Whereas 1147 Alu elements were significantly upregulated in young OPCs, only 221 were upregulated in aged OPCs (adjusted p-value < 0.05).

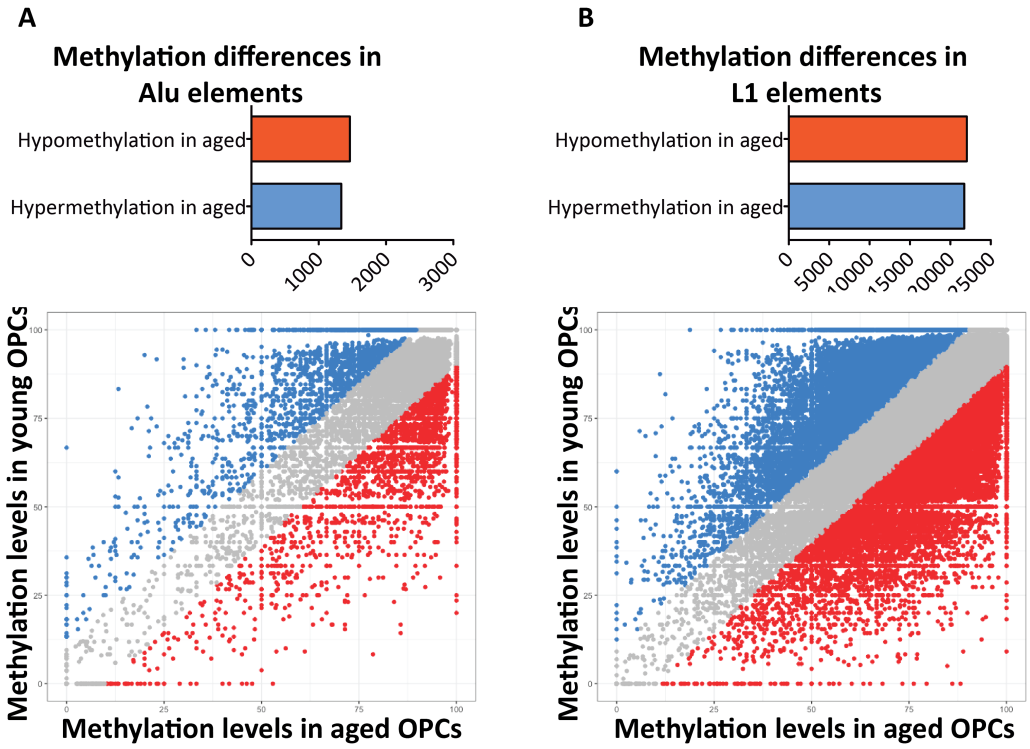

Fig. 4.19 **Age related changes in methylation of Alu and L1 elements (A)** A bar plot and scatter plot summarising the number of Alu elements which exhibited methylation changes (above 10% change between aged and young). Similar number of elements exhibited hyper hypo methylation. **(B)** A bar plot and scatter plot summarising the number of Alu elements which exhibited methylation changes (above 10% change between aged and young). Similar number of elements exhibited hyper hypo methylation.

4.4 Discussion

4.4.1 Aged OPCs more differentiated than young counterparts

Using RNA sequencing I was able to obtain a full and complete transcriptional profile of aged and young adult OPCs. This tool enabled me to begin exploring the internal processes that underlie OPC ageing, and might lead to their reduced differentiation capabilities in aged animals. One of the first things I noticed were the changes in transcription of key OPC signature genes. These included *Pdgfra, Cspg4, Hes1, Hes5* and *Ascl1* (some of which have been confirmed in qRT-PCR by Neumann et al.,). This is in line with previous studies which have compared neonatal and young adult OPCs (Moyon et al., 2015) and have shown that when compared to neonatal OPCs, adult OPCs have a more differential transcriptional signature. It is important to note that additional studies which have used the same mouse line (PPDGFRa-GFP) to

isolate OPCs, have shown that the GFP in this mouse line can also make mature OLs (Marques et al., 2016). It could be argued that at least some of the adult OPCs isolated in that study were in fact OLs. A similar claim can be made against my database, i.e. my aged OPC sample is contaminated by OLs. As I would explain in the following paragraphs, I believe this is highly unlikely. I used the recently published single cell RNA sequencing database of OL lineage cells by Marques et al., (Marques et al., 2016) to create signature gene lists for OPCs and OL lineage cells. When comparing the expression of OPC signature genes in young and aged OPCs, it is clear that aged OPCs show reduced expression of many of those genes (fig4.3B and fig4.4A). This was further confirmed when I used the data collected from sequencing adult OLs (see chapter 4). Figure 4.4C shows the clustering of aged young OPCs in relation to adult OLs, using the transcriptional signature of OL lineage cells established by Marques and colleagues (Marques et al., 2016). This heatmap supports two main claims: firstly, aged and young OPCs cluster together; secondly, aged OPCs cluster closer to OLs than young OPCs. This refutes a possible claim that the aged OPCs that were isolated were in fact OLs, and shows that they are OPCs that are in a more advance stage of the lineage. Moreover, the fact that the aged OPCs do not show upregulation of any of the myelin genes (e.g. *Mbp, Mog, Mag, Mobp* and *Plp1*) supports this claim as well. The upregulation of these genes in OLs is striking, as can be observed by data collected by me (chapter 4) and others (Zhang et al., 2014). Thus, I would claim that the main feature of aged OPCs is their loss of progenitor characteristics, which renders them less prepared to be activated in states such as remyelination.

The upregulation of OL genes in aged OPCs could be seen as a advantage in cases of demyelination, when OPCs are required to differentiate into OLs in order to remyelinate nude axons. But during remyelination OPCs are required not only to differentiate, but also to migrate and proliferate. This pre-mature differentiation profile can be seen as rendering the aged OPCs as less flexible than their young counterparts. Moyon et al., have shown that in an event of demyelination, young adult OPCs are activated and revert to the neonatal transcriptional profile. One can assume that since aged OPCs show even less expression of OPC genes they would be less capable to revert back to neonatal activated state which is required for successful remyelination, since this will require more dramatic changes in transcription and epigenetic states.

The premature differentiation of aged OPCs also renders them vulnerable to other dangers of the ageing brain. For example, inflammation levels increase with ageing (Norden & Godbout, 2013), and include the increase in expression of TNFα in the aged brain. At the same time, studies have shown the mature OPCs are more vulnerable to TNFα toxic effects (Cammer & Zhang, 1999). This increased sensitivity to TNFα

levels is also supported by my data showing an increase in the expression of TNF receptors (*Tnfrsf1a*) in aged OPCs. Moreover, 'late OL progenitors' have also been shown to be more vulnerable to hypoxia-ischemia conditions (S. A. Back et al., 2002). Therefore, it seems that the pre-mature differentiation of aged OPCs renders them both inadequate to efficiently address demyelination lesions, but also place them in more vulnerable position when confronting other challenges the aged brain exhibit.

4.4.2 Nuclear receptors

Retinoic acid (RA) receptors (and specifically retinoid x receptors; RXR) have emerged in recent years as a potential therapeutic target for enhancing remyelination. Early study by Huang and colleagues from 2011, has shown that there is an upregulation of RA receptors during remyelination by comparing microarrays of different stages in remyelination (Huang et al., 2011). This study have focused on the role of RXR-γ as the main receptor that its activation can enhance remyelination. Another study from 2015, has supported this notion by enhancing remyelination in the optic nerve by specifically activating RAR receptors by using either all-trans retinoic acid (ATRA; metabolised Vitamin A) or by using a synthetic agonist of RAR-α/β (Am80).

These two studies have shown that the beneficial effects of RA agonists relate to both the immune system as well as the OPCs themselves. Moreover, RXR activation has been shown to reverse the age related decline in myelin phagocytosis by macrophages/microglia (Natrajan et al., 2015).
It is important to note though that a recent study have concluded that even though human NSCs upregulate RARs when they become OPCs, a long-term exposure to RA isoforms can result in upregulation of differentiation inhibitors, such as *Hes5* and *Id4* (young Kim et al., 2017).

In contrast to the studies which have shown the beneficial effects of RXR activation both *in vivo* and *in vitro* (de la Fuente et al., 2015; Huang et al., 2011), experiments conducted in our lab (Neumann et al., 2017, Neumann, unpublished data) have shown that treating aged OPCs RXR agonists do not enhance their differentiation capacity. Exploring my RNA-seq data, I found that aged OPCs express lower levels of multiple nuclear receptors related to RA pathway, including ones which have been specifically important for OPC differentiation, such as *Rar*-$\alpha/\beta/\gamma$ and *Thr*-α/β. As RXR are known to work as hetrodimers (de la Fuente et al., 2015), it is clear that the downregulation of many of RXR binding partners would reduced their activation and potential to enhance differentiation.

Therefore, it possible that the failure of RA agonists to enhance aged OPCs

differentiation *in vitro*, is due to the fact that these cells do not express sufficient levels of these receptors and their essential binding partners. It is possible that higher doses of RA would have been more efficient in enhancing OPC differentiation, and these experiments should be conducted in order to assess properly the therapeutic effect of these molecules.

Another known enhancer of OPC differentiation *in vivo* and *in vitro* is T3 (Thyroid hormone) (B. A. Barres, Lazar, & Raff, 1994), which is used by many researchers (including myself) to accelerate OPC differentiation *in vitro*. T3 acts on cells through nuclear thyroid receptors, *Thra/b* to activate a set of genes that promote OPC differentiation, mainly through the activation of *Klf9*. Similarly to the RA, the effect of T3 on aged OPC differentiation is extremely limited (Neumann et al., 2017). The RNA sequencing data I show here revealed that both thyroid receptors (*Thra/b*) are significantly downregulated in aged OPCs (fig4.6). Similarly, the main target of T3 in OPC differentiation, *Klf9*, is also significantly downregulated in aged OPCs. This can at least partially explain why T3 has such reduced effect on aged OPCs. The lack of receptors for T3 reduces its ability to activate and promote the differentiation programme in aged OPCs.

Exploring nuclear receptors expression in aged OPCs reveals important changes between aged and young OPCs and highlights the significance of experimenting with aged cells, when studying ageing. The encouraging results described by past papers regarding the potential of RXR activation in the enhancement of OPC differentiation, myelin debris removal and ultimately remyelination led to clinical trials being currently conducted using RXR agonist bexarotene. The results I show in this chapter suggests that these trials might encounter severe difficulties, due to the downregulation of key genes in aged OPCs relevant for the activity of nuclear receptors. This could be true of other potential therapeutic targets identified by *in vitro* and *in vivo* essays that were performed using young and neonatal rodents, such as endothelin 2. This inflammatory factor was identified by Yuen et al., (Yuen et al., 2013) as a one which is important in promotion of OPC differentiation mainly through the activation of Endothelin Receptor Type B (*Ednrb*). Similar to the RA and T3 receptors, Endothelin receptors *Ednra/b* are both found to be significantly downregulated in aged OPCs (adjusted p-value $<$ 0.05). This is similar to the situation found in chronically demyelinated lesions in MS patients (Yuen et al., 2013). Thus, *Ednrb* low expression in aged OPCs precisely exemplified the connection and relevance between the study of aged OPCs and chronic MS patients.

4.4.3 mTOR pathway is dysregulated in Aged OPCs

Using Ingenuity Pathway Analysis (IPA) to characterise the upregulated genes in aged OPCs revealed multiple pathways which are upregulated in aged OPCs, one of those is the mTOR pathway (figures 4.3 and 4.5). mTOR (mammalian target of rapamycin) is a well studied pathway which is known to integrate extracellular signals to induce many cell responses, such as cell differentiation and proliferation. mTOR has also been studied extensively in relation to ageing in multiple cells and organisms (Johnson, Rabinovitch, & Kaeberlein, 2013), as the inhibition of mTOR activity, either by administering rapamycin or by mutating mTOR genes lead to extended life span in multiple organisms, including mice (Roizen, 2010). Moreover, activation of Wnt pathway in hair follicles stem cells, leads to hyper proliferation and depletion of the stem cell pool. This process can be blocked by the administration of rapamycin, again supporting the notion that inhibition of mTOR pathway in stem cells can protect from stem cells exhaustion and senescence (Castilho, Squarize, Chodosh, Williams, & Gutkind, 2009).

These reports, combined with the results shown in this chapter, suggest that aged OPCs are no different than stem cells in other tissues, in which hyper activation of mTOR pathway lead to stem cell exhaustion and cell senescence. It is therefore not surprising that the addition of rapamycin to aged OPCs, prior to differentiation, enhanced their differentiation abilities. (Neumann et al., 2017).

The relation between Wnt and mTOR pathways in regard to ageing stem cells was suggested by a paper from Castilho et al., (Castilho et al., 2009), in which mTOR signalling was activated by Wnt signalling pathway, and this led to stem cells exhaustion. In OPCs, dysregulated Wnt signalling is known to inhibit OPC differentiation (Fancy et al., 2009). Since mTOR signalling is necessary for OPC differentiation, these results seem to contradict each other. I suggest that there is a need to separate between the different states of OPCs and the effects of these pathways. Thus, overexpression of mTOR can promote OPC differentiation and this explains why aged OPCs are more differentiated than young ones. But at the same time, this renders them less equipped for challenging situations which emerge in demyelinating lesions, as well as can lead to stem cell exhaustion, similar to what has been describes in other tissues.

4.4.4 OPCs exhibit specific changes in methylation with ageing

One of the hallmarks of ageing is the changes in epigenetic landscape, which is also referred to as an 'epigenetic drift'. Even though, epigenetic mechanisms and their direct relation to transcription have yet to be fully understood, it clear that a specific epigenome is required for a proper cell function. The importance of the epigenome is even more relevant for progenitor cells, such as OPCs which are required to initiate complex transcriptional programs in a timely manner.

Unlike past publications (Issa, 2014), I could not find marked global changes between the epigenome of aged and young OPCs (figure 4.7). This could be due to different cell types used, as well as differences in the methods used by different researchers. Since no significant global changes were identified, I decided to explore specific changes, by examining specific genes which hare known to play a role in OPC biology. In both groups of genes (OPC genes and nuclear receptors), the same pattern emerged: a marked decrease in promoter methylation. Examining the changes in DNA methylation in aged and young OPCs in the promoters of nuclear receptors revealed a surprising result. In contrast to what is described in previous literature, where promoter hypermethylation is correlated with reduced mRNA expression, when exploring the methylation of these promoters, most promoters lose their methylation markings in aged OPCs. This can be a good example to loss of epigenetic memory and actual 'epigenetic drift'. As was mentioned before, some studies do describe DNA methylation as epigenetic memory marker rather than an actual inhibitor of transcription. Thus, the loss of these markings with age leads to the loss of 'correct' methylome in particular and epigenetic memory in general in ageing cells. This is extremely relevant to cycling cells such as OPCs, as they are unable to properly inherit their epigenetic landscape after proliferation. This is another aspect in which, my database shown here differ from older studies: even though, 'classical' notion of promoter methylation suggests that promoter hyper methylation leads to decreased gene expression. My data suggests that this is not the case, not only when assessing all promoters but also when comparing specific groups of genes (figure4.8). I would suggest that this supposed discrepancy is the result of differences in cell type, and more likely differences in methods. Past studies have focused on specific, single genes, but now, with advanced technology it is possible to sequence the whole genome in sufficient depth to explore most if not all promoters.

These changes in methylome states between young and aged OPCs, resemble findings from stem cells in other tissues. For example, Liu and colleagues (L. Liu et al., 2013) show that muscle stem cells exhibit dramatic changes in chromatin formations in

ageing. They further report reduction in histone expression with ageing, and increase in H3K27me3 at the histone loci. This could be observed in similar manner in OPCs, as they seem to lose methylation patterns in key gene sets, such as OPC genes and nuclear receptors, both important to OPC biology (figure4.8).

Another set of evidence to the epigenetic drift exhibited by aged OPCs are the changes in methylation within gene bodies (figure4.10). In similar fashion to what have been shown by previous studies, methylation levels in intragenic regions is not conclusively correlated to gene expression (P. a. Jones, 2012). This ambiguity regarding the role of DNA methylation, makes it difficult to predict the transcriptional results of the changes in methylation. In either case, there is clear change in methylation patterns in hundreds of gene bodies. Interestingly, majority of these genes ($\sim$70% of the genes) are downregulated in aged OPCs. GO analysis of these genes (data not shown) show that these genes are involved in multiple cellular processes, mainly specific metabolic processes.

These results support the notion of epigenetic drift in ageing cells. Even though we cannot yet correlate specifically these changes in methylation to gene expression, it can be added to an increasing set of evidence showing that aged OPCs, similarly to other tissue specific stem cells, show dysregulated epigenome which could reduce their capability to be activated in due time.

4.4.5 Alternative splicing in ageing OPCs

When analysing the changes in alternative splicing in aged and young OPCs, I used several approaches. Firstly, I filtered the list of genes so it won't include genes which I previously detected to exhibit significant changes in transcript expression. GO analysis of these genes showed enrichment in metabolic processes and regulation of biological processes. This shows that alternative splicing alone can be accounted for changes in aged OPCs, without showing effects in full transcript expression. For the second analysis, I listed again all the genes which exhibit significantly differential exon expression (adjusted p-value < 0.05) and plotted them according to their frequency of appearance, i.e. how many significant splicing events occur in each gene. Interestingly, the top gene which emerged was *Kif1b*. This gene showed significant differential exon usage in 15 exons, but did not show significant change in transcript expression (young/aged log2 fold change = 0.09, p-value = 0.633). *Kif1b* has been shown previously to have an important role in myelination. *Kif1b* encodes for a kinesin motor, and in OLs it is required for the localisation of *Mbp* mRNA transcripts in OL processes (Lyons et al., 2009). Lyons and colleagues have shown that a mutation in this

gene disrupts its interaction with microtubule, which leads to improper myelination and CNS development. Since aged OPCs exhibit high ratios of alternative splicing events in comparison to young ones, it is possible that this also explain some of the phenotypes exhibit by aged OPCs *in vitro*, specifically their failure to differentiate to fully mature, MBP positive oligodendrocytes.

Another gene which exhibited high rate of significant alternative splicing events was *Myrf* (total of 9 significantly differentially expressed exons). *Myrf* is OL lineage specific nuclear protein, which is required for OPC differentiation and myelination (Emery et al., 2009). MYRF is DNA binding transcription factor which is responsible to the activation of myelin genes in differentiating OPCs. Similar to *Kif1b*, *Myrf* does not exhibit changes in full transcript expression between aged and young OPCs (young/aged log fold2 change = -0.222, adjusted p-value = 0.387). Using the ensemble genome browser, I was able to map the the exons which exhibit downregualted expression, and show that they are part of two critical domains in *Myrf*. The main part of MYRF that seemed to be affected is its C-terminal. This part of the protein is left in the ER after auto-cleaving by the ICA domain. Even though it does not translocate into the nucleus and act as a transcription factor, MYRF C-terminal seems to have a role in expression of myelin genes, since MYRF lacking this part is not as efficient as the full version in inducing myelin genes transcription in CG4 cells (Z. Li et al., 2013). Moreover, disruptions in the ICA domain (which are also possible to the downregualtion of exon 14) can lead to inefficient auto-cleavage of MYRF, and will prevent it from translocating to nucleus. Lack of either of these domains renders MYRF non functional (Emery et al., 2009; Z. Li et al., 2013). This is in line with the differentiated phenotype of aged OPCs I have reported above. Aged OPCs show the expression of several differentiation genes, but none of the myelin genes. This could be explained by the lack of 'young' version of *Myrf* that is required to activate these genes. This could be especially relvant for adult animals, as MYRF is required for myelin maintenance in adult mice (Koenning et al., 2012).

Unfortunately, I could not specifically correlate between the methylation of intragenic regions to differential exon expression, in contrast to other reports (P. A. Jones, 2012; Moyon, Huynh, et al., 2016). This might be due to several technical reasons: firstly, the fact that DNA was extracted from FACS A2B5 positive OPCs, in comparison to the RNA that was isolated from MACS A2B5 sorted OPCs. Secondly, It could be that the potential relations between methylation and alternative splicing are very subtle, and as such a very scrutinising view should be used to analyse methylation rate. I.e. correlate specific splice sites methylation to splicing events. In this study a more global approach was taken, and therefore these correlations might have been

overlooked.

In summary, these examples show the potential of research into alternative splicing as serving a role in the reduced capacity of aged OPCs to differentiate properly. Further studies will be required in order to specifically investigate which splicing events are actually relevant in the decreased differentiation ability of aged OPCs. This can be done by artificially expressing 'young' gene versions of specific target genes in aged cells. Further research would need to be done in order to understand better what are the underlying causes to these changes in exons expression. This can be done by a thorough examination of methylation states of specific splice sites, as well as expression of specific chromatin marks in those areas.

4.4.6 Transposable elements in OPCs

Transposable elements (TEs) are DNA sequences which have a capability of self replication and insertion into the genomic DNA. There are two main types of TEs - DNA transposons encode an enzyme named transposase. This allow them to be 'cut and pasted' within the genome. The other group of TEs, are are the retrotransposons. These TEs use an intermediate RNA molecule to mobilise throughout the genome. The RNA is reverse transcribed to cDNA and can therefore be 'copied and pasted' in multiple sites in the genome. Both modes of mobilisation can lead to mutations and genomic instability (Sturm, Ivics, & Vellai, 2015). Therefore, cells have devised multiple mechanisms in order to inhibit TE activation. These include epigenetic mechanisms, such as DNA methylation and RNA silencing (Sturm et al., 2015). In ageing somatic tissues, there is evident that specific TEs become active, thus leading to increased genomic instability (De Cecco, Criscione, Peterson, et al., 2013; Maxwell, Burhans, & Curcio, 2011).

Therefore, it seems that exploring the behaviour of TEs in ageing OPCs could lead to some insightful observations. Surprisingly, my results shown in this chapter are not in line with some of the previous reports regarding the activity of TEs. Firstly, I observed downregulation of multiple TEs in aged OPCs, when compared to young ones (4709 TEs). In comparison, only a 1168 TEs exhibited an increase in expression in aged OPCs. A similar trend of increased expression of TEs in young OPCs in comparison to aged ones was detected even when I have focused on specific TE families, such as Alu and L1 (figure 4.18). I chose these two families since they have been specifically indicated to increase in ageing cells (De Cecco, Criscione, Peckham, et al., 2013; De Cecco, Criscione, Peterson, et al., 2013). Besides the technical differences between these studies and mine, I can again point out to the difference in cell types, as previous

studies used fully differentiated cells in comparison to stem cells which were used in the current study (i.e., OPCs). These results are in line with a smaller number of reports regarding the role of TEs in CNS cells, and specifically their role in neural progenitor cells (NPC) (Coufal et al., 2009; Singer, McConnell, Marchetto, Coufal, & Gage, 2010). A study from 2009 by Coufal et al., shows evidence that neuronal progenitors activate L1 retrotransposons preferentially to other somatic cell types from the same animal. Thus leading to a mosaic of somatic neural cells. The cells used for that study were identified as SOX2, NESTIN, MUASASHI-1 and SOX1 cells. At least two of these genes (*Sox2* and *Msi1*) are significantly increased in my young OPCs in comparison to the aged ones. SOX2 is especially interesting in this context, as Coufal et al., show through immunoprecipitation, that it can bind to the 5'-UTR of L1 elements, and show similar pattern of expression to L1 (Coufal et al., 2009).
Furthermore, when trying to correlate methylation levels and TE expression, no correlation was found (figure 4.17). This is again in contrast to previous reports describing the relation between DNA methylation and TE expression (Slotkin & Martienssen, 2007). I cannot fully explain these differences between previous studies and the results presented here. The most obvious explanation regarding the lack of relation between TE expression and methylation is that these analysis were done on different cells. I.e. RNA was isolated from MACS sorted OPCs while DNA was isolated from FACS sorted OPCs. Clearly, finding a link between the databases will be difficult. Since I found similar relations when analysing changes in DNA methylation and RNA expression in another dataset (chapter 4, OL lineage cells) where the DNA and RNA were isolated from the same cells, I find that this explanation is insufficient. It is possible that the relation between RNA expression and DNA methylation of TEs (and other genomic elements) is much more complex than was previously thought to be. It is possible that many correlations previously reported were due to the authors tendency to focus on specific element at the genome. Thus, a more global genomic view that I used might obscure these specific correlations.

In summary, my results suggest that TEs might play a crucial role in OPC biology and maintenance, and are highly activated in these cells, similarly to what have been shown in NPCs. This is in striking contrast to TEs in other tissues and cell types. This strengthens the concept that specific features can play very different roles in different cell types.

Chapter 5

Ageing Mononuclear Phagocytes and their effects on OPC differentiation

5.1 Introduction

Microglia, the resident immune cells of the CNS play a pivotal role in both normal as well as during pathological conditions. As described in chapter 1, previous studies have been conducted in relation to role of microglia in ageing in general, and in remyelination specifically. With regards of their role in remyelination, studies have mostly focused on the decreased phagocytosis capability of aged microglia (Natrajan et al., 2015; Ruckh et al., 2012). Other studies have shown that aged microglia exhibit a delayed response to demyelination, and thus create a delayed and lingered response to demyelinating lesion (Zhao et al., 2006). In parallel, the field of microglia biology has advanced considerably, and now there are multiple transcriptomic studies that reveal the changes between young and aged microglia (Hickman et al., 2013; Holtman et al., 2015). Most studies though, have mainly focused on the changes in the secretion profile (secretome) of aged microglia and did not focus on other aspects of aged microglia, such as changes in their surface antigens and their contribution to the ECM in the normal and pathological CNS. Study of microglia in the relation of remyelination has also neglected the potential role of of various surface antigens expressed by microglia, and focused only on a handful of secreted cytokines. The effect of the ECM and surface antigens on OPC differentiation is evident from studies which explored the various surface antigens on neurons in order to identify which cues limit myelin wrapping to axons, such as JAM2 (Redmond et al., 2016). Moreover, experiments using *in vitro* astrocytes and OPCs cultures show that proteoglycans expressed by activated astrocytes inhibit OPC differentiation (Keough et al., 2016).

Despite these findings, there has been very little research regarding the changes in microglia surface antigen expression and its potential relation to OPC differentiation. This is surprising, since in the early days of a demyelinating lesion, the main cell type which populates the lesion is from a microglial origin. Thus, it will be reasonable to assume that the main contributor to microenvironment and ECM in the lesion area will be the microglial cells.

Another aspect of aged microglia which has not been fully explored in relation to remyelination, is their changes in their secretome. Previous studies have only focused on specific cytokines and the changes in their levels in remyelination. These usually focus on the well studied pro and anti inflammatory cytokines. But these studies overlook the complete secretome of microglia, and the fact that even though these cell originated from primitive myeloid cells (Ginhoux et al., 2010), they are an integral part of the CNS and perform multiple non-immune roles, by secreting various factors, such as BDNF for promoting synapse formation (Parkhurst et al., 2013).

In light of the these findings and the knowledge gaps described above, I set out to address the following questions:

1. Do microglia surface antigens directly affect OPC differentiation capacities?
2. Do microglia surface antigens change with ageing?
3. What is the driving factor behind the changes in microglia surface antigens?
4. Are the effects of microglia surface antigens identical to the effects of microglia secreted factors?

5.2 Experimental strategy

In order to study microglia and their effects on OPCs, I first had to establish microglia cultures. I have used the vast experience that our lab has in culturing aged OPCs and put it into use in isolating and culturing microglia. I isolated microglia using anti CD11B labeled magnetic beads (fully detailed protocol can be found in the Materials and Methods chapter). Since CD11B does not identify microglia exclusively but can be found on other phagocytic cells (Lavin et al., 2014) I cannot be certain that the cells that were isolated were pure microglia population, and are not contaminated by other macrophages. Since this does not alter the results or my interpretations of my results, I will abbreviate the CD11B+ cells isolated in this method as mono-nuclear phagocytes, or MNPs. In order to study the role of the MNP surface antigens separately

from the roles of their secreted factors, I created MNP 'ghosts', in a technique similar to that used before with astrocytes (Keough et al., 2016) and with OPCs in my lab (Segel 2017, unpublished). In short, MNPs were cultured for 48 hours, and then were submerged in ddH_2O and then immediately placed into dry ice. The short culturing time allowed the attachment of most microglia to the plates on one hand, but also to retain their age associated properties as much as possible. The addition of ddH_2O resulted in immediate death of the microglia, while maintaining their cell morphology and surface antigens (which was evident by light microscopy). The ghosts were then kept in -80°C for use at a later stage. Next, freshly isolated OPCs were plated on top of these ghosts, and their differentiation capabilities were assessed. This protocol is illustrated in figure 5.1.

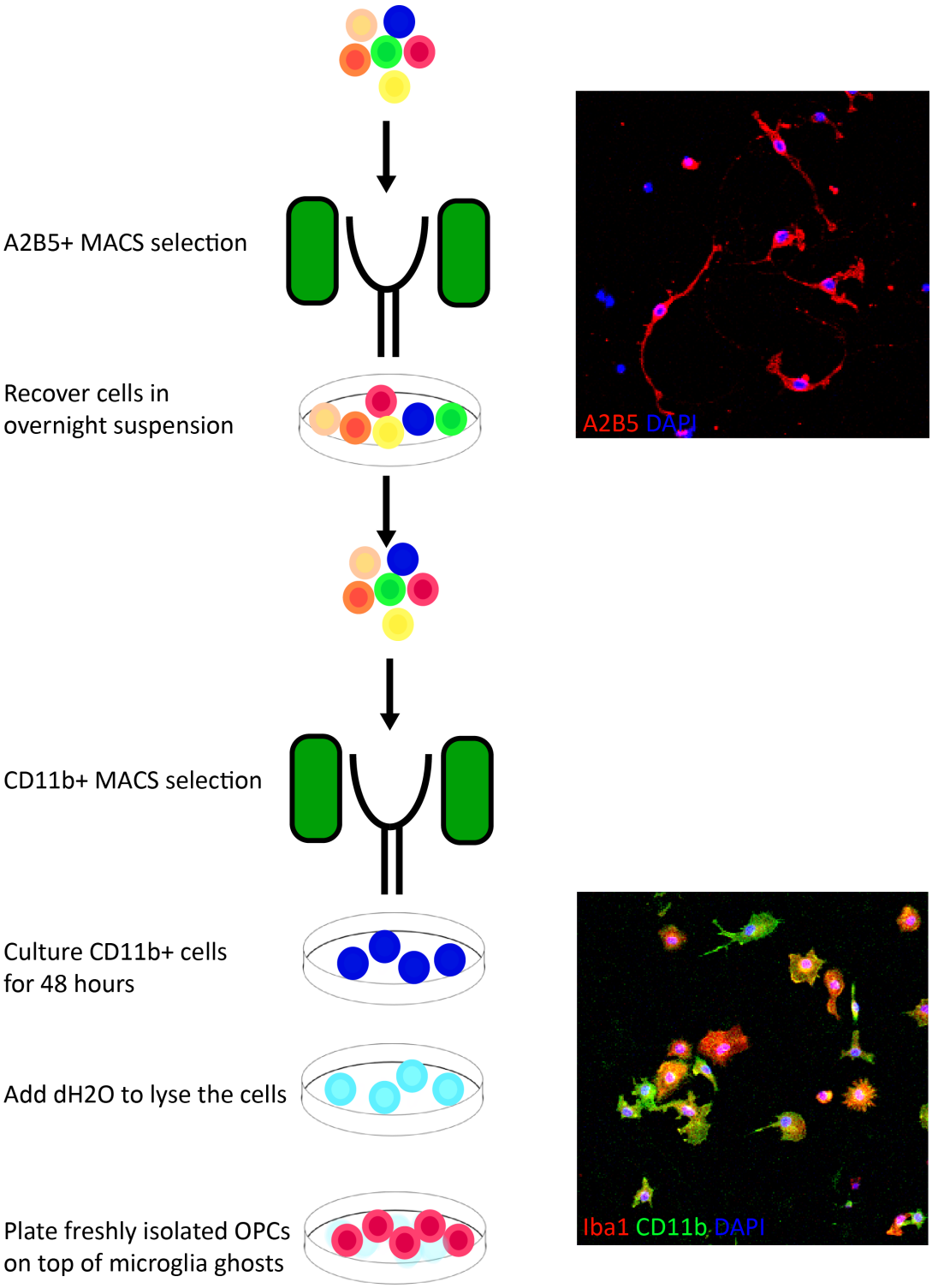

Fig. 5.1 **Microglia ghost protocol** Schematic illustration of the protocol used to generate microglia ghosts. Following the generation of single cell suspension (as described in section 2.3.1) A2B5 MACS was performed to remove OPCs. A2B5 negative cells were then cultured overnight in suspension plates. The next day, MNPs were isolated using CD11B MACS. Cells were plated in PDL pre-coated plates, and after 48 hours *in vitro* were lysed using dH_2O. Plates were frozen immediately to preserve the ghosts. When needed, plates were defrosted and OPCs (A2B5+) were plated on top of the ghosts.

5.3 Results

5.3.1 Isolating and culturing adult MNPs

Using the protocol described above, and in combination with the experience I gained in isolating adult OPCs and other OL lineage cells (chapters 3 and 4), I used CD11B MACS in order to isolate MNPs (mono0nuclear phagocytes). MNPs cells were isolated after overnight recovery in suspension plates after A2B5 MACS was performed on the cells.

Isolated MNPs usually recovered very quickly and assumed microglia-like typical cell morphology within a few hours (Fig5.2). Following culturing, in order to test the purity and health of the culture, I stained the cells using CD11B and IBA1 (known markers for microglia and macrophages (Zhang et al., 2014)). In all conditions tested, cultures were composed of mainly MNPs (>80%) (Fig5.2). This was expected, as FACS sorting using CD11B conjugated antibody (see Fig5.4) yielded very clear population separation, suggesting this is a suitable antibody.

Following 48 hours of culture, MNPs were 'ghosted' as described before or media was collected to be used for conditioned media experiments. Microglia used as ghosts were cultured on top of PDL (figures 5.3 and 5.5) coated wells, while those used for conditioning media were cultured on non coated wells (figure 5.8). Cells that were treated with ddH_2O show very little nuclear staining, and do not regrow when fresh media is added later on. This eliminated the possibility that the results observed in later stages of the experiments were due to live microglia and not due to OPCs plated on top of the ghosts. Moreover, as the first step in the ghost protocol was to isolate A2B5+ cells, it is unlikely that the ghosts were composed out cells which are not MNPs (Fig5.1).

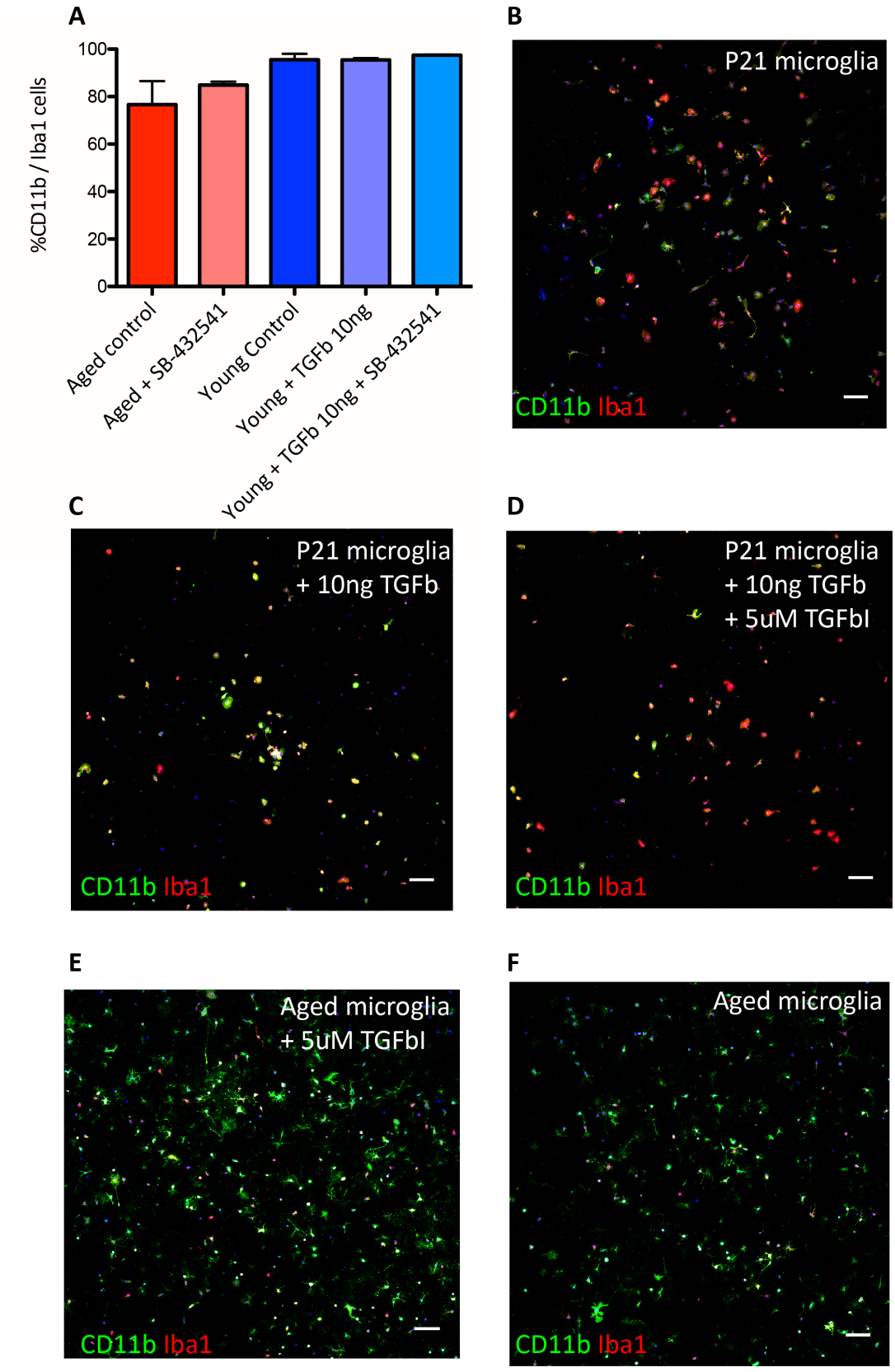
A
%CD11b / Iba1 cells
100
80
60
40
20
0
Aged control
Aged + SB-432541
Young Control
Young + TGFb 10ng
Young + TGFb 10ng + SB-432541
B
P21 microglia
CD11b Iba1
C
P21 microglia
+ 10ng TGFb
CD11b Iba1
D
P21 microglia
+ 10ng TGFb
+ 5uM TGFbI
CD11b Iba1
E
Aged microglia
+ 5uM TGFbI
CD11b Iba1
F
Aged microglia
CD11b Iba1

Fig. 5.2 ***In vitro*** **culture of adult MNPs** Following CD11B MACS sorting, MNPs cells (young and aged) were plated in high density plating (50,000 cells per 1cm^2). **(A)** After 48 hours in serum free media (OPCM), majority of cells were positive for either CD11B and/or IBA1 staining (>80%) and exhibited microglia-like cell morphology. This did not change between ages, and TGFb1 treatment. **(B-D)** Representative images of MNPs of different ages, treated or untreated with TGFβ and/or SB-431542 (scale bar represents 100μm).

5.3.2 OPCs cultured on aged MNPs ghosts alter their differentiation fate

Following A2B5 MACS isolation, neonatal A2B5 positive cells were plated on either PDL coated wells, or on top of ghost microglia (prepared as illustrated in figure 5.1). Following overnight recovery in proliferation conditions (OPCM supplemented with 20ng/ml b-FGF and PDGF), media was changed completely into differentiation media (OPCM + 40ng/ml T3). Cells were cultured in differentiation media for 6 days (media was changed every second day), then fixed and stained. To assess the fate of the differentiating cells, I used GFAP to mark astrocytes, and NG2 to mark OPCs. OLIG2 was used to mark cells of the OL lineage.

OPCs which were cultured on aged microglia ghosts showed an increase in astrocyte formation, as well as changes in NG2 cells to more astrocytic form, characterised by long processes in comparison to normal NG2 cells, which usually show short and branched processes.

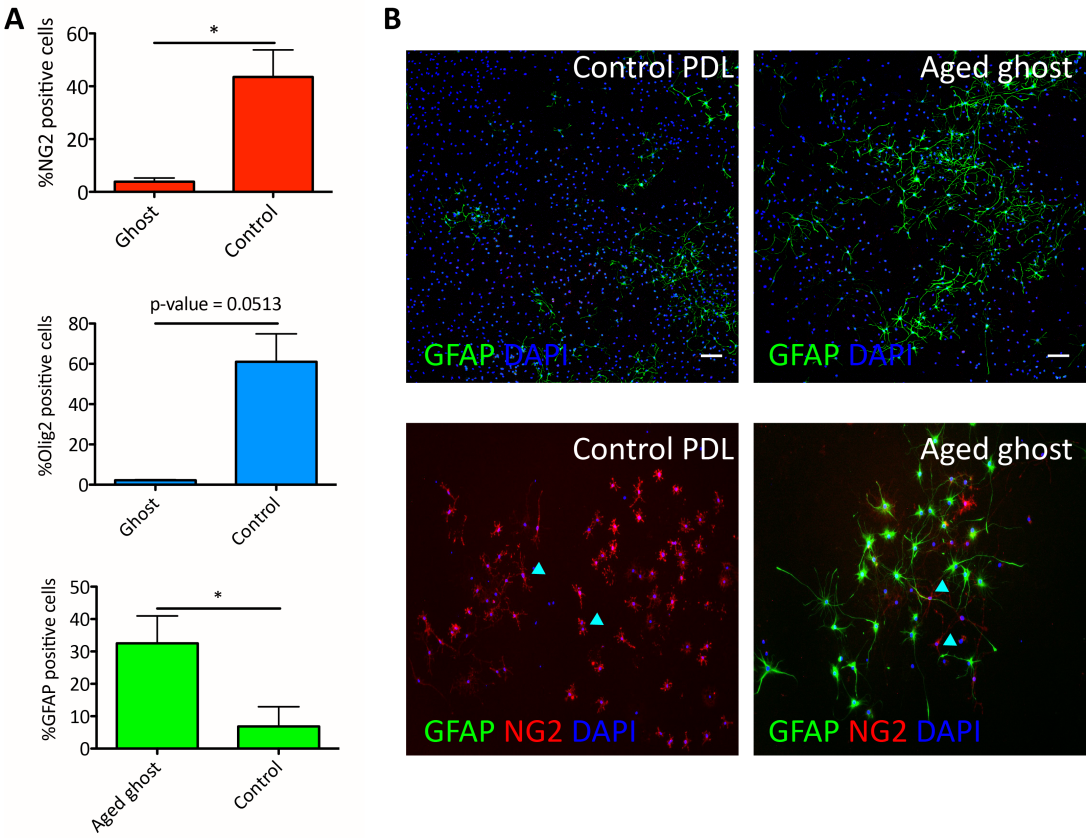

Fig. 5.3 **Culturing OPCs on aged MNPs ghosts** Neonatal OPCs were cultured on either control PDL or on top of aged microglia ghosts in serum free media (OPCM) for 6 days *in vitro* before fixed and stained. **(A)** OPCs cultured on top of aged MNPs ghosts have show significant increase in astrocyte formation, marked by percentage of GFAP positive cells (p-value < 0.05, n = 4). These OPCs also show reduced percentage of NG2 positive cells (p-value < 0.05, n = 4) and OLIG2 positive cells (p-value = 0.0513, n = 2) in comparison to cells cultured on PDL. **(B)** Representative images of cells grown either on aged ghosts or control PDL. The lower panel shows a digital magnification in which changes in cell morphology can be spotted in NG2 positive cells (marked by cyan arrows) (scale bar represents 100µm).

5.3.3 Changes in aged MNPs surface antigens

Following the results described in figure 5.3 I investigated potential candidates for molecules on the surface on aged MNPs that could explain part of the effect. Using a published RNA sequencing data base (Hickman et al., 2013) I identified multiple matrix associated molecules that are known to affect OPC differentiation and also show an increase in expression with ageing microglia (fig5.4A). One of the upregulated genes I identified was *Cspg4*, which encodes the NG2 proteoglycan. Using FACS

analysis, I confirmed that in aged animals a higher percentage of MNPs (labeled by CD11B) are also NG2 positive (fig5.4C). FACS analysis also revealed an increase in the total percentage of microglia cells in animals (fig5.4B). This indicates that any changes that is observed within the aged MNPs population could have important effects due to the total increase in MNPs cell numbers with ageing.

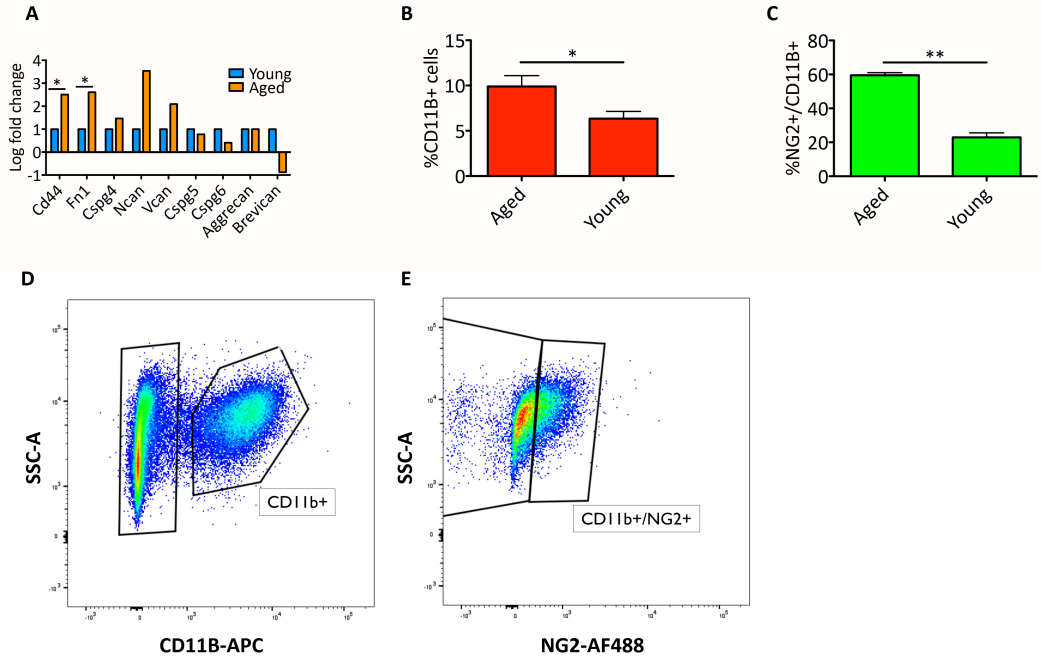

Fig. 5.4 **Changes in aged MNPs surface antigens (A)** Data adapted from published RNA sequencing (Hickman et al., 2013) showing an array of surface antigens which are upregulated in aged microglia. Data is shown in log fold change normalised to expression levels in young microglia. Young microglia expression levels are marked in orange, while aged microglia levels are marked in blue. Significant changes (p-value < 0.05) are marked. **(B)** Results of FACS analysis using CD11B antibodies showing a significant increase in percentage of MNPs in aged rats (p-value < 0.05, n = 7). **(C)** FACS results showing percentage of MNPs which are double positive for NG2 and CD11B. Aged MNPs showed a significant higher proportion of NG2 positive cells than young ones (p-value < 0.005, n = 3). **(D-E)** Representative FACS plots of both CD11B separation (D) and NG2 (E) expression exemplifying the results described in above graphs.

5.3.4 Aged MNPs ghost effect can be recapitulated by TGFβ-treated neonatal MNPs

Following the findings described in figure 5.3 using aged MNPs, I wondered if this effect is due to the age of the MNPs or any type of microglia will elicit the increase in astrocyte formation. In order to test wether or not this effect is due to MNPs

ageing, I repeated the ghost experiments with neonatal MNPs. As shown in figure 5.5, there was no significant increase in astrocyte formation when OPCs were cultured on top of neonatal MNPs ghost. In order to test if there are factors that could alter the surface antigens of young microglia I decided to treat neonatal microglia with TGFβ in increasing concentrations. TGFb was chosen as it has previously been shown to promote the expression of NG2 in microglia (Sugimoto et al., 2014). Moreover, TGFβ levels have been shown to increase with ageing in both humans and mice (Carlson et al., 2009). Treating neonatal MNPs with TGFb1 (0-20 ng/ml) for 48 hours prior to the ghost protocol have resulted in a steady and significant increase in astrocyte formation by cultured OPCs. This led me to hypothesise that TGFβ might be one of the driving forces behind the changes in microglia surface antigens.

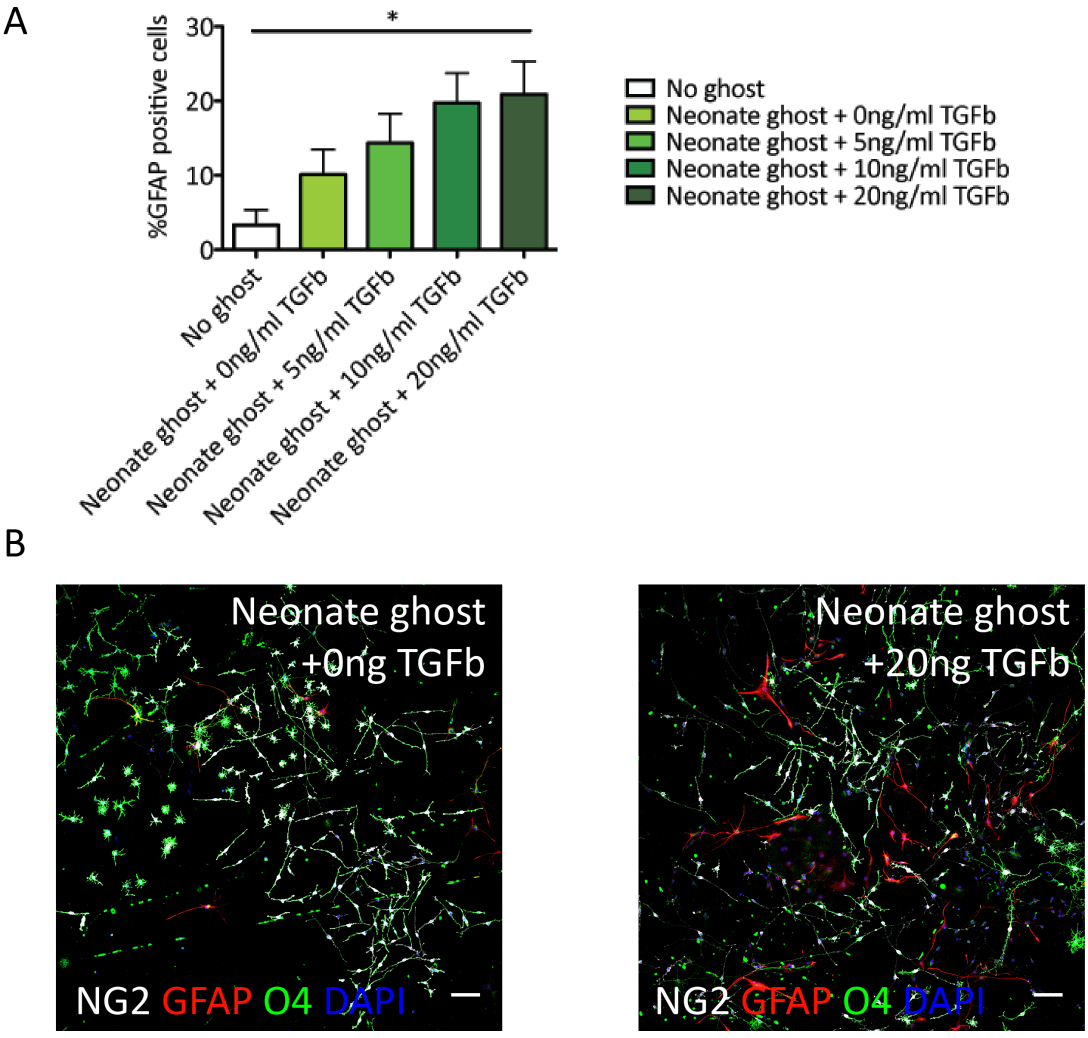

Fig. 5.5 **Culturing OPCs on neonatal MNPs ghosts** Neonatal OPCs were plated on either control PDL, or on top of P21 MNPs ghosts. Prior to the ghost protocol, MNPs were cultured for 48 hours in 10% FBS media with increasing concentrations of TGFb1 (0-20 ng/ml). **(A)** Astrocyte formation (measured by GFAP staining) by OPCs is increased when cultured on top of microglia ghosts treated with TGFβ in a concentration dependent manner (one-way ANOVA, p-value < 0.05, $n = 2$). **(B)** Representative images of OPCs cultured on top of neonatal ghost MNPs with different concentrations of TGFβ. Cells were fixed and stained for GFAP (red), NG2 (white) and O4 (green) (scale bar represents 100µm).

5.3.5 Treating young MNPs with TGFβ enhances the expression of multiple surface antigens

To identify a factor that could be responsible to the changes in aged MNPs surface antigens, I identified TGFβ as a possible candidate. TGFβ levels has been shown to

increase with ageing in both mice and humans (Carlson et al., 2009). Furthermore, TGFβ has been shown to enhance the expression of multiple surface molecules which are known to be related to OPC differentiation, such as fibronectin in multiple cell types, including astrocytes, (Ignotz & Massagué, 1986; Laping et al., 2002; Yu, Wang, Katagiri, & Geller, 2012) and NG2 in microglia (Sugimoto et al., 2014) . Moreover, in order to inhibit the production of CSPGs in astrocytes (which as described before are inhibitory to OPC differentiation) Keough and colleagues also used a TGFβ inhibitor (Keough et al., 2016). Therefore, I set out to test whether TGFβ can play a role in the modification of MNPs extracellular markers. In order to to do so, I cultured neonatal MNPs in the presence of rising concentrations of TGFβ (0-20ng/ml). As shown in figure 5.6. The addition of TGFβ to MNPs culture resulted in the upregulation of mRNA of multiple surface antigens, specifically of *Cspg4*, *Fn1* and *Cd44*. This upregulation in transcription was reversed in the presence of a small molecule TGFβ inhibitor (SB-432542, (Laping et al., 2002)). This is in line with transcriptional data I gathered from published RNA sequencing of aged and young microglia, which shows an increase in these antigens with ageing. In order to eliminate any global effect of transcription up-regulation by TGFβ, I tested the effect of TGFβ on the expression of several cytokines, which yielded no change in expression, in oppose to the global effect of LPS, which resulted in up-regulation of multiple genes in a global effect (data not shown).

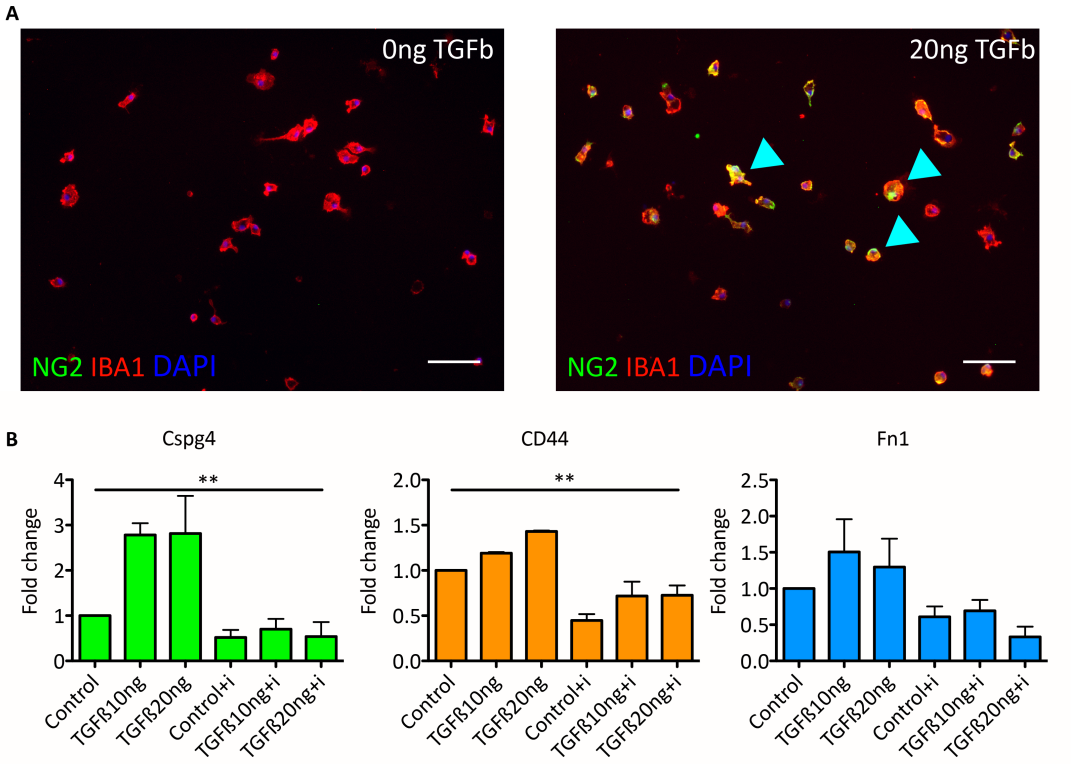

Fig. 5.6 **Upregulation of aged MNPs surface antigens in neonatal MNPs treated with TGF**β In order to recapitulate the ageing effects, neonatal MNPs were cultured in presence of TGFβ (0, 10, 20 ng/ml) with or without 5uM of SB-431542 (i) for 48 hours. **(A)** MNPs (IBA1 positive, red) treated with TGFβ (right image) show co-localisation with NG2 (stained in green) in contrast to non treated MNPs (left image) (scale bar represents 100µm). **(B)** Summary of qRT-PCR results for three selected surface antigen genes, show significant increase in the expression of *Cspg4* and *Cd44*, which was blocked in the presence of TGFβ inhibitor SB-431542 (one-way ANOVA, p-value < 0.005, n = 2). Similar trend was shown for *Fn1*, albeit not significant (one-way ANOVA, p-value = 0.06, n = 2).

5.3.6 TGFβ directly affects OPC differentiation

After observing the effects of TGFβ on MNPs, I asked whether TGFβ can affect OPC differentiation directly. Past papers have claimed that TGFβ promotes OPC differentiation, but after reviewing this in more depth, I realised that no direct evidence was shown in these papers (McKinnon, 1993).

In order to test the effects of TGFβ, neonatal OPCs were isolated using MACS (as described before, chapter 4) and were kept in differentiation media with rising concentration of TGFβ1 and SB-431542 (TGFβ pathway inhibitor), or LDN-193189 (BMP pathway inhibitor). I used two types of inhibitors in order to clarify more

precisely the pathway in which TGFβ might affect OPC differentiation (fig5.7D). When cultured with TGFβ, OPCs showed reduce differentiation capacity, exhibited by lower percentages of CNPase positive cells. This effect was blocked completely when SB-431542 was added to the culture together with TGFβ (fig5.7B). TGFβ effect was not blocked by the addition of LDN-193189 (fig5.7C), which suggests that the effects of TGFβ is through its canonical pathways, and more specifically through the activation of ALK4/5/7 and their downstream targets, SMAD2/3 as described in fig5.7D.

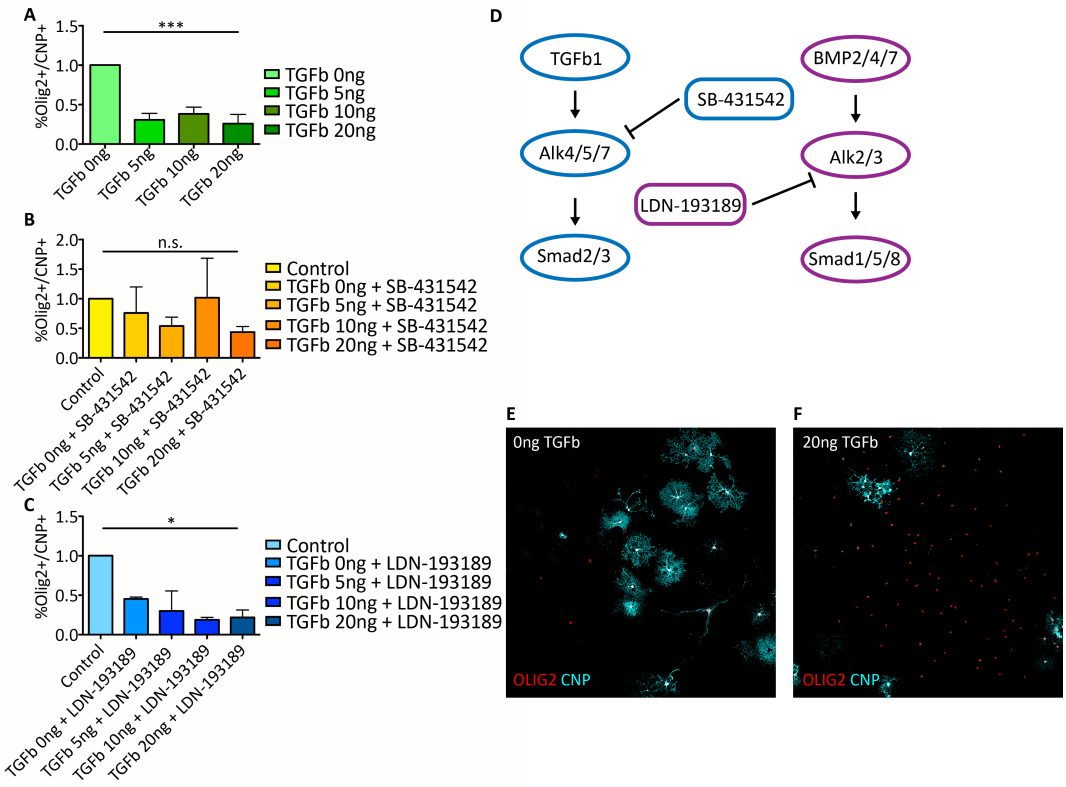

Fig. 5.7 **Direct effects of TGFβ on OPC differentiation *in vitro*** Neonatal OPCs were isolated using A2B5 MACS and plated in PDL coated plates. After 24 hours recovery in proliferation media, differentiation media was introduced with TGFb1 +/- SB-431542 or +/- LDN-193189. **(A)** OPC differentiation *in vitro* is inhibited by the addition of TGFb1 (one way ANOVA, n = 3, p-value = 0.0007). **(B)** effects of TGFb1 were ameliorated by the addition of 5μM of TGFβ pathway small molecule inhibitor, SB-431542 (one way ANOVA, n = 3, p-value = 0.728). **(C)** Addition of BMP pathway inhibitor (LDN-193198, 200nM) does not replicate the effects of TGFβ pathway inhibition, as TGFβ still inhibited OPC differentiation rate (t-test, p-value < 0.05, n = 2). **(D)** Diagram illustrating the different targets of SB-431542 and LDN-193198, and the different SMADs activated in both pathways. **(E-F)** Representative images of differentiating OPC with or without the addition of TGFb1. Cells were fixed and stained for OLIG2 (red) and CNP (cyan).

5.3.7 OPCs cultured with aged MNPs-conditioned media do not increase astrocyte production

After establishing that microglia surface antigens affect OPC differentiation, I tested whether molecules secreted from MNPs are related to this effect, or was the effect only due surface antigens expressed on the cell bodies of the MNPs ghosts and deposited in the plate matrix. To test this, I cultured aged microglia in serum free media (OPCM,

similar to the one I used to culture the OPCs) and collected the media after 48 hours. I then used the different conditioned media to culture OPCs, by mixing the conditioned media with fresh media in a 50/50 ratio.
Neonatal OPCs were cultured for 6 days with conditioned media supplemented with T3 (40ng/ml). Cells were then fixed and differentiation capabilities were assessed using CNPase and GFAP.
Unlike the clear changes expressed by the OPCs cultured on top of ghost MNPs, OPCs cultured in presence of conditioned media did not show a significant increase in astrocyte numbers (fig5.8A). No difference was detected when OPC differentiation into OLs (marked by CNPase expression) was tested.

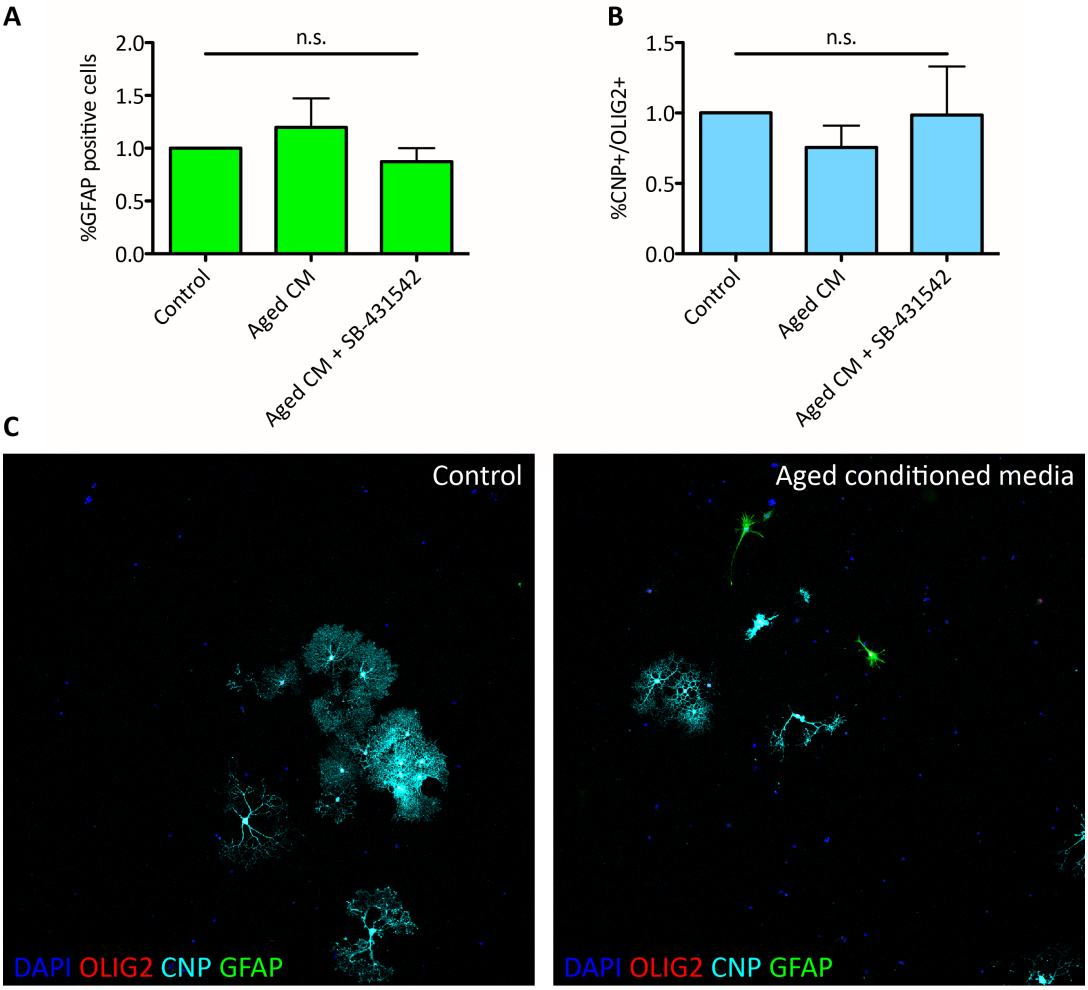

Fig. 5.8 **OPCs cultured with aged microglia MNPs media** Neonatal OPCs were cultured in media containing 50% media previously conditioned by aged MNPs. The 1:1 ratio was chosen according to previously published studies which have also experimented with MNPs conditioned media (Miron et al., 2013). OPCs were cultured for 6 days in conditioned media before stained for astrocyte and oligodendrocyte markers. **(A-B)** OPCs cultured with MNPs conditioned media with or without the presence of SB-431542 TGFβ inhibitor. No significant change was observed in both astrocyte (A) or oligodendrocyte (B) numbers (p-value > 0.05, n = 3). **(C-D)** Representative images of young adult OPCs cultured with or without conditioned media. Cells were fixed and stained for GFAP (green), OLIG2 (red) and CNP (cyan).

5.4 Discussion

5.4.1 Changes in microglia surface antigens lead to OL inhibitory environment

Usually, culturing OPCs *in vitro* in relatively low densities (10,000 cells / cm^2) on PDL coated cell culture plates, results in low numbers of astrocytes (figure 5.3 and 5.5). In contrast, when I cultured OPCs on MNPs ghosts (especially aged ones), I observed a significant increase in the number of astrocytes. As this phenomena was not replicated by using media conditioned with aged MNPs, one can assume that the increase in astrocytes was due to the direct effects of the microglia surface antigens on the OPCs.

The effect of direct cell-to-cell contact on OPCs has been shown in relation to other cell types. For example, neurons control their own myelination, both by secreting neurotransmitters, but also by expressing specific antigens that inhibit OLs from wrapping them with myelin. For example, recent paper published by Redmond and colleagues (Redmond et al., 2016) show that expression of JAM2 on neuronal dendrites play a role in inhibiting myelination of these parts in the neuron. Similarly, experiments with astrocytes show that ghost activated astrocytes prevent OPC differentiation *in vitro* (Keough et al., 2016). In this paper, astrocytes activated by LPS were ghosted and OPCs were plated on top. This resulted in decreased OPC differentiation which was attributed to upregulation of multiple surface antigens by the activated astrocytes, especially various CSPGs. Inhibiting the production of these antigens neutralised this effect. In this chapter, I show that MNPs surface antigens can also influence and play a role in OPC differentiation. More importantly, I show that this effect is more profound in aged MNPs than in young ones. This adds another layer to how aged MNPs inhibit OPC differentiation, and contribute to the failure of remyelination in ageing.

The results described in figures 5.3 and 5.5 suggest that the formation of astrocytes by OPCs is a result of the matrix components deposited by the MNPs cultured in the same well and are now ghosts. There could multiple other explanations for my results, which I will present, and will try to refute:
Firstly, the ghost MNPs might not be dead. This way, aged MNPs (and TGFβ treated neonatal MNPs) could influence the OPCs through active secretion of different factors. I find that the possibility for this is extremely low. During the ghost protocol, cells are fully submerged in ddH_2O. Due to the osmotic pressure, the cells die immediately. Moreover, cells were immediately frozen using dry ice. Imaging plates of ghosts with no OPCs on them showed only very faint nuclear staining, if any. This also eliminates

the second possible explanation: the original cells I plated included astrocytes which have survived the ghost protocol and then took over the culture once 'revived'. Staining for GFAP on ghosts with no OPCs on top did not show any positive cells (data not shown). This explanation is also highly unlikely, as prior to CD11B isolation, A2B5+ cells were removed using MACS, making it unlikely to have contamination of non MNPs (especially OPCs). A third explanation could be that leftover ingredients from the microglia media, especially the serum which was used to culture microglia, affected the OPCs. This is unlikely for three main reasons: firstly, control wells had media in them and did not produce any effect. Secondly, if the effect was mainly due to serum components lingering on, then young MNPs would have had the same effect, regardless of the TGFβ treatment (fig5.5). Thirdly, during the ghost protocol, all media was removed, and then 300µl of ddH_2O was added, thus washing off any leftover components of the serum.

My experiments with aged MNPs conditioned media (fig5.8) did not seem to yield any effects, in contrast to the effects shown by aged MNPs ghosts. This result was not expected, as according to published datasets, aged MNPs harbour many differences in their secretome (Hickman et al., 2013), and some of genes which have been shown to change with ageing are known to affect OPC differentiation, including pro-inflammatory cytokines. This surprising lack of effect could be due to multiple reasons. Firstly, in order to perform the experiments, I used conditioned media in 1:1 ratio with fresh media. Although Miron et al., (Miron et al., 2013) have used similar ratio in previous study with microglia/macrophages conditioned media, the conditioned media might have been too diluted to elicit effects in my experiments. The difference could stem from the fact that Miron et al., used media conditioned by activated microglia/macrophages. At an activated state, the cells are prone to secrete higher amount of various factors, especially in comparison to non activated cells (which I have used). Secondly, in order to prepare the conditioned media, I have cultured microglia in OPCM for 48 hours. It is possible that this was not enough time for substantial concentration of factors to be released into the media (especially as the media was then diluted in a factor of 2 as explained above). Preliminary experiments with adult OPCs (isolated from 7 months old rat) show that OPCs cultured in conditioned media show less complex branches when stained for CNPase (fig5.8C-D), but this was not quantified and needs to be repeated. However these experiments do support my initial conclusions regarding the origin of the effects observed in the ghost experiments, i.e. that it is the deposition of aged MNPs matrix that affects the differentiation of OPCs into astrocytes and not secreted molecules.

Future experiments will have to address the issues raised above, i.e. testing higher

ratios of conditioned media and longer conditioning times by the microglia. I have also begun a set of experiments using neonatal MNPs conditioned media. I would expect that conditioned media by young/MNPs microglia will promote the differentiation of OPCs, as one of the roles of MNPs during remyelination is the secretion of pro differentiation factors. Moreover, I would also expect that media conditioned by young MNPs would especially be useful in promoting the differentiation capacities of aged OPCs. As was described before, heterochronic parabiosis studies which involved young and aged mice, revealed that invasion of young macrophages to the aged CNS enhances remyelination (Ruckh et al., 2012). This effect was explained as mainly stems from enhanced myelin debris clearance by the young macrophages (compared to the resident old ones). This does not rule out other potential beneficial effects that could have been exerted by the young macrophages population, including enhanced secretion of differentiation factors, which can be similar in young microglia due to the similarities between these two cell types.

Exploring previously published RNA sequencing data (Hickman et al., 2013), revealed to me that aged microglia upregulate multiple surface antigens. Analysing the surface antigens which are upregulated in aged microglia, reveal that they include a variety of CSPGs, as well as other molecules which are known to affect OPC differentiation. The effect of the MNPs surface antigens as seen in the ghost experiments is most likely due to a unique combination of surface molecules, and does not stem from a single factor. Despite that, I would describe some specific effects of a few of the upregulated molecules and how they might affect OPCs according to known literature.

Fibronectin, a glycoprotein, has been shown to play multiple roles in remyelination and MS. In toxin induced demyelinating lesions, fibronectin is upregulated in the first 3-5 days post lesion (the main demyelination time period) and then cleared during later stages (10 and 15 days post lesion) (Stoffels et al., 2013). In demyelinated MS lesions, fibronectin is visible in the form of aggregates (Stoffels et al., 2013), and these aggregates can inhibit OPC differentiation *in vitro*. Further experiments using fibronectin coated plates will be required in order to assess if this is enough to elicit the production of astrocytes by OPCs *in vitro*.

Another matrix molecule upregulated in aged microglia is **NG2** (encoded by the *Cspg4* gene). NG2 is mainly known to be expressed by OPCs and is not commonly expressed by microglia. Despite this, there are multiple reports showing that in certain conditions, microglia can express NG2 (Pouly, Becher, Blain, & Antel, 1999). Following stroke, microglia express NG2 and this is due to effects elicited by TGFβ. (Sugimoto et al., 2014). These cells were considered helpful for recovery from stroke, as they seem to present important phagocytosis abilities that aid in removal

of debris and dead cells. In contrast, directly testing the phagocytosis capacities of NG2 microglia by Zhu et al., (Zhu et al., 2012) revealed that NG2 microglia show reduced phagocytic abilities. Moreover, a recent study have shown that inhibiting the upregulation of NG2 in microglia after LPS induction promotes the expression of multiple neurotrophic factors, such as GDNF and NGF (Zhu et al., 2016).
Considering that aged microglia upregulate NG2, and at the same time show decreased phagocytosis capabilities (Natrajan et al., 2015), it is likely that the conversion of more and more microglia into NG2 positive microglia is part of their phenotype that inhibits their regenerative aspects, including efficient phagocytosis and secretion of pro regenerative molecules. Thus aged microglia exert a less effective support for the remyelination process in multiple levels: firstly, as NG2 microglia they exhibit decreased myelin phagocytosis capabilities. Secondly, shifting their expression of multiple matrix associated molecules supports a less pro regenerative ECM in the lesion site. According to this theory, treating neonatal microglia with TGFβ should render them less efficient in phagocytosis, as it promotes the expression of NG2 (fig5.6).

Despite the results I describe in this chapter, I cannot completely rule out that other changes in ageing MNPs can also contribute to the effects I observed. Aged microglia upregulate various pro-inflammatory cytokines, including IL1β and TNFα (Hickman et al., 2013). Both of these cytokines have shown to promote OPC proliferation and migration, but not necessarily differentiation (Arnett et al., 2001; Moyon et al., 2015). Thus, it can be proposed that aged microglia in culture deposit higher amount of these cytokines in the culture matrix. These cytokines are then detected by the OPCs seeded on top of the culture matrix, thus inhibiting their differentiation into OLs.

5.4.2 Rising levels of TGFβ with ageing alters MNPs expression of surface antigens

After observing the effects aged microglia exert on OPCs, I turned to explore what could be the underlying cause to these changes witnessed in aged MNPs surface antigens. I have focused on TGFβ, a well known and potent cytokine. I have decided to focus on TGFβ for multiple reasons:

1. TGFβ levels have been shown to increase with ageing in both mice and humans (Carlson et al., 2009). Secondly, TGFβ is known to play a pivotal role in microglia biology (Butovsky et al., 2014) and microglia highly express TGFβ

receptors in comparison to other type of macrophages (Lavin et al., 2014), as well as in comparison to other cell types in the CNS (Zhang et al., 2014).

2. TGFβ has been shown to promote fibronectin and other matrix related proteins expression in other cells (Ignotz & Massagué, 1986; Laping et al., 2002; Roberts et al., 1988)

3. microglia have been shown to upregulate NG2 after stroke as a response to elevated TGFβ levels.

This positions TGFβ a major candidate to affect microglia and change its matrix associated molecules.
Figure 5.5 supports the role of TGFβ in the effects seen by aged OPCs, as culturing neonatal microglia (P21) in the presence on rising concentrations of TGFβ prior to ghost protocol, results in an increase in of astrocyte formation by OPCs. My qRT-PCR results (as shown in fig5.6) also supports the claim that TGFβ is sufficient to induce changes in microglia matrix related proteins expression. Culturing microglia in the presence of TGFβ resulted in the upregulation of multiple surface genes, and as this was blocked by using specific small molecule inhibitor, it suggests that this is a specific effect unique to TGFβ.

Therefore, I suggest the following mechanism of action: with ageing, there is an increase in both microglia numbers (due to repeating inflammation incidents), as well as an increase in TGFβ levels. Elevated TGFβ levels elicit changes in the expression profile of aged microglia, and specifically alter their expression of matrix related molecules. This chronic exposure to TGFβ renders the aged microglia to express multiple anti regenerative surface molecules which contribute to the formation of an anti regenerative matrix in aged animals lesion sites. This process is summarised and illustrated below in figure 5.9.
Further experiments would be needed to test other possible effects of TGFβ on microglia, starting from the effect of TGFβ on myelin phagocytosis by microglia, i.e. TGFβ transforms microglia to NG2+ microglia which are less efficient in debris clearance. Moreover, I'm currently in the process of assessing the potential of using TGFβ inhibitor (SB-431542) on the neonatal microglia treated with TGFβ ghosts in order to prevent the unwanted effects of TGFβ. Similar experiments will be conducted with aged microglia ghosts, in which using SB-431542 could potentially block the expression of anti regenerative surface antigens.

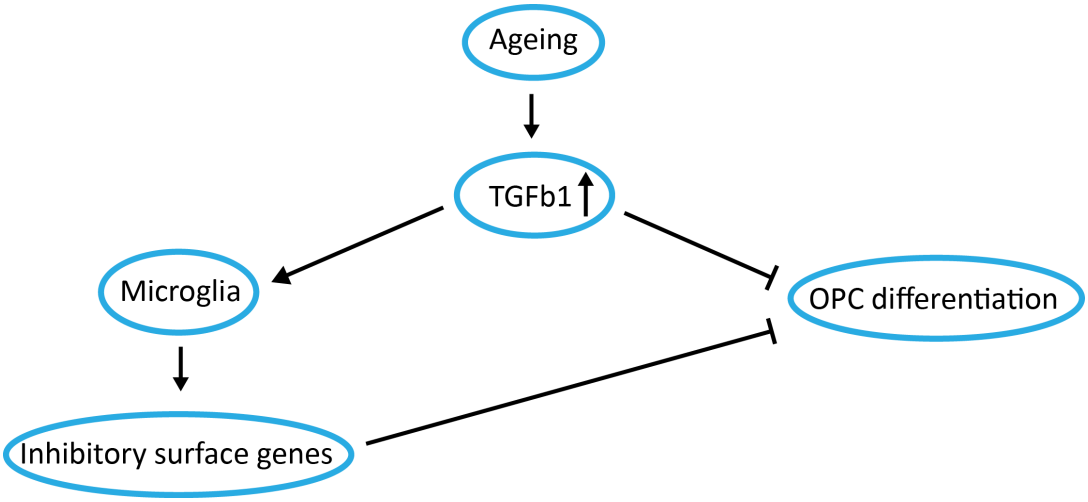

Fig. 5.9 **Schematic summary illustrating the effects of TGFβ on OPC differentiation** This schematic illustration shows the ways in which TGFβ affects the potential of OPC to differentiate, both directly and indirectly.

5.4.3 TGFβ inhibits OPC differentiation *in vitro*

Following the results obtained by adding TGFβ to MNPs, and the reports that TGFβ levels rise with ageing, I tested the potential direct effects of TGFβ on OPC differentiation, as this can also give an alternative explanation for the effects seen by TGFβ treated neonatal MNPs ghosts. I.e., treating MNPs with TGFβ enhances the expression of TGFβ (Butovsky et al., 2014), it is possible that this lead to increased deposition of TGFβ in the culture matrix, and in turn affected OPC differentiation.

Surveying current the literature revealed a consensus that TGFβ enhances OPC differentiation. Exploring the references that led to this conclusion revealed only a single report by McKinnon et al., that shows a direct connection between TGFβ and OPC differentiation (McKinnon, 1993). A more scrutinising view of this paper revealed that no differentiation assay was actually conducted. Only proliferation assays were done, and TGFβ was only shown to inhibit OPC proliferation. Other studies, such as (Hinks & Franklin, 2000) rely on indirect evidence. For example, delay in TGFβ expression in aged animals leads to decreased OPC differentiation. In summary, it seems that the evidence for the supportive roles of TGFβ in OPC differentiation is lacking, and following my own experiments (fig5.7) I would propose a different effect for TGFβ in OPC differentiation.

As shown in figure 5.7, addition of TGFb1 inhibits OPC differentiation *in vitro*, while blocking TGFβ signalling pathway using small molecule inhibitor, SB-431542, cancels this effect. SB-431542 is a direct inhibitor of Alk4/5/7 (Inman et al., 2002), and thus

prevents the phosphorylation of SMAD2/3 (Laping et al., 2002). This prevents the downstream activation of genes usually activated by phosphorylated SMAD2/3.
These results are in contrast to results shown by Palazuelos and colleagues (Palazuelos, Klingener, & Aguirre, 2014). They show opposite results for the effects of TGFβ on OPCs, both *in vitro* and *in vivo*, as the addition of TGFβ OPCs in their cultures accelerated OPC differentiation, and administering TGFβ *in vivo* promoted myelination. As I did not test the effects of TGFβ *in vivo*, I cannot say for certain what will be the systematic effects of TGFβ administration to animals. I believe that the main reasons for the differences in the *in vitro* results stem from two main factors: the identity of the cells and the concentration/duration of the TGFβ administration. Firstly, Palazuelos et al., used CNP-EGFP in order to sort OPCs. This is in contrast to the A2B5+ cells I used. CNP+ cells are at best pre-myelinating OLs, and cannot be considered real progenitors cells. Secondly, Palazuelos et al., administered TGFb1 in a concentration of 5ng/ml, which is half of the concentration I used. As TGFβ pathway are complex and include multiple feedback loops, I would expect that different doses will have different effects. As shown with the MNPs experiments, only at 10 ng/ml a significant change was achieved in MNPs gene expression. The role of TGFβ in stem cells biology is not yet fully known. Several reports have shown that inhibiting TGFβ pathway results in induction and maintenance of pluripotency. For example, Ichida and colleagues show that TGFβ inhibition can replace *Sox2* overexpression in reprogramming adult fibroblasts (Ichida et al., 2009), while Tan et al., show that inhibiting TGFβ pathway in combination with inhibiting HDACs (using Valporic Acid, VPA) can substitute *Oct4* overexpression in reprogramming (Tan, Qian, Tang, Abd-Allah, & Jing, 2015).
In the OL lineage, Schultz and colleagues have shown that TGFβ and Activin A induce cell death in OL cell line (OLI-neu) (Schulz, Vogel, Dressel, & Krieglstein, 2008; Schuster et al., 2002). I cannot rule out that cell death was the reason behind the decline of differentiation in my experiments, but unlike the studies with OLI-neu I could eliminate the effects of TGFβ on OPCs by using small molecule inhibitors. This is also in contrast to the effect of TGFb1 administration by Palazuelos et al. (Palazuelos et al., 2014). It is also noteworthy to point out that more recent studies by Miron et al., have pointed out that Activin A secreted by microglia/macrophages promotes OPC differentiation *in vitro*, and adding Activin A blocking antibody eliminated the effect of the microglia conditioned media (Miron et al., 2013). These latest results cast doubts on the results shown by Schuster et al.

TGFβ favourable role in OPC differentiation was further enhanced by studies that claimed that at least part of the delay in OPC differentiation in aged animals is due to changes in TGFβ expression by microglia/macrophages. Hinks and Franklin

(Hinks & Franklin, 2000) described the delayed kinetics of several growth factors (including TGFβ) during remyelination in aged animals and concluded that this leads to delayed remyelination. According to the results I show in this chapter I would suggest a different mechanism for the delayed OPC differentiation in relation to TGFβ kinetics in aged animals. In aged animals, the duration of exposure to TGFβ is longer and delays OPC differentiation. According to this paper, in young animals, who exhibit successful remyelination, TGFβ levels peak very early after the lesion (10 days post lesion), but then also decline very rapidly. In contrast, in aged animals TGFβ levels peak later on, but also remain high for a longer duration. A similar phenomena is described by Doyle and colleagues when investigating stroke in aged and young animals, where aged animals show a higher baseline of TGFβ levels before and after stroke. This is thought to lead to enhanced astrogliosis, which in turn inhibits regeneration (Doyle, Cekanaviciute, Mamer, & Buckwalter, 2010).
Therefore, I suggest that in aged animals, delayed appearance and more importantly delayed removal of TGFβ, result in direct inhibition of OPC differentiation.

In summary, I show here results that suggest that TGFβ actually plays an inhibitory role in OPC differentiation, both directly and indirectly (through microglia surface antigens). This adverse effect is only enhanced with age, as TGFβ levels rise.

This situates TGFβ as a potential therapeutic target for enhancement of remyelination in aged patients. To test this, aged animals would be subjected to demyelinating lesion while being treated with a TGFβ pathway inhibitor. A good candidate for this could be the FDA approved compound Losartan. Losartan, which is mainly known as Angiotensin type receptor 1 (*Agtr1*, AGTR1) antagonist, has been shown to be effective in blocking TGFβ signalling in multiple pathological conditions, including Marfan syndrome models where it has been shown to inhibit TGFβ dependent activation of signal regulated kinase (ERK) (Holm et al., 2011; Habashi et al., 2011). Even tough *Agtr1a* is not highly expressed in microglia (Zhang et al., 2014), a report from 2008 by Miyoshi and colleagues showed that Losartan can attenuate the activation of microglia by LPS, measured by reduced expression of IL1B and NO (nitric oxide) (Miyoshi, Miyano, Moriyama, Taniguchi, & Watanabe, 2008). Furthermore, Losartan has been shown to be an effective suppressor of TGFβ related epilepsy (Bar-Klein et al., 2014). Most importantly, in the study from 2014, although Losartan was administered intraperitoneally and orally, it was successful in affecting epileptic processes in the CNS, suggesting it can cross the BBB. Future studies would involve the direct effects of Losartan on aged and young microglia, as well as the effects of Losartan on OPC differentiation.

Chapter 6

Final discussion and conclusions

6.1 DNA methylation in adult and aged OPCs

The epigenetic landscape and DNA methylation in particular, has been studied for many decades. In recent years, with the emergence of new genomic tools, it has been clear that the original notions regarding the role of DNA methylation were at best simplistic, and even incorrect in some respects. One of the most repeated statements relates to the inhibitory role of DNA methylation in transcription (A. P. Bird, 1985; A. Bird, 2002). This inhibition can act in two main ways - firstly, direct effects of DNA methylation on the affinity of the transcription machinery to the promoter sites; secondly, through recruitment of histone modifiers which inhibit transcription (Fuks, 2005).

In this research I studied the potential connection between DNA methylation and transcription in OL lineage cells, in both young adult and aged cells. In both cases, I could not find a strong correlation between changes in DNA methylation and changes in transcription. This was true when exploring full transcripts expression, differential exon usage and expression of transposable elements (TEs). In all these cases, no direct correlation was detected. I would like to explain this discrepancy by looking at the following aspects:

1. **Technical differences in comparison to past papers** -
   My research included Whole Genome Bisulfite Sequencing (WGBS). This allowed me to observe changes in methylation in specific CpGs in a global genome view. Many of the older studies regarding DNA methylation function used more limited techniques (such as Reduced Representation Bisulfite Sequencing - RRBS) that focus on specific locations in the genome and thus enable the view of patterns more easily. Moreover, as recently reviewed by Tirado-Magallanes

et al., (Tirado-Magallanes, Rebbani, Lim, Pradhan, & Benoukraf, 2017) identification of correlation between DNA methylation and gene transcription is greatly influenced by the window size chosen when analysing the methylation state. For this study promoters were analysed with a window size of 1000bp (-900...+100), which might not be the optimal window size, especially as there are no big changes between the samples. This correlation can also be influenced by the exact region that is analysed (i.e. different parts of the transcription start site). This being said, exploring a recent publication regarding DNA methylation in developmental OPCs (Moyon, Liang, & Casaccia, 2016) I could identify similar patterns detected in my results and theirs. In both cases, the correlation between DNA methylation and transcription was not perfect. Moyon et al. focused only on the genes which showed the expected behaviour. Focusing only on promoters that show correlation in methylation-transcription restricts the data that can be inferred. For example, most of the genes identified in the Moyon paper as being hypomethylated in OLs were myelin related. This could have also been inferred by just observing the transcriptional data as OLs increase the expression of myelin genes.

2. **Novel role for DNA methylation in OL lineage cells** - As opposed to the original role set out for DNA methylation as an inhibitory cue for gene transcription, my data suggests that DNA methylation plays a much more complex role in in gene transcription than a binary state, at least in the OL lineage cells. A publication by Raynal et al., (Raynal et al., 2012) supports this notion, as they show that treating cells with HDAC inhibitors is sufficient for activation gene transcription without altering DNA methylation states.
   Thus, when observing promoter methylation levels of several key OPC genes in aged cells, it is possible to hypothesise that DNA methylation acts more as a fine-tuner than as a simple inhibitor. This loss of methylome landscape observed in key genes in aged OPCs (some of which show significant changes in expression as well) might be a contributing factor to the changes in transcriptome and cell maintenance in aged OPCs. In order to fully investigate these changes, further experiments will need to be conducted. This could include the comparison of aged dietary restricted OPCs to young ones, similarly to the effects shown for the transcription of key genes (Neumann et al., 2017),

In conclusion, my results show that the relation between DNA methylation and gene transcription is more complex then a simple binary switch. Therefore, further

research will need to be conducted in order to fully understand the role of DNA methylation in gene transcription in general and in adult OPCs in particular.

6.2 Adult OL lineage cells are inherently different from their neonatal counterparts

To date, the majority of *in vitro* studies involving OPCs have used neonatal OPCs. This has generated substantial amounts of data regarding the developmental myelination occurring in the early weeks postnatally in rodents. It is still unknown how much of this is relevant to adult OPC biology and the transition they undergo during remyelination (Fancy, Chan, Baranzini, Franklin, & Rowitch, 2011). Using a newly developed isolation method I was able to isolate OLs and OPCs from adult rats in order to further study the unique transcriptome of adult OPCs. When comparing my results to previously published studies comparing neonatal OPCs and OLs (Zhang et al., 2014) I found very few similarities. I believe that the main reason for this is the extensive differences between neonatal and adult OPCs.

While the role of neonatal OPCs is to facilitate the developmental myelination process, thus being in a constant state of activation, adult OPCs are not constantly activated. Adult OPCs seems to reside in a quiescent state, characterised by a slow cycling of the cells (Young et al., 2013). The differences between neonatal and adult OPCs raise two main questions - are adult OPCs in fact adult stem cells residing in the adult CNS? And what is the main role of adult OPCs in CNS homeostasis?

The discussion regarding the question whether or not OPCs can be considered adult stem cells has been reviewed previously (A. Crawford et al., 2014; Franklin & Ffrench-Constant, 2008). When examining the criteria for stem cells, one would point out multipotency, self-renewal, asymmetric division and quiescence state when not activated. Adult OPCs show multipotency both *in vitro* as well as *in vivo*, as they can differentiate into oligodendrocytes, astrocytes, neurons and schwann cells (Belachew et al., 2003; Raff et al., 1983; Zawadzka et al., 2010). OPCs can also undergo asymmetrical division (Sugiarto et al., 2011) and can self renew themselves (Neumann, unpublished). Using the transcriptional data presented in this study I can add further evidence to this argument by pointing out that adult OPCs express specific quiescence markers shared by other stem cells, such as *Lgr5* (Jaks et al., 2008). Adult OPCs also upregulate the expression LKB1 (*Stk11* (fold change = 4.39, p-value = 0.051). LKB1 is a master regulator which maintains homeostasis in haematopoietic stem cell (HSC), and its deletion leads to their depletion (Gan et al., 2010). Therefore,

OPCs seems to activate quiescence genes similar to other adult quiescence stem cells. This raises another question - if OPCs are quiescent stem cells, what is their stem cell niche? OPCs are usually described as spread across the CNS following their initial migration waves (A. H. Crawford, Tripathi, Richardson, & Franklin, 2016), and do not seem to encompass any specific niche beyond the original blood vessels they follow in their initial stages of development (Tsai et al., 2016). This will have to be investigated in future studies. My data presents future researchers with some key findings for identifying quiescent adult OPCs. As mentioned in chapter 3, adult OPCs do not upregulate 'classical' markers in comparison to adult OLs, including *Pdgfra* (fold change = 0.956, adjusted p-value = 0.937). Therefore, I suggest that in order to accurately study quiescent adult OPCs, new markers should be used, such as *Lgr5*, which is used to identify quiescent stem cell in other niches (Barker et al., 2007; Basak et al., 2017; Jaks et al., 2008) and *Hes1* which is a known marker for neural stem cells (Nakamura et al., 2000). Discovering the stem cell niche of OPCs is essential to combat the external effects of ageing, as it has been shown in other tissues that the ageing niche can inhibit the function of young stem cells (Carlson & Conboy, 2007).

Another question that arises from the distinct differences between neonatal and adult OPCs involves the role of adult OPCs in CNS homeostasis. In the adult CNS, OPCs comprise 5-10% of the cells and the majority of the dividing cells (BrdU positive) (Dawson et al., 2003; Horner et al., 2000); yet their role remains unknown. As yet, the function of adult OPCs has been investigated mainly in the relation of remyelination following pathological demyelinating events (Franklin & Ffrench-Constant, 2008). Adult OPCs also play a role in *de novo* myelination in learning processes (Mckenzie et al., 2014) and modulate myelin in the adult CNS (Young et al., 2013). Both of these behaviours are to be expected by the nature of OPCs, as **oligodendrocyte** progenitors. But both of these examples are primarily instances of OPC differentiation into OL in order to facilitate their functions (remyelination and myelination). These do not account for the activity of OPCs in their progenitor state. Do OPCs have an active function while still in their progenitor state? In other tissues, the reason for the existence of quiescence stem cells is to maintain a pool of cells ready to differentiate when needed. The quiescent state is thought to protect the cells from genomic damages that will occur if these cells divide rapidly (Richmond, Shah, Carlone, & Breault, 2016). But unlike the stem cells of the haematopoietic or intestinal systems, in which large numbers of new differentiated cells need to be generated daily, adult OPCs do not differentiate rapidly every day, but mainly proliferate and differentiate in response to injury or outside stimulation (as described above). Future research would be required to determine if adult OPCs participate in normal brain homeostasis (similar to the

part played by astrocytes, for example), or if they only play a role when needed to differentiate (similar to stem cells in the hair follicle, for example (Ito et al., 2005)).

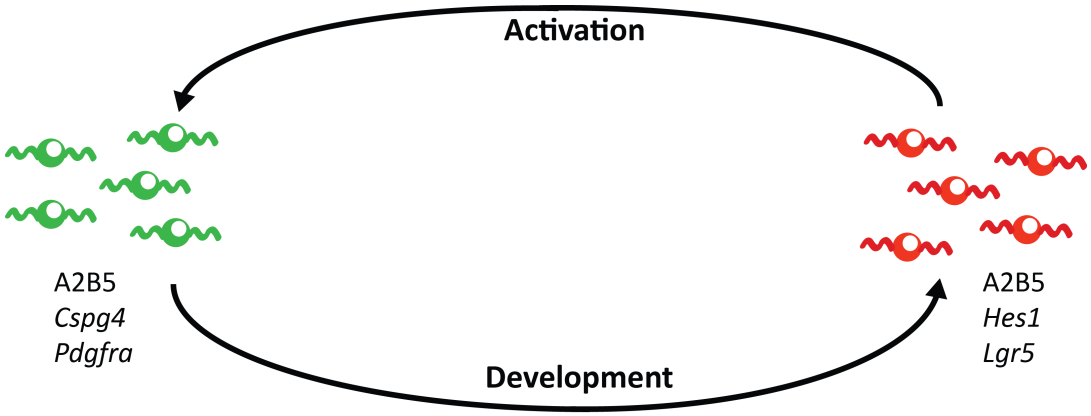

Fig. 6.1 **Summary of differences between adult and neonatal OPCs** Schematic illustration describing the differences between neonatal and young adult OPCs. Neonatal OPCs (green) are in activated state, characterised by the high expression of *Pdgfra* and *Cspg4*. As developmental myelination ends, OPCs transform into a more quiescent state (red cells) which is characterised by the expression of quiescent stem cell genes such as *Hes1* and *Lgr5*. Once activated, either *in vitro* by PDGF or following demyelinating lesion, adult OPCs are activated and revert to a more neonatal state, in similar way to what has been described in previous studies (Moyon et al., 2015).

6.3 Transcriptional changes in aged OPCs inhibit their differentiation capabilities

Remyelination is a regenerative process which is essential for the restoration of myelin sheaths onto demyelinated axons. This enables adequate saltatory conduction, as well as providing essential trophic support to axons, thus preventing axonal degeneration (Franklin & Ffrench-Constant, 2008). This process seems to play a critical role in the relapsing remitting phases of Multiple Sclerosis (MS) in humans (Compston & Coles, 2008). Unsuccessful remyelination is usually thought to result from the failure of OPCs to differentiate in the lesion site (Kuhlmann et al., 2008), and not due to insufficient numbers of OPCs in ageing (Woodruff et al., 2004). In demyelinating animal models, there is a marked decrease in the remyelination rate, making this process virtually unsuccessful (Shields et al., 1999). Since age is correlated to the transition to progressive phase in MS patients, it is clear that ageing must be considered as a factor in remyelination therapies being developed. Unfortunately, due to technical

limitations, most studies regarding the enhancement of OPC differentiation capabilities up to this day have used *in vitro* neonatal OPCs.

In line with recent reports from the Franklin lab (Neumann et al., 2017), I show here transcriptional and epigenetic evidence to support the notion that aged OPCs are inherently different to neonate and young adult OPCs. This difference is extremely relevant to the decrease in differentiation capacities of aged OPCs. Aged OPCs exhibit decreased expression levels of several nuclear receptors previously shown to enhance OPC differentiation, including Thyroid hormone receptors and Retinoic Acid receptors (B. A. Barres et al., 1994; Huang et al., 2011). These results raise concerns that drugs developed to enhance OPC differentiation in aged MS patients might be of limited efficacy.

Furthermore, I show here evidence that aged OPCs lose their progenitor state when compared to young OPCs. This includes the upregulation of several OL markers (*Cnp* and *Enpp6*) but none of the myelin genes. This state is even more evident when considering the fact that downregulation of multiple OPC genes are tightly connected to maintaining the cell's progenitor status (including *Pdgfra, Hes1, Hes5, Ptprz1, Sox2* and *Ascl1*). When recruited to demyelinating lesions, adult OPCs revert back to neonatal activated state (Moyon et al., 2015). Aged OPCs, which show downregulation of many progenitor genes, are therefore less likely to be able to revert back to neonatal progenitor state. Moreover, expression of progenitor genes is essential in order to maintain the quiescence state of OPCs. Mainataing cells in a quiescence state is key in protecting cells from DNA damage as forced exit from quiescence can lead to extensive DNA damage (Beerman, Seita, Inlay, Weissman, & Rossi, 2014) and maintaing pool of stem cells for lineage differentiation (Cheung & Rando, 2013).

Using WGBS I was able to study change in methylation status in aged OPCs. Although no global changes were found, when observing specifically OPC related genes (which are necessary for the progenitor phenotype of OPCs) I detected a significant loss of methylation in many promoters. This suggests a loss of epigenetic memory in aged OPCs and an epigenetic drift. Mechanisms behind epigenetic inheritance are still unknown, but DNA methylation presents a good candidate since it is transmitted in a semi-conservative way during DNA replication and hence can be fully copied to daughter cells. DNMT1 is responsible for this cellular inheritance, and, as expected, is downregulated in aged OPCs (log2 fold change = 0.4, adjusted p-value < 0.05). This provides the basis for the reduction of methylation observed in several key OPC gene promoters. It is not yet known how these changes directly affect the biology of aged OPCs and their ability to differentiate and respond to external stimuli. Since calorie restriction results in restoration of expression of key genes in aged OPCs to

similar levels as those exhibited by young OPCs (Neumann et al., 2017), similar results might be shown by performing WGBS on aged OPCs derived from calorie restricted animals. Changes in multiple genes reduces the chances that interventions which are based on specific DNA methylation alterations, including using CRISPR mediated DNA methylation (McDonald et al., 2016), will be effective in correcting the aged methylome, as it includes multiple targets. Therefore, a more global approach that will reverse the epigenetic landscape of aged OPCs in a global way should be favoured, as described below.

Ingenuity Pathway Analysis (IPA) has revealed that one of the main pathways which is upregulated in aged OPCs is the mTOR (mammalian target of rapamycin) pathway. mTOR has been described as a major effector in ageing related processes (Johnson et al., 2013). Inhibition of the mTOR pathway leads to increased life span in multiple organisms, ranging from yeast, nematode Caenorhabditis elegans, fruitfly Drosophila and mice (Roizen, 2010; Johnson et al., 2013). More specifically, upregulation of mTOR has been detected in aged HSCs, and inhibiting mTOR activation in these cells using rapamycin restored their function and improved the overall immune system activity of aged mice (C. Chen, Liu, Liu, & Zheng, 2009). Besides mTOR inhibition, the only other mechanism that has been shown to increase life expectancy throughout the animal kingdom is calorie restriction (CR) (Johnson et al., 2013). CR rejuvenation effects are thought to be mediated, partially, through the inhibition of mTOR activation (specifically mTORC1 inhibition), and have been shown to enhance aged stem cell function (Cerletti, Jang, Finley, Haigis, & Wagers, 2012). mTOR is inhibited by AMPK (AMP-activated protein kinase). As expected, one of the catalytic sub units of AMPK, encoded by *Prkaa2*, is downregulated in aged OPCs (log2 fold change = 0.745, adjusted p-value = 0.005). Therefore, this could at least partially explain the dysregulation in mTOR signalling. Similar to what have been shown in HSCs, aged mice which have been subjected to CR show enhanced remyelination capacities, and OPCs isolated from these mice show enhanced differentiation capabilities (Neumann et al., 2017). This rejuvenation of aged OPCs can also be achieved by inhibiting mTOR directly using rapamycin. Unfortunately, this probably cannot be used as therapeutic strategy for two main reasons: firstly, mTOR activation is also needed for OPC differentiation (Tyler et al., 2009). Secondly, chronic exposure to rapamycin can inhibit mTORC2 activity, impairing glucose tolerance and insulin function (Lamming et al., 2012). Therefore, the use of metformin in order to activate AMPK was favoured, and has been shown to reproduce the effects of CR (Neumann et al., 2017). I expect that by altering major pathways in aged OPCs as described here, it will be possible to reverse at least some of the changes I observed in the methylation

patterns in aged OPCs.

In conclusion, aged OPCs show dramatic changes in their transcriptional landscape. These changes render them less equipped to address challenges posed by demyelinating lesions in the aged CNS. Therapeutic interventions can potentially rejuvenate aged OPCs, and these will be most effective if done in a global, systematic fashion.

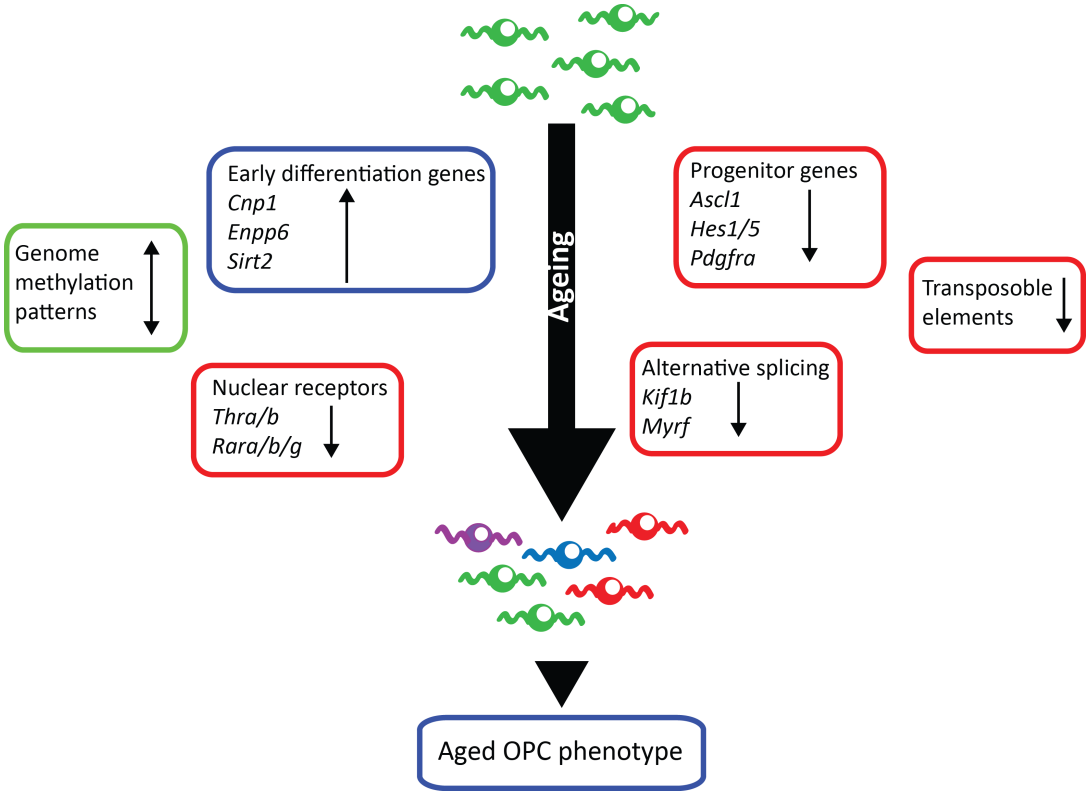

Fig. 6.2 **Summary of intrinsic changes in aged OPCs** Schematic summary of intrinsic changes in aged OPCs described throughout this study. Young adult OPCs (labelled in green) are an homegenous population stem cells which are capable to self renew and differentiate once activated. During ageing, these cells exhibit several intrinsic changes, as illustrated. This results in heterogeneous population which is less effective in responding to injury and activation cues. Red text boxes describe changes which include downregualtion of elements, whereas blue text box exhibit upregulations. The green text box describe changes which include and up and down-regulation. Each text box includes a few specific examples of the process which is mentioned.

6.4 Microglia ECM molecules affect OPC differentiation

OPC differentiation is a complex process which is governed by both internal and external factors. In the previous sections I focused on the internal gene networks in

play in adult OPCs and how they are affected by the process of ageing. In this final section I will focus on some external effectors which mediate OPC differentiation, focusing on microglia and their contribution to the lesion microenvironment, with emphasis on their effect on the extracellular matrix. The microenvironment has a profound role on the function of stem cells, and this has been shown for multiple tissue and cell types (Carlson & Conboy, 2007).

Previous studies have explored the ways in which the microenvironment can affect OPC differentiation, with emphasis on the inhibitory effects of CSPGs (chondroitin sulfate proteoglycans), myelin debris and more. CSPGs are known to be inhibitory for axonal regeneration in general and OPC differentiation in particular (Lau et al., 2012; Pendleton et al., 2013). More recent investigations have focused on the role astrocytes play in demyelinating lesions, by the expression of inhibitory CSPGs, and how the mere presence of these molecules can ameliorate all effects OPC differentiation drugs might exert (Keough et al., 2016). These studies emphasise the pivotal part the lesion substrate plays in the dynamics of OPC differentiation. In contrast to astrocytes which encompass the rim of the demyelinating lesion, the core of the lesion - where remyelination will eventually take place - is mainly populated by microglia and macrophages. These cells are usually the first to arrive at the lesion, and are responsible for the clearance of inhibitory myelin debris and the recruitment of OPCs to the site lesion using specific chemokines (McMurran, Jones, Fitzgerald, & Franklin, 2016).

The results I show in chapter 5 describe (for the first time to my knowledge) an additional role for microglia in remyelination as they shape the extracellular matrix (ECM) of the lesion by the expression of multiple surface antigens that in turn affect OPC differentiation. Aged microglia upregulate multiple surface antigens, including NG2 and fibronectin (Hickman et al., 2013), which in turn can affect OPC differentiation. Using *in vitro* cultures, I show that aged microglia antigens can alter the fate of OPCs, and promote astrogenesis. I can further show that this process can reproduced in neonatal microglia by adding TGFβ, similarly to what have been shown in other cell types (Ignotz & Massagué, 1986).

In vivo experiments have shown that microglia in demyelinating lesions express fibronectin and this can potentially inhibit remyelination (Stoffels et al., 2013). Therefore, I suggest here that elevated levels of TGFβ in aged animals alters the expression of surface antigens on microglia to a phenotype which does not supports OPC differentiation, and might even inhibit it.

6.5 Conclusions

This thesis presents novel methods and new data regarding the transcriptome of adult OPCs, and the changes they encounter during ageing. The discussion also emphasises the differences between neonatal and adult progenitor cells, and the importance of studying adult and aged cells in order to develop therapeutic targets in human diseases. I provide here evidence for multiple changes in the aged OPC transcriptome which can lead to inefficient differentiation; these include alterations in full gene expression as well as changes in alternative splicing. I also supply transcriptional evidence to support the notion that OPCs are in fact similar to other tissue resident adult stem cells. Furthermore, the data reveals a previously undiscovered contribution of microglia to the ageing ECM, and its effect on OPC differentiation. I go on to explore the possible mechanism behind the changes in microglia ECM molecules, and identify TGFβ as a potential therapeutic target in aged animals and humans. Collectively, these findings will provide a framework for further research that will unravel the biology of adult and aged OPCs, aiding in the pursuit of new strategies to enhance remyelination in adult and aged human patients.

> In summary, all great work is the fruit of patience and perseverance, combined with tenacious concentration on a subject over a period of months or years.
>
> *Santiago Ramon y Cajal*
> *Advice for a Young Investigator*

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