Oligodendrocyte Population Dynamics: Insights from Transgenic Mice

Andrew Robert Calver

MRC Laboratory of Molecular Cell Biology and Department of Biology,
University College London,
Gower Street,
London.
WC1E 6BT.

A thesis submitted to the University of London in part fulfillment of the degree of Doctor of Philosophy.
ABSTRACT

The control of cell population size is a critical part of the development of any organism, and is a complex process involving cell division, migration and death. In addition to absolute cell numbers, the control of relative population sizes is of fundamental importance, as the different parts of an organism must develop in proportion to one another. I have attempted to address these questions using an in vivo model system; the oligodendrocyte lineage in the developing mouse spinal cord.

Oligodendrocytes myelinate axons in the central nervous system (CNS). Their precursors originate in a restricted region in the ventral part of the developing spinal cord, and subsequently proliferate and migrate away from there to populate the entire spinal cord before differentiating into mature oligodendrocytes. It is not known what controls the number of proliferating progenitor cells, the timing and site of differentiation, or the number of their mature progeny.

Platelet-derived growth factor-AA (PDGF-AA) is a potent mitogen for oligodendrocyte progenitor cells in vitro, but it was not known whether PDGF is important for oligodendrocyte development in vivo. I have shown by analysis of 'knockout' and transgenic mice that PDGF-AA is crucial for proliferation of oligodendrocyte progenitor cells in vivo, and that a decline in PDGF signaling is responsible for slowing of the cell cycle, withdrawal from the cycle and initiation of oligodendrocyte differentiation.
I have also demonstrated that the number and location of mature spinal cord oligodendrocytes is controlled by selective cell survival, and is independent of progenitor cell proliferation. Excess and ectopic differentiating progenitors are cleared during normal development by programmed cell death, and as-yet-unidentified survival factors control the temporal and spatial appearance of mature oligodendrocytes. These data demonstrate the overriding importance of cell survival controls compared to cell proliferation controls for determining the final number of post-mitotic oligodendrocytes. It seems possible that many differentiated cell populations in the developing animal might be similarly regulated by selective cell survival and death.
ACKNOWLEDGMENTS

I would like to thank all the members of the Department of Biology and, latterly, the Laboratory for Molecular Cell Biology for making the years I spent at UCL so enjoyable. I would particularly like to thank Anita, Caroline, Carol, Hazel, Nigel, Paul, Marcus, Nathalie, Valerie, Bernie, Steve, Costas, the Moss lab, the Raff lab and the rest of the Richardson crew both past and present for advice, encouragement, friendship and accompanying me to Huntley Street for the odd ‘quick half after work’. Special mention also goes to Gary, honorary member of the Richardson lab, for his friendship and support, and for not betting against me finishing this before the end of the millennium.

A huge debt of gratitude is due to Bill Richardson, not only for putting up with me in the lab for so long, but also for his vast insight and input into the work described in this Thesis, and for making the lab such a stimulating and pleasurable place to work.

My utmost thanks and love go to Juliette, for keeping me going through this difficult year, and for her encouragement, support and patience throughout.

Lastly, and with all my heart, I’d like to thank my mother and father, without whom none of this would ever have been possible, and to whom I extend my deepest thanks and respect.
To the Memory

of my Father

(1922-1998)
### TABLE OF CONTENTS

ABSTRACT ............................................................................................................................ 2  
ACKNOWLEDGMENTS .......................................................................................................... 4  
DEDICATION ........................................................................................................................ 5  
TABLE OF CONTENTS ....................................................................................................... 6  
LIST OF FIGURES & TABLES ........................................................................................ 12  
ABBREVIATIONS .............................................................................................................. 14  

1. CHAPTER ONE - GENERAL INTRODUCTION ................................................................ 17  
   1.1 SIZE CONTROL IN ANIMAL DEVELOPMENT ........................................................ 18  
   1.2 VERTEBRATE NEURAL DEVELOPMENT .................................................................... 20  
   1.3 THE OLIGODENDROCYTE LINEAGE ........................................................................ 24  
      1.3.1 OLIGODENDROCYTES MYELINATE CNS AXONS ........................................... 24  
      1.3.2 OLIGODENDROCYTES DEVELOP FROM BIPOTENTIAL PROGENITOR CELLS IN VITRO ................................................................. 25  
      1.3.3 MITOGENS FOR O-2A PROGENITORS .......................................................... 27  
      1.3.4 PDGF AND ITS RECEPTORS: STRUCTURE AND POSSIBLE ROLES IN VIVO ................................................................. 28  
      1.3.5 PDGF IN OLIGODENDROCYTE LINEAGE DEVELOPMENT: A CHRONOLOGY ............................................................................. 31  
      1.3.6 A VENTRAL ORIGIN FOR THE SPINAL CORD OLIGODENDROCYTE LINEAGE ........................................................................... 33  
   1.4 OLIGODENDROCYTE POPULATION CONTROL .................................................... 36  
      1.4.1 CONTROL OF O-2A PROGENITOR PROLIFERATION ...................................... 36  
      1.4.2 CONTROL OF O-2A PROGENITOR DIFFERENTIATION .................................. 39
14.3 CONTROL OF O-2A PROGENITOR AND OLIGODENDROCYTE SURVIVAL

1.5 SUMMARY AND AIMS OF THESIS

2. CHAPTER TWO - MATERIALS & METHODS

2.1 BACTERIOLOGY
  2.1.1 BACTERIAL STRAINS, GROWTH AND STORAGE
  2.1.2 PREPARATION AND TRANSFORMATION OF COMPETENT BACTERIA

2.2 MOLECULAR BIOLOGY
  2.2.1 EXTRACTION OF DNA WITH PHENOL/CHLOROFORM/ISO-AMYL ALCOHOL
  2.2.2 PRECIPITATION OF DNA WITH ETHANOL OR ISOPROPANOL
  2.2.3 AGAROSE GEL ELECTROPHORESIS AND GEL PURIFICATION OF DNA
  2.2.4 DNA-MODIFYING ENZYME REACTIONS
  2.2.5 DENATURING POLYACRYLAMIDE (SEQUENCING) GEL ELECTROPHORESIS OF DNA/RNA
  2.2.6 PURIFICATION OF $^{32}$P-LABELED RNA PROBES ('RIBOPROBES') FROM SEQUENCING GELS
  2.2.7 SMALL SCALE PREPARATION OF PLASMID DNA BY ALKALINE LYSIS ('MINIPREPS')
  2.2.8 SCREENING BACTERIAL COLONIES BY FILTER HYBRIDIZATION ('COLONY LIFTS')
  2.2.9 LARGE-SCALE PREPARATION OF PLASMID DNA BY CESIUM CHLORIDE EQUILIBRIUM CENTRIFUGATION ('MAXIPREPS')

2.3 MAMMALIAN CELL CULTURE
2.3.2 TRANSFECTION OF COS CELLS .............................................................65
2.3.3 RADIOACTIVE LABELING OF COS CELL PROTEINS ................. 65

2.4 PROTEIN ANALYSIS ...........................................................................66
2.4.1 IMMUNOPRECIPITATION ...............................................................66
2.4.2 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE). 67
2.4.3 GEL FLUOROGRAPHY .................................................................68

2.5 TRANSGENIC MICE PRODUCTION AND SCREENING ................. 68
2.5.1 TRANSGENE PLASMID CONSTRUCTION ......................................68
2.5.2 PURIFICATION OF TRANSGENE DNA ........................................68
2.5.3 PRODUCTION OF TRANSGENIC MICE ........................................69
2.5.4 ISOLATION OF GENOMIC DNA ..................................................69
2.5.5 SOUTHERN BLOT ANALYSIS OF GENOMIC DNA ....................70
2.5.6 RANDOM-PRIMER LABELING OF DNA PROBES .......................70

2.6 ANALYSIS OF TRANSGENIC MICE .................................................. 71
2.6.1 STAGING OF EMBRYONIC MICE ...............................................71
2.6.2 AVOIDANCE OF RNASE CONTAMINATION ...............................72
2.6.3 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR) .......................................................................................................................72
2.6.4 RNase PROTECTION ASSAYS ......................................................77
2.6.5 IN SITU HYBRIDIZATION (ISH) USING RADIOLabeled PROBES ..........................................................................................................................78
2.6.6 IN SITU HYBRIDIZATION USING DIGOXYGENIN-Labeled PROBES ..........................................................................................................................86
2.6.7 IN VIVO BrdU LABELING AND SPINAL CORD CULTURE ..........89

2.7 HISTOCHEMISTRY, CYTOCHEMISTRY AND IMAGING ............... 90
2.7.1 TISSUE CULTURE CELLS ........................................................... 90
2.7.2 TISSUE SECTIONS ...................................................................... 92
2.7.3 MICROSCOPY .............................................................................94
3. CHAPTER THREE - A TRANSGENIC APPROACH TO MODULATE PDGF ACTIVITY IN THE CNS ................................................................. 96

3.1 INTRODUCTION .......................................................................................................................... 97

3.2 RESULTS ......................................................................................................................................... 98
  3.2.1 Construction of in vitro expression vectors ........................................................................... 98
  3.2.2 Expression of pHYKamyce and pHYKAKDEL in Cos cells ................................................. 99
  3.2.3 Construction of PDGF-A transgene vectors ......................................................................... 105
  3.2.4 Results of oocyte injections .................................................................................................. 106
  3.2.5 PDGF expression in the normal developing CNS ................................................................. 108
  3.2.6 Expression of the transgenes ............................................................................................... 109
  3.2.7 Analysis of oligodendrocyte progenitor cell numbers in NSE-PDGF transgenic mice .......... 116

3.3 DISCUSSION .................................................................................................................................. 120
  3.3.1 Transgenic mice which over-express and inhibit PDGF in the nervous system .................. 120
  3.3.2 Over-expression of PDGF-A by neurons results in an increase in the number of oligodendrocyte progenitors ...................................................... 121
  3.3.3 Neuronal expression of the dominant negative PDGF isoform PDGF-AKDEL has no effect on the number of oligodendrocyte progenitors .................. 122

4. CHAPTER FOUR - CONTROL OF OLIGODENDROCYTE PROGENITOR CELL NUMBER BY COMPETITION FOR LIMITING AMOUNTS OF PDGF ................................................................................................................. 124

4.1 INTRODUCTION ............................................................................................................................ 125

4.2 RESULTS ......................................................................................................................................... 126
4.2.1 Oligodendrocyte lineage development depends on PDGF-AA, but not -AB or -BB ................................................................................................................................126
4.2.2 Proliferation of O-2A progenitors in the spinal cord ceases before birth .... 128
4.2.3 O-2A progenitor proliferation arrest reflects both a slowing of the cell cycle and an increase in cell death................................................................................................................................132
4.2.4 PDGF becomes limiting in the embryonic spinal cord and causes slowing of the O-2A progenitor cell cycle ....................................................................................139
4.2.5 O-2A progenitor cell number is proportional to the level of expression of PDGF-A........................................................................................................................141

4.3 DISCUSSION................................................................................................................................................147
4.3.1 PDGF-AA is necessary for proliferation of O-2A progenitors in vivo...... 147
4.3.2 O-2A progenitor proliferation and final cell number is controlled by the supply of PDGF ................................................................................................................................148
4.3.3 Why does the progenitor cell cycle slow down?......................................................149
4.3.4 Autocrine inhibition of O-2A progenitor proliferation......................................... 150
4.3.5 Decreasing cellular response to PDGF stimulation............................................. 152
4.3.6 Decreasing extra-cellular concentration or activity of PDGF ......................... 154
4.3.7 Competition for limiting mitogens in vivo - a calculation ......................... 155
4.3.8 Controls on cell cycle time are a general feature of embryonic development .................................................................................................................................................156

5. CHAPTER FIVE - OLIGODENDROCYTE NUMBER AND DISTRIBUTION IS CONTROLLED ENTIRELY BY SELECTIVE CELL SURVIVAL ..........................................................................................................................157

5.1 INTRODUCTION........................................................................................................................................158

5.2 RESULTS...................................................................................................................................................160
5.2.1 Overexpression of PDGF causes a sustained increase in steady-state numbers of O-2A progenitors.............................................................................................................. 160
5.2.2 Excessive and ectopic production of oligodendrocytes in NSE-PDGF-A transgenic mice ................................................................. 160
5.2.3 Elimination of excess oligodendrocytes by programmed cell death .......... 164
5.2.4 Superfluous oligodendrocytes are eliminated at a distinct, immature developmental stage ................................................................................... 167
5.2.5 Elimination of excess, ectopic, newly-formed oligodendrocytes is a normal developmental process ........................................................................ 169

5.3 DISCUSSION ......................................................................................................... 172
5.3.1 Overproduction of oligodendrocyte progenitors in NSE-PDGF-A transgenic mice ................................................................................................................................. 172
5.3.2 Secondary over-production of oligodendrocytes in NSE-PDGF-A spinal cords ................................................................................................. 172
5.3.3 Oligodendrocyte number and distribution is controlled by programmed cell death ................................................................................................. 173
5.3.4 Extending the clearance time of apoptotic cells displays the true extent and distribution of oligodendrocyte death during normal development .......... 175

6. CHAPTER SIX - GENERAL DISCUSSION ............................................................. 178
6.1 POPULATION DYNAMICS OF OLIGODENDROCYTE DEVELOPMENT: A MODEL .................................................................................... 179
6.1.1 Lineage specification .................................................................................. 179
6.1.2 Progenitor cell proliferation and migration ................................................ 180
6.1.3 Stochastic differentiation of oligodendrocyte progenitors ......................... 181
6.1.4 Abortive oligodendrocyte differentiation .................................................. 181
6.1.5 Long term oligodendrocyte survival and myelination ............................. 182

6.2 FURTHER EXPERIMENTS .................................................................................. 185

7. REFERENCES .............................................................................................................. 188
LIST OF FIGURES & TABLES

Figure 1.1 - Transverse sections through dorsal ectoderm/mesoderm of developing embryos during neurulation ................................................................. 22

Figure 1.2. Structure and binding specificities of PDGF and its receptors. ........... 30

Figure 1.3 Positive and negative regulators of G1 progression ......................... 43

Table 2.1 - Riboprobes used for RNase protection assays and in situ hybridization .. 82

Figure 3.1 - PDGF-A<sub>KDEL</sub> protein expressed in Cos cells is retained in the endoplasmic reticulum .............................................................. 102

Figure 3.2 - PDGF-A<sub>KDEL</sub> can form dimers with wild-type PDGF-A .............. 103

Figure 3.3 - PDGF-A<sub>KDEL</sub> acts as a dominant negative to sequester PDGF-A inside the cell ................................................................................. 104

Figure 3.4 - Structure of the NSE-PDGF-A transgenes .................................. 106

Figure 3.5 - Transgene copy numbers among the four independent NSE-PDGF-A lines generated ................................................................. 107

Figure 3.6 - PDGF-A transcripts in the developing mouse spinal cord .......... 110

Figure 3.7 - Expression of the human PDGF-A transgenes in the retina ............... 113

Figure 3.8 - Analysis of expression of NSE-PDGF-A transcripts in the spinal cord by RT-PCR ................................................................. 114

Figure 3.9 - Expression of PDGF-A in wild-type and NSE-PDGF-A transgenic spinal cords ................................................................. 115

Figure 3.10 - Numbers of oligodendrocyte progenitors are increased in the spinal cords of NSE-PDGF-A transgenic mice ........................................ 118

Figure 3.11 - Numbers of oligodendrocyte progenitors are not greatly affected in the spinal cords of NSE-PDGF-A<sub>KDEL</sub> transgenic mice ................. 119

Figure 4.1 - PDGF-A is required for normal proliferation of oligodendrocyte progenitors in the spinal cord ................................................................. 129

Figure 4.2 - Proliferation of oligodendrocyte progenitors in normal and transgenic spinal cords ceases after E15 ................................................................. 130
Figure 4.3 - Numbers of PDGFRα⁺ progenitors in sections of spinal cords of hemizygous transgenic NSE-PDGF-A mice and their wild-type littermates. .......... 131
Figure 4.4 - The progenitor cell division cycle slows down markedly before birth. ... 133
Figure 4.5 - The entire progenitor cell population is cycling as a single population... 134
Figure 4.6 - Labeling of a dividing cell population by BrdU incorporation........... 137

Figure 4.7 - Cumulative labeling of a dividing cell population by BrdU

Figure 4.8 - PDGF dose dependency of oligodendrocyte progenitor proliferation in vivo................................................................. 142

Figure 4.9 - Progenitor cell number is proportional to the PDGF supply in a range of transgenic backgrounds................................. 145
Figure 4.10 - Numbers of oligodendrocyte progenitors continue to rise in response to a step-wise increase in the supply of PDGF............... 146
Figure 4.11 - Two models to explain the slowing-down of the cell cycle. .......... 153

Figure 5.1 - Excessive and ectopic production of oligodendrocytes in NSE-PDGF-A spinal cords.......................................................... 162
Figure 5.2 - The number of oligodendrocytes surviving postnatally is independent of the number of oligodendrocyte progenitor cells............ 163
Figure 5.3 - Superfluous oligodendrocytes in NSE-PDGF-A transgenic spinal cords are eliminated by programmed cell death....................... 165
Figure 5.4 - Many more dying oligodendrocytes can be detected in NSE-PDGF-A spinal cords than in wild-type................................. 166
Figure 5.5 - Excess oligodendrocytes die at an early stage of differentiation......... 168
Figure 5.6 - Death of excess spinal cord oligodendrocytes is a normal developmental process................................................................. 171

Figure 6.1 - A tentative model of spinal cord oligodendrocyte development........ 184
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>APES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>(k)bp</td>
<td>(kilo)base pairs</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl-phosphate</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2′-deoxyuridine</td>
</tr>
<tr>
<td>cdk</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CNP</td>
<td>2′,3′-cyclic nucleotide 3′-phosphodiesterase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>(c)DNA</td>
<td>(complementary) deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle's-buffered salt solution</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GC</td>
<td>galactocerebroside</td>
</tr>
<tr>
<td>GGF</td>
<td>glial growth factor</td>
</tr>
<tr>
<td>GTC</td>
<td>guanidinium isothiocyanate</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>ISH</td>
<td>in situ hybridization</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>KDEL</td>
<td>amino acid sequence ‘lysine-aspartate-glutamate-leucine’</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NBT</td>
<td>nitroblue tetrazolium salt</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NSE</td>
<td>neuron-specific enolase</td>
</tr>
<tr>
<td>NT</td>
<td>neurotrophin</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleotide triphosphate</td>
</tr>
<tr>
<td>ODₙ</td>
<td>optical density at a wavelength of ‘n’ nm</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>programmed cell death</td>
</tr>
<tr>
<td>PDGF(Rα)</td>
<td>platelet-derived growth factor (alpha receptor)</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PLP</td>
<td>proteolipid protein</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl-methyl-sulphonyl fluoride</td>
</tr>
<tr>
<td>PNK</td>
<td>polynucleotide kinase</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPA</td>
<td>RNase protection assay</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Tₛ</td>
<td>length of S-phase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetraethylmethylenediamine</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TH</td>
<td>thyroid hormone</td>
</tr>
<tr>
<td>TRITC</td>
<td>Texas Red isothiocyanate</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial cell growth factor</td>
</tr>
<tr>
<td>VZ</td>
<td>ventricular zone</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
</tbody>
</table>
1. CHAPTER ONE
GENERAL INTRODUCTION
1.1 SIZE CONTROL IN ANIMAL DEVELOPMENT

Animal development can be summarized as the sum of the processes by which a single fertilized egg goes on to become the immensely complex collection of cells, tissues and organs of the mature animal. These processes, which proceed well into postnatal life in mammals, include division, migration, differentiation and survival of cells, all of which are controlled by a myriad of cell-intrinsic and cell-extrinsic signals.

Throughout animal development cells proliferate to form different populations and sub-populations that assemble with one another to form tissues and organs. Proliferation of these different cell populations is presumably interdependent, as numbers of different cell types must be matched to each other to create a functional unit. For example, in the adult human kidney there are approximately two million nephrons, which are the functional units that make up the kidney. Each nephron consists of about twelve different cell types, and contains a total of over ten thousand cells, so clearly the number of individual but interacting cell populations in a fully developed kidney is vast. Therefore the control of relative cell population sizes is crucial for the regulated development of a normal nephron, a normal kidney, or indeed any other organ or tissue in the body. In addition, the relative sizes of different organs, which depends upon the number of constituent cells, must be tightly regulated to ensure the correct functionality of the animal as a whole. Finally, not only relative cell numbers, but absolute numbers must be under strict genetic and environmental control, in order to endow each animal species with its own characteristic size.
This tight regulation of cell numbers is not only fundamentally important during the normal development of an animal, it must also be maintained over its entire adult life, as the majority of cells in the body are continually being turned over - cells are constantly dying and being replaced from a dividing progenitor population. In addition, during repair processes such as wound healing, numbers of cells in each depleted population must be replenished to their normal levels, at which point they must again cease proliferating.

Control can also be exerted at the levels of cell migration into or out of a tissue, cell differentiation, and selective cell survival or death - often known as programmed cell death (PCD) or apoptosis.

Selective PCD has been known for many years to be involved in specific cases of normal development, such as removal of the tadpole's tail during metamorphosis and mammalian digit formation, where fingers and toes are sculpted from initial limb 'paddles'; however the full extent to which PCD plays a role in development has for a long time been overlooked. A possible reason for this is that the clearance time for apoptotic (dying) cells is usually very short. It is now thought that by the time a cell is visibly undergoing apoptosis, in most instances it has already been engulfed by a neighbouring cell. Thus, although at any one instant the number of dying cells that can be detected in a developing tissue may be small, the real number of dying cells per unit time may in fact be much greater.
One system where the role of PCD in normal development has been studied in great detail is the neurons of the peripheral nervous system (PNS). These are initially produced in large excess with respect to their target cells, and then the superfluous neurons are subsequently removed by PCD as a result of competition for specific target-derived survival signals such as nerve growth factor (reviewed in Oppenheim, 1991). In addition, it is now known that oligodendrocytes in the developing optic nerve are produced in excess, and that the population is trimmed back by PCD, presumably as a result of competition for limiting survival factors from neurons (Barres et al., 1992a; Barres et al., 1992b). The control of cell number in the oligodendrocyte lineage is the subject of this Thesis.

In conclusion, the control of relative and absolute cell numbers is fundamental to the process of development, and is a complex process involving the interplay of division, migration, differentiation and death. In the remainder of this general introduction, I will first outline the process of vertebrate neural development, then I will introduce the oligodendrocyte lineage and its origins, and describe what is known about oligodendrocyte number control.

1.2 VERTEBRATE NEURAL DEVELOPMENT

The mature vertebrate nervous system consists of a bewildering array of structures and cell types, including a large number of sub-classes of neurons, various types of astrocytes, microglia, fibroblasts, epithelial cells and cells of the immune system. Some of these cells migrate in from elsewhere, but the majority develop from a specialized
unicellular sheet of primitive neuroectoderm, the neural plate, which is induced to become neuroectoderm by underlying mesodermal tissue (Mangold, 1933). The neural plate can consist of up to 50 percent of the total embryonic ectoderm, and initially has the potential to give rise to any neural cell type.

Subsequently, the neural plate starts to invaginate along its rostro-caudal axis at the midline to form the neural groove, while its edges thicken and lift up to form the neural folds. This process continues until the lateral neural folds come together and fuse, thus 'pinching off' what is now called the neural tube (Figure 1.1; reviewed in Schoenwolf and Smith, 1990). The cells immediately adjacent to the dorsal neural tube become neural crest cells, which migrate away from this region and give rise to neurons and glia of the PNS as well as pigment cells (melanocytes) in the skin, connective tissue in the face and many other cell types (Gilbert, 1994). At first the walls of the neural tube consist of a seemingly uniform neuroepithelium surrounding a fluid filled space (lumen) which will eventually give rise to the ventricles of the mature brain and the central canal of the spinal cord. Around this time a specialized rod of mesoderm lying just ventral to the neural tube - the notochord - induces the ventralmost cells in the neural tube to differentiate and form a structure known as the floor plate. This process is initiated by an extracellular signaling molecule, Sonic hedgehog; this and other molecules produced by the notochord, and later by the floor plate itself, are now known to be crucial for the correct dorso-ventral patterning of the nervous system and the specification of many neural cell types and sub-types (for review see Tanabe and Jessell, 1996).
Figure 1.1 - Transverse sections through dorsal ectoderm/mesoderm of developing embryos during neurulation.
From the neural plate stage onwards, the neuroepithelial cells begin to proliferate rapidly along the entire length of the neural axis, initially generating more neuroepithelial cells by symmetric division. At some time after neural tube closure proliferation of neuroepithelial cells slows down, as the cells begin to divide asymmetrically, each cell generating a replacement neuroepithelial cell which remains at the ventricular surface, and in addition a daughter cell that migrates away from the ventricular surface as either a post-mitotic (non-dividing) neuronal progenitor or, later in development, a glial progenitor (reviewed in Huttner and Brand, 1997). There is evidence that the switch from symmetric to asymmetric division depends on the re-orientation of the neuroepithelial cell cleavage plane (Chenn and McConnell, 1995).

Differential cell proliferation at the anterior end of the neural tube results in the formation of a series of swellings and boundaries which define the prosencephalon (forebrain vesicle), mesencephalon (midbrain vesicle) and rhombencephalon (hindbrain vesicle), while more posterior to this is the spinal cord. As these anterior structures continue to expand and fold, they begin to form recognizable structures such as the cerebral cortex (from the forebrain) and the cerebellum (from the hindbrain) (Cowan, 1978; Cowan, 1979). Morphogenesis of these various structures depends on localized proliferation of the neuroepithelium, implying regionalization, or patterning, of the neuroepithelial response to mitogens and/or survival factors. Hence, understanding the local control of cell proliferation will be essential before we can understand brain morphogenesis.
Although the vast majority of neurons are born long before birth, the process of vertebrate neural development proceeds well into post-natal (or post-hatch) life because many of the supporting cells which add functionality to this ‘neuronal scaffold’, such as astrocytes and oligodendrocytes, are not generated until much later. I now turn my attention to oligodendrocytes, which are the focus of this Thesis, and outline their function and origin.

1.3 THE OLIGODENDROCYTE LINEAGE

1.3.1 OLIGODENDROCYTES MYELINATE CNS AXONS

Oligodendrocytes are the myelinating cells of the central nervous system (CNS); they are the functional equivalent of Schwann cells in the peripheral nervous system. At present, their only known function is to ensheathe CNS axons, by wrapping layer upon layer of their own plasma membrane around the axons, then tightly compacting these layers to form the characteristic electron-dense “myelin sheath”. The myelin sheath is contiguous with the plasma membrane of the myelin-forming cell, and even after it is fully formed the sheath remains in metabolic equilibrium with the rest of the cell. Thus the myelin sheath can be viewed as a specialized organelle unique to oligodendrocytes and Schwann cells, in the same way as axons and dendrites can be regarded as specialized organelles unique to neurons.

Myelin is not laid down continuously along axons, but in relatively short segments each connected individually to the oligodendrocyte cell body via a thin cytoplasmic process.
The gaps between adjacent segments are known as “nodes of Ranvier” and the myelin segments themselves as “internodes”. The myelin sheath allows fast axonal transmission of action potentials by saltatory conduction, where the action potential effectively ‘jumps’ across internodes from one node to the next, facilitated by the efficient insulating properties of myelin and clustering of ion channels at the nodes. Not only does this enable faster transmission, it also conserves metabolic energy and space, as for a given speed of transmission, a myelinated fibre need only be a fraction of the diameter of an unmyelinated fibre.

The major difference between oligodendrocytes and their PNS counterparts, the Schwann cells, is in the stoichiometry of myelinating cells to axons. In the PNS, one Schwann cell will only contribute to a single internode, whereas in the CNS an oligodendrocyte is able to form sheaths around twenty or more axons (Butt and Ransom, 1989). It therefore requires less oligodendrocytes than Schwann cells to myelinate the same number of axons, and this further space saving might be a driving force behind the evolutionary transition from Schwann cell-like myelination, thought to be the phylogenetically older process, to myelination by oligodendrocytes.

1.3.2 OLIGODENDROCYTES DEVELOP FROM BIPOTENTIAL PROGENITOR CELLS IN VITRO

Proliferating cells which can differentiate into oligodendrocytes in culture were first isolated from rat optic nerve by Raff, Miller and Noble (1983) who coined the name "O-2A progenitors". This was in view of the fact that these cells could give rise to two
entirely different glial cell types, depending on the culture conditions. When cultured in defined medium and low serum (≤0.5% fetal calf serum, FCS), O-2A progenitors rapidly differentiate into post-mitotic oligodendrocytes, whereas in the presence of 10% FCS they stop dividing and become type-2 astrocytes, these cell types being defined by their different morphologies and antigenic phenotypes (Raff et al., 1983). Type-2 astrocytes are also generated when O-2A progenitors are cultured in ciliary neurotrophic factor (CNTF) in the presence of cortical astrocyte-derived extracellular matrix factors (Lillien and Raff, 1990). Oligodendrocyte differentiation seems to be the default pathway, as it occurs in defined medium when O-2A progenitors are cultured as single cells (Temple and Raff, 1985). Type-2 astrocytes have so far not been identified in vivo (Fulton et al., 1991) so they might represent an in vitro artifact; nevertheless in this Thesis I retain the term 'O-2A progenitor' for simplicity and to acknowledge the possibility that type-2 astrocytes might one day be identified in some part of the CNS, or in some pathological or physiological situation.

Optic nerve O-2A progenitors are not generated within the nerve itself, but migrate in from the brain through the optic chiasm starting around embryonic day 15 to 18 (E15-18), reaching the retinal end of the nerve a few days later. The first myelinating oligodendrocytes appear around the day of birth (E21 in the rat) (Mudhar et al., 1993; Small et al., 1987) and continue to accumulate during the first postnatal month or so (Vaughn, 1969). If embryonic optic nerves are dissociated and cultured in defined medium, the O-2A progenitors rapidly stop dividing and differentiate into oligodendrocytes; however if they are cultured on a layer of cortical astrocytes or in astrocyte-conditioned medium they continue to divide and differentiation is delayed until the in vitro equivalent of the day of birth, when they would first have appeared in vivo (Noble and Murray, 1984; Raff et al., 1985). This suggests that the timely
development of oligodendrocytes in vivo is controlled by mitogens for O-2A progenitor cells. A lot of work has therefore been devoted to identifying mitogens for these cells in vitro and in vivo. This work is described in the next section.

1.3.3 MITOGENS FOR O-2A PROGENITORS

When purified O-2A progenitors are cultured in defined medium containing no growth factors, either singly in microwells (Temple and Raff, 1985) or as a monolayer (Barres et al., 1992b), they stop dividing and differentiate, suggesting that they require diffusible signals from other cell types in order to proliferate. What are the natures and identities of these signals?

Several polypeptide growth factors have been shown to promote DNA synthesis in O-2A progenitors in vitro: PDGF (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988), FGF-2 (bFGF) (Bögl er et al., 1990; McKinnon et al., 1990), neurotrophin-3 (NT-3, Barres et al., 1993b, 1994b), insulin-like growth factor-1 (IGF-1, McMorris and Dubois-Dalcq, 1988) and glial growth factor-2 (GGF-2/neuregulin, Canoll et al., 1996). Although none of these factors is capable of independently causing continued proliferation of purified O-2A progenitors in vitro, it has been shown that with a combination of PDGF, NT-3 and IGF-1, the pattern of proliferation and differentiation of purified optic nerve O-2A progenitors closely resembles that of cells in the developing nerve (Barres et al., 1994b). This also seems to be the case for the combination of PDGF, CNTF and high insulin (Barres et al., 1996). Therefore it seems likely that O-2A progenitors, at least in vitro, require multiple growth factors in order to proliferate to their full extent.
Nevertheless, there is now a large body of evidence that PDGF plays a major role during embryonic development. Before discussing this evidence, I review briefly the structure/function relationships of PDGF and PDGF receptors.

### 1.3.4 PDGF AND ITS RECEPTORS: STRUCTURE AND POSSIBLE ROLES IN VIVO

As the name suggests, platelet-derived growth factor, or PDGF, was discovered as a mitogen for fibroblasts which is secreted by blood platelets at the site of tissue injury; it is, in fact, the major mitogenic component of blood serum (Ross et al., 1974). It was also discovered independently as a mitogen for glial cells in culture (Heldin et al., 1977) and has subsequently been implicated not only in normal physiological roles, such as wound healing (reviewed in Pierce et al., 1991) and normal development of the lung (Boström et al., 1996; Lindahl et al., 1997b), retina (Fruttiger et al., 1996), kidney and vascular system (Leveén et al., 1994; Lindahl et al., 1997a, 1998), but also in the course of a number of disease processes, such as atherosclerosis, neoplasia and bone marrow fibrosis (reviewed in Ross et al., 1990). When it was purified, PDGF was found to be a 30KDa secreted dimeric glycoprotein, held together by intrachain disulphide bonds (Antoniades, 1981; Deuel et al., 1981; Heldin et al., 1979, 1981; Raines and Ross, 1982). The monomers are either A or B isoforms, which are coded for by separate but related genes (Antoniades and Hunkapiller, 1983; Waterfield et al., 1983); the PDGF-B chain gene turned out to be the oncogene c-sis, confirming a role for PDGF in oncogenesis (Johnsson et al., 1984; Waterfield et al., 1983). Therefore there are three possible forms of PDGF (AA, AB, or BB; Figure 1.2), and all these occur in nature. For
example porcine platelets predominantly secrete PDGF-BB, human platelets mainly make PDGF-AB, and a number of human gliomas have been shown to express PDGF-AA (for review of PDGF biology see Ross et al., 1986).

PDGF exerts its action by binding to and cross-linking cell surface receptors (for review see Heldin and Westermark, 1989). There are two isoforms of PDGF receptors, PDGFRα and PDGFRβ, again coded for by separate but related genes. They consist of an extracellular ligand-binding region, containing five immunoglobulin-like domains, a single membrane-spanning region, and an intracellular region containing a split tyrosine kinase domain (Figure 1.2); thus the PDGF receptors are in the same receptor family as c-Kit (the Steel factor receptor) and the receptor for vascular endothelial cell growth factor (VEGF), Flk-1. The three receptors PDGFRα, c-Kit and Flk-1 are also clustered in tandem on chromosome 5 in the mouse (Brunkow et al., 1995), and PDGFRβ, c-fms and Flt-4 are clustered on chromosome 11, suggesting a common origin through gene duplication. In a prototypic way for this family, PDGF receptors are stimulated when a PDGF dimer binds two PDGF receptor subunits at the cell surface, causing activation of the intracellular tyrosine kinase domains, receptor auto-phosphorylation and interaction of a number of downstream effector proteins, usually resulting in cell division (for review of tyrosine kinase receptors, see Ullrich and Schlessinger, 1990).

PDGFRα and PDGFRβ have different affinities for the two isomers of PDGF. PDGFRα will bind both PDGF-A and PDGF-B, whereas PDGFRβ will only bind PDGF-B (Hart et al., 1988). Therefore cells expressing only PDGFRα can respond to
Figure 1.2 - Structure and binding specificities of PDGF and its receptors.
all three forms of PDGF dimers, whereas cells expressing only PDGFRβ are only responsive to PDGF-BB dimers (Figure 1.2; for review of PDGF and its receptors see Heldin and Westermark, 1989; Williams, 1989). It was shown a number of years ago that O-2A progenitors express PDGFRα but not PDGFRβ (Hart et al., 1989b; Pringle et al., 1989), and can therefore respond to all three forms of PDGF in vitro (Pringle et al., 1989).

1.3.5 PDGF IN OLIGODENDROCYTE LINEAGE DEVELOPMENT: A CHRONOLOGY

There is now a large body of evidence both from in vitro and in vivo studies that PDGF plays a central role in oligodendrocyte development. A chronology of the main lines of evidence is given on the following page.
- 1983 O-2A progenitors first described (Raff et al., 1983)
- 1984 Mitogenic activity for O-2A progenitors present in astrocyte conditioned medium (ACM) (Noble and Murray, 1984)
- 1985 Astrocyte-derived mitogen is able to recapitulate the in vivo timing of O-2A progenitor differentiation (Raff et al., 1985)
- 1988 PDGF is a mitogen for O-2A progenitors in vitro, astrocyte-derived mitogen is PDGF, probably the PDGF-AA isoform, and PDGF-A transcripts are present in the developing CNS. PDGF is able to recapitulate the in vivo timing of O-2A progenitor differentiation. (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988)
- 1989 In situ hybridization demonstrates presence of PDGF-A mRNA in optic nerve cells (presumably astrocytes) (Pringle et al., 1989)
- 1989 O-2A progenitors in vitro express α-receptors for PDGF (Hart et al., 1989b)
- 1989 Cerebellar interneurons in vitro secrete a mitogen and survival factor for O-2A progenitors which can neutralized by anti-PDGF antibodies (Levine, 1989)
- 1991 In situ hybridization demonstrates expression of PDGF-A in vivo by neurons and presumptive astrocytes (Yeh et al., 1991)
- 1991 PDGF is chemotactic for O-2A progenitors (Armstrong et al., 1991)
- 1992 PDGF-A is a survival factor for O-2A progenitors and newly-formed oligodendrocytes in vitro. Exogenous supply of PDGF-A to the post-natal rat optic nerve greatly enhances the survival of oligodendrocyte lineage cells (Barres et al., 1992a)
- 1996 Evidence for a single PDGFRα+ oligodendrocyte lineage in spinal cord (Hall et al., 1996)
- 1998/9 Genetic proof from PDGF knockout mice that PDGF-AA is crucial for normal oligodendrocyte lineage development (Calver et al., 1998; Fruttiger et al., 1999; this Thesis)

There is therefore much circumstantial evidence both in vitro and in vivo which was pointing to a role for PDGF in the development of the oligodendrocyte lineage,
culminating in direct genetic evidence, some of which is presented in this Thesis, that PDGF is necessary for normal oligodendrocyte development in vivo.

Not only is PDGF-AA important for O-2A lineage development, but the robust expression of PDGFRα mRNA and protein by O-2A progenitors provides us with a convenient molecular marker for these cells, allowing us to visualize and study their development in vivo.

1.3.6 A VENTRAL ORIGIN FOR THE SPINAL CORD OLIGODENDROCYTE LINEAGE

O-2A progenitors are found in other parts of the CNS in addition to the optic nerve, such as cerebellum, cerebral cortex and spinal cord. Using PDGFRα as an in situ hybridization marker for O-2A progenitors, Pringle et al. (1992) presented evidence that during late neurogenesis in the rat CNS (from E16 onwards), PDGFRα mRNA seemed only to be expressed by cells of the oligodendrocyte lineage, despite its widespread expression outside the nervous system (Orr-Urtreger et al., 1992; Orr-Urtreger and Lonai, 1992; Pringle et al., 1992). Subsequently it was discovered that, in the rat spinal cord at E14, PDGFRα mRNA is restricted to two narrow longitudinal ribbons of neuroepithelial cells in the ventricular zone (VZ) on either side of the central canal, in the ventral part of the cord near the floor plate (Pringle and Richardson, 1993). These ribbons, which initially are only a few cells wide, extend from the caudal spinal cord to the midbrain, and suggested a possible origin of oligodendrocyte lineage cells in the CNS. As the spinal cord develops, these foci disappear as PDGFRα⁺ cells appear to
proliferate and migrate away from the VZ to populate the entire spinal cord by around E18 in the rat. This focal origin of presumptive oligodendrocyte progenitors in the ventral neural tube was consistent with earlier work by Warf et al (1991). These workers dissociated ventral and dorsal halves of E14 spinal cord and cultured them separately; they found that only the ventral cultures subsequently gave rise to oligodendrocytes. When a similar experiment was repeated with E16 spinal cords, oligodendrocytes were generated in both ventral and dorsal cultures. Similar results have since been confirmed several times with chicken spinal cord at equivalent developmental stages (Pringle et al., 1998; Pringle et al., 1996; Trousse et al., 1995). These results suggest that spinal cord oligodendrocytes may originate from this focal region of PDGFRα+ cells in the ventral part of the cord.

The idea of a focal ventral source of oligodendrocyte progenitors is supported by in situ labeling studies using four other putative markers of the oligodendrocyte lineage, 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP; Yu et al., 1994), antigens recognized by monoclonal antibody O4 (Ono et al., 1995), NG2 proteoglycan (Nishiyama et al., 1996) and the myelin proteolipid protein (PLP/DM-20; Peyron et al., 1997; Timsit et al., 1995), all of which recognize a discrete microdomain of cells in the ventral spinal cord at similar developmental stages. In addition, the results obtained with PDGFRα in the rat have since been reproduced in the chicken (Pringle et al., 1996), Xenopus (N. Pringle, unpublished), human (Hajihosseini et al., 1996) and mouse (Pringle et al., 1996) spinal cords.
Confirmation that spinal cord oligodendrocytes arise from ventrally-derived progenitor cells, and that these cells express PDGFRα in vivo, came from studies which confirmed and extended the observations of Warf et al. (1991, see above). When PDGFRα⁺ cells were immunoselected from E17 spinal cords, all these cells gave rise to oligodendrocytes when cultured in defined medium without PDGF. In addition, when A2B5⁺ cells (specific for O-2A progenitors in vitro), which overlapped almost completely with PDGFRα⁺, were removed from suspensions of dissociated E17 spinal cord by antibody-mediated complement lysis, oligodendrocytes were not generated in these cultures (Hall et al., 1996). In another study, Pringle et al. (1998) used the chick-quail chimera system (Le Douarin, 1993) and chick-quail co-culture to show both in vivo and in vitro that oligodendrocytes are generated from neuroepithelial precursors in the ventral, but not the dorsal half of the spinal cord.

In conclusion, the evidence is overwhelming that all PDGFRα⁺ cells in the pre- and perinatal spinal cord are oligodendrocyte precursors, and all spinal cord oligodendrocytes are derived from PDGFRα⁺ precursor cells.

It is important to note here that all the work described above has been carried out using the spinal cord as a model for CNS development. It is quite possible that the brain has a more complicated process of oligodendrocyte development, involving one or more separate lineages (Peyron et al., 1997; Timsit et al., 1995). Also, there are reports that PDGFRα is expressed by a subset of mature spinal cord neurons in the postnatal (Nait-Oumesmar et al., 1997) and adult (Vignais et al., 1995) mouse, although other groups
have failed to confirm this in rodent brain (Butt et al., 1997; Ellison and de Vellis, 1994; Nishiyama et al., 1996). We do not understand the reason for this discrepancy; in any case it does not affect the conclusions of the work in this Thesis.

1.4 Oligodendrocyte Population Control

1.4.1 Control of O-2A Progenitor Proliferation

1.4.1.1 In vivo mitogens for O-2A progenitors

In vivo, no factor apart from PDGF (this Thesis) has been shown to be crucial for the normal proliferation of the oligodendrocyte lineage, although exogenous delivery of antibodies against NT-3 into the developing CNS was reported to cause a decrease in the number of O-2A progenitors in the optic nerve (Barres et al., 1994b). In addition, the number of mitotic O-2A progenitors is reduced in the optic nerves of CNTF knockout mice at P14, and this number can be increased to wild-type levels by supplying exogenous recombinant CNTF (Barres et al., 1996). IGF-1 knockout mice exhibit a reduction in oligodendrocyte numbers (Beck et al., 1995; Liu et al., 1993) and, conversely, oligodendrocyte numbers are increased in mice which ubiquitously over-express IGF-1 (Ye et al., 1995); however in both these cases there is a corresponding decrease or increase in axon numbers, and therefore an indirect effect is possible. There is also no evidence in any of these cases to indicate a direct effect on cell division as opposed to cell survival. A great deal more in vivo information will be gained in future from other transgenic approaches, an example of which is provided by this Thesis.
1.4.1.2 Neuronal control of O-2A progenitor proliferation

There is in vitro evidence to suggest that neurons provide mitogens for O-2A progenitors in vivo (reviewed in Hardy and Reynolds, 1993). Transection of the rat P8 optic nerve, with or without subsequent degeneration (Barres and Raff, 1993; David et al., 1984; Fulcrand and Privat, 1977), causes a 90% decrease in mitotic figures in the nerve 4 days later. This effect can be reproduced by the injection of tetrodotoxin (TTX) into the eye, which silences the sodium channels of the retinal ganglion neurons, suggesting that electrical activity of optic nerve axons is important for progenitor cell proliferation (Barres and Raff, 1993). Providing exogenous PDGF overcame the TTX-induced inhibition of mitosis, suggesting that neuronal activity somehow is required for release of PDGF or other mitogens into the nerve. It is possible that electrical activity induces release of PDGF from the axons themselves, or from astrocytes within the nerve.

1.4.1.3 Inhibitors of O-2A progenitor proliferation

It is also known that extracellular growth inhibitors exist in vivo that antagonize the effects of mitogenic signals on proliferating cells. Mutations in the recently identified myostatin gene, a transforming growth factor-β (TGF-β) homologue expressed in differentiated muscle, result in hypertrophy and hyperplasia of muscle cells in both mice (McPherron et al., 1997) and cattle (Grobet et al., 1997; McPherron and Lee, 1997), although this is an effect of increased cell size as well as number, and it is not known whether this is a direct or indirect effect. It is known that TGF-β family members are expressed by oligodendrocytes in vitro and can inhibit the proliferative
effects of PDGF on O-2A progenitors (McKinnon et al., 1993). This suggests that feedback inhibition of proliferation might play a role in O-2A progenitor growth control. However, such a role has yet to be demonstrated in vivo.

It has also been shown both in primary culture (Gallo et al., 1996) and cerebellar tissue slices (Yuan et al., 1998) that non-NMDA glutamate receptor agonists can inhibit both proliferation and differentiation of O-2A progenitors, seemingly through the specific blockage of voltage-gated K⁺ channels. Glutamate is known to be released by axons in white matter (Kriegler and Chiu, 1993; Weinreich and Hammerschlag, 1975; Wheeler et al., 1966), so it could have a role in the timing of oligodendrocyte development in vivo.

The response of cells to extracellular proliferative signals must ultimately be regulated by intracellular effectors, such as elements of the cell cycle control mechanism. It is known, for example, that the relative intracellular levels of cyclin-dependent kinase (cdk) inhibitors can adjust the sensitivity of cells to extracellular mitogens (Coats et al., 1996; Fero et al., 1996), and O-2A progenitors purified from mice with a targeted disruption in the cdk inhibitor p27Kip1 (p27) exhibit a greater response to the mitogenic effect of PDGF than wild-type cells (Durand et al., 1998). The possible roles of cyclins, cdk's and cdk inhibitors in oligodendrocyte development is discussed in a later section.
1.4.2 CONTROL OF O-2A PROGENITOR DIFFERENTIATION

The mechanisms by which cells in vivo decide when to stop dividing and differentiate are still largely unknown, although this occurs in many vertebrate cell lineages on a strict developmental time course. In this section I review what is known about the control of O-2A progenitor cell differentiation, which has been studied in vitro for a number of years, mainly with cells derived from the rat optic nerve.

1.4.2.1 A cell-intrinsic clock controls O-2A progenitor differentiation in vitro

When embryonic optic nerves are cultured in defined medium containing a low (≤0.5%) concentration of FCS, O-2A progenitors rapidly drop out of division and differentiate into oligodendrocytes (Raff et al., 1983). If PDGF-AA is included in these cultures, O-2A progenitors initially proliferate; however on the equivalent of the day of birth they begin to differentiate, irrespective of the age at which the optic nerves were dissociated (Raff et al., 1988). Adding excess PDGF cannot prevent this differentiation (Raff et al., 1988), nor is it caused by a loss of functional PDGF receptors (Hart et al., 1989a; Hart et al., 1989b; Hart et al., 1992). This is also the case if single O-2A progenitors are cultured on a monolayer of astrocytes (Temple and Raff, 1986), or if purified O-2A progenitors are cultured in defined medium containing PDGF, NT-3 and IGF-1 (Barres et al., 1994b). In addition, if a single isolated progenitor is allowed to divide once in a microwell, and the two daughter cells are separated and cultured on their own, the progeny of these two cells tend to divide the same number of times before
differentiating (Temple and Raff, 1986). These results suggest the presence of a cell intrinsic ‘clock’ which counts either time or cell divisions, limiting the duration of cell proliferation before differentiation to a maximum of about 8 divisions in optic nerve progenitors, although since the progenitors are born elsewhere in the brain and migrate into the nerve (Small et al., 1987), they will presumably have undergone an unknown number of divisions before entering the nerve. Some recent evidence suggests that O-2A progenitors may in fact count time, not number of divisions, because when they are cultured at 33°C instead of 37°C, the cells divide more slowly but differentiate after fewer divisions (Gao et al., 1997).

1.4.2.2 The ‘counting’ mechanism of the clock is independent of the ‘effector’ mechanism

There is evidence to suggest that the ‘clock’ consists of two components; a counting mechanism presumably driven by extracellular mitogens and possibly involving elements of the cell cycle control machinery (see below), and an effector mechanism which causes the cells to differentiate. If O-2A progenitors are cultured in defined medium in the presence of both PDGF and FGF-2 (bFGF), differentiation is inhibited, but when FGF-2 is subsequently removed the cells rapidly differentiate into oligodendrocytes (Bogler et al., 1990; McKinnon et al., 1990), and the same has been shown for O-2A progenitors cultured in GGF-2/neuregulin and low serum (Canoll et al., 1996). It has been suggested that the effector mechanism is dependent on hydrophobic signaling molecules such as thyroid hormone (TH) or retinoic acid (Ahlgren et al., 1997; Barres et al., 1994a; Gao et al., 1998); however others have reported that such
molecules may only enhance the probability of oligodendrocyte differentiation in vitro and in vivo (Ibarrola et al., 1996).

The counting aspect of the cell intrinsic clock, which appears to be independent of the effector mechanism, is presumably dependent on the extracellular mitogens that drive proliferation by interacting with the intracellular cell cycle machinery. Therefore before I discuss what is known about the counting mechanism in O-2A progenitors, I will briefly discuss cell cycle regulation, and its involvement in the control of cell differentiation.

1.4.2.3 Cell cycle regulation by cyclins, cdks and cdk inhibitors

Control of the mammalian cell cycle by growth factors is exerted mainly during a short period in G1 known as the ‘restriction point’, equivalent to START in yeast, after which DNA replication (S phase) and cell division (M phase) occur independently of mitogenic stimulation (Pardee, 1989). To a large extent, this control is governed by the G1 cyclins and their associated kinases (cdks) (Pines, 1993; Sherr, 1993; Sherr, 1994) and cdk inhibitors of the p21cip/p27kip family (el-Deiry et al., 1993; Harper et al., 1993; Polyak et al., 1994; Toyoshima and Hunter, 1994) and the Ink4 family (Serrano et al., 1993).

As cells enter G1 phase, there is sequential synthesis of cell-specific combinations of the cyclin D family, and subsequently cyclin E. These associate with cdk4/6 and cdk2 respectively, and these kinase holoenzymes are then activated by phosphorylation with
a cdk-activating kinase (CAK). The active holoenzymes now sequentially phosphorylate the retinoblastoma tumour-suppressor protein (pRb), and this phosphorylation in turn causes the dissociation of pRb from its bound transcription factors such as E2F, and subsequent activation of S phase specific genes and DNA replication (Figure 1.3; for review see Sherr and Roberts, 1995).

These processes clearly need to be controlled, as without a ‘braking’ mechanism cell proliferation would continue unchecked. Most steps in these pathways have now been shown to be governed, at least in part, by members of the G1 cdk inhibitor families such as p21^{Cip} (p21), p27^{Kip} (p27) and Ink4. Thus the Ink4 proteins compete with the cyclin D family for binding to cdk4/6, and also inhibit the phosphorylation of pRb by the cyclin D/cdk4 enzyme complex. The p21/p27 family of proteins, in addition to inhibiting the phosphorylation of pRb by cyclin D/cdk4 and cyclin E/cdk2, also inhibit the initial activation of the cyclin/cdk complexes by CAK-mediated phosphorylation (Figure 1.3; for review see Sherr and Roberts, 1995). Therefore progression through the cell cycle can be controlled by regulating the stoichiometry of cell cycle activators to cell cycle inhibitors.
Figure 1.3 - Positive and negative regulators of G1 progression. The length of G1 is indicated by the arrow at the bottom with the restriction point indicated. The question mark represents other possible cyclin E/cdk2 substrates involved in S phase transition. Adapted from Sherr and Roberts (1995).
1.4.2.4 Regulation of proliferation and differentiation by cdk inhibitors

There is now accumulating in vivo evidence to suggest that cyclins/cdkks and cdk inhibitors are important for regulating progenitor cell proliferation and the timing of differentiation. For example in the Drosophila epidermis, cyclin E levels control progression through S phase, and its down-regulation is necessary and sufficient to limit progenitor cell proliferation (Knoblich et al., 1994). Epidermal precursor cells from mutants in the Drosophila p21/p27 homologue dacapo undergo an extra cell division during development, resulting in an increase in the final epidermal cell density, although differentiation does occur normally after this extra cycle (de Nooij et al., 1996; Lane et al., 1996). In the mouse, the expression pattern of p21 correlates with terminal differentiation of multiple cell lineages including skeletal muscle, cartilage, skin, and olfactory neurons (Parker et al., 1995), and over-expression of p21 in the developing liver cause a dramatic inhibition of hepatocyte proliferation and a correspondingly runted liver (Wu et al., 1996). Finally, mice carrying a targeted deletion in the p27 gene are larger than wild-type littermates as a result of at least a 20% hyperplasia in multiple organs; this seems to be a non-systemic, cell-intrinsic mechanism as serum levels of growth hormone and IGF-1 are normal, consistent with a role for p27 in limiting progenitor cell proliferation in vivo (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996).

1.4.2.5 Control of O-2A progenitor differentiation by cell cycle regulators

Could the timing of differentiation of O-2A progenitors be in some way controlled by relative levels of positive and negative intracellular regulators of the cell cycle?
Consistent with this idea is the observation that cyclin D1 and cdc2 are down-regulated in post-mitotic oligodendrocytes, and that levels of p27 steadily rise as O-2A progenitors divide in vitro, remaining high in post-mitotic oligodendrocytes (Durand et al., 1997). In the absence of TH, or in the presence of both PDGF and FGF-2 (bFGF), p27 levels continue to rise even though differentiation is inhibited (Durand et al., 1997), again consistent with previous findings that the 'clock' still runs in the absence of differentiation (Barres et al., 1994a; Ibarrola et al., 1996), and demonstrating that the presence of p27 on its own is not sufficient to cause cell cycle withdrawal.

Two subsequent studies have shown that O-2A progenitors derived from p27 'knockout' mice have a greater proliferative capacity in vitro than their wild-type counterparts, going through on average one to two more divisions before differentiation (Casaccia-Bonnefil et al. 1997; Durand et al. 1998). When deprived of mitogens, however, optic nerve progenitors still differentiate normally, indicating that p27 is not necessary for the differentiation process. The fact that O-2A progenitors from p27−/− mice do differentiate, albeit belatedly, is consistent with the results described above from the dacapo flies and the p27−/− mice, indicating that the cell cycle exit and differentiation pathways use multiple and possibly redundant components.

Taken together, these results suggest that levels of cdk inhibitors and/or cyclin/cdks may have a role in the control of O-2A progenitor differentiation, at least in vitro. Various growth factors and serum have been shown to influence intracellular levels of cyclin D, cdk4 and Ink4 (Matsushime et al., 1991; Sherr and Roberts, 1995);
conversely, the differential expression of these cell cycle control genes is likely to affect the sensitivity of cells to stimulation by extracellular growth factors (Sherr and Roberts, 1995). Therefore the levels of intracellular regulators might change with time in a cell-autonomous fashion, or their levels might be controlled by changing environmental factors, or a combination of both.

1.4.3 CONTROL OF O-2A PROGENITOR AND OLIGODENDROCYTE SURVIVAL

1.4.3.1 Programmed cell death in normal development

Normal programmed cell death (PCD) is known to be an integral part of animal development, and has been observed for many years in most cell lineages (Glucksmann, 1951). The tissue in which it has been most widely studied is the developing vertebrate nervous system, particularly neurons. In both the CNS and PNS, neurons are over-produced with respect to their target tissues, such as muscle cells for example. These target tissues supply survival, or trophic, factors to the innervating neurons, but only in limiting quantities. Therefore there is competition for these limited supplies of trophic signals, and neurons which do not receive sufficient signaling will undergo PCD and be removed. This achieves two goals: first, the number of neurons is matched to the number of their respective target cells and second, neurons which make inappropriate projections can be removed as they will receive inappropriate survival signals (for reviews see Barde, 1989; Cowan et al., 1984; Levi-Montalcini, 1987; Oppenheim, 1991; Purves et al., 1988).
1.4.3.2 PCD occurs during development of the oligodendrocyte lineage

PCD is now known to occur in the oligodendrocyte lineage in vivo; in fact 50% of all optic nerve oligodendrocytes are removed by PCD soon after they differentiate from O-2A progenitors (Barres et al., 1992a). When O-2A progenitors or oligodendrocytes are cultured as single cells in defined medium, they rapidly die by PCD (Barres et al., 1992a; Gard and Pfeiffer, 1993), and the same is true for immunopurified monolayers of O-2A progenitors or oligodendrocytes (Barres et al., 1992a), suggesting that continuous paracrine signaling from other cell types is necessary for oligodendrocyte lineage cells to survive. This is supported by the observations in vitro that these cells can be saved, at least in the short term, by conditioned medium from either type-1 astrocyte cultures or optic nerve cultures from which O-2A lineage cells have been removed (Barres et al., 1992b). So what are the signals required for oligodendrocyte lineage cell survival, both in vitro and in vivo?

1.4.3.3 In vitro survival factors for O-2A progenitors

Immunopurified optic nerve O-2A progenitors in defined medium can be kept alive in the short-term by PDGF or IGF-1 (or its relatives insulin and IGF-2), and the insulin-like factors can also act as a short-term survival signals for oligodendrocytes. PDGF receptors are rapidly down-regulated when O-2A progenitors differentiate (Hart et al., 1989b; McKinnon et al., 1990), so although PDGF is a survival factor for newly formed oligodendrocytes, they soon become unresponsive to its actions (Barres et al., 1992a).
It has also been shown that NT-3 (but not NGF or BDNF) and CNTF (or its homologues LIF and IL-6) can support short-term survival of oligodendrocyte lineage cells in vitro, although any of these factors on their own (including the insulin-like factors) can only support survival of one half to two thirds of oligodendrocytes after 3 days in culture, and survival rates rapidly fall beyond this time (Barres et al., 1993b). However, together these survival factors are much more potent, to the extent that a combination of IGF-1, NT-3 and CNTF can promote survival of up to 95% of purified oligodendrocytes in culture for 2 weeks or longer (Barres et al., 1993b). GGF-2/neuregulin, expressed by both neurons (Canoll et al., 1996) and oligodendrocytes (Raabe et al., 1997) in culture, also enhances survival of O-2A progenitors in vitro, although in this study cell survival was only followed for 12-24 hours after serum withdrawal (Canoll et al., 1996). Finally, an unidentified LIF-related protein present in astrocyte-conditioned medium is able to support long-term survival of oligodendrocytes in vitro in the presence of insulin (Gard et al., 1995).

1.4.3.4 Inhibition of oligodendrocyte lineage PCD in vivo

Death of newly-formed optic nerve oligodendrocytes can be inhibited, at least in the short term, by exogenous delivery of PDGF (Barres et al., 1992a), IGF-1 (Barres et al., 1993a), CNTF (Barres et al., 1993a) or NT-3 (B. Barres, personal communication). This suggests that all these factors are present in limiting quantities for oligodendrocyte survival during optic nerve development, and that survival is dependent on competition for these factors, as is the case for neurotrophins and neurons. This is circumstantially supported by the finding that brain size, brain DNA and amounts of CNS myelin are
increased in transgenic mice which over-express IGF-1 (Carson et al., 1993; Ye et al., 1995), although this may be a proliferative or indirect effect. The effect of continuous over-expression of any of these factors on long-term survival of oligodendrocytes in vivo has yet to be addressed in detail; I will return to this later in my Thesis with respect to PDGF.

1.4.3.5 Axonal control of oligodendrocyte lineage survival

The only known function of oligodendrocytes is to myelinate axons in the CNS, and so their numbers need to be critically matched to the numbers of axons, in the same way that numbers of neurons are matched to their targets. Intact axons are known to be required for O-2A progenitor division. Axons are also required for survival of differentiated oligodendrocytes. When postnatal rat optic nerves are transected behind the eye, causing the RGC axons to degenerate, virtually all oligodendrocyte lineage cells in the nerve are lost by PCD (Barres et al., 1993a; David et al., 1984; Fulcrand and Privat, 1977). This death does not occur in transected nerves from mutant animals whose axons do not degenerate after transection (Ola mice), suggesting that the survival signal is contact-mediated, nor does death occur in intact nerves after intraocular injection of TTX, indicating that the survival signal, unlike the proliferative signal, is independent of axonal electrical activity (Barres et al., 1993a).

A tentative model for oligodendrocyte survival and axon number-matching has therefore been proposed, such that when an O-2A progenitor differentiates, it loses its dependence on certain survival factors, such as PDGF, and becomes dependent on a
trophic activity associated with axons. It therefore has a limited time to find an unmyelinated stretch of axon and ensheath it, and if it is unsuccessful its death program will be activated (Barres and Raff, 1994). Another line of evidence which supports this is that optic nerve oligodendrocyte death is reduced in bcl-2 transgenic mice whose optic nerves contain 80% more axons than wild-type nerves (Burne et al., 1996).

1.5 SUMMARY AND AIMS OF THESIS

In this chapter I have introduced the concept and relevance of size control in animal development, a concept which despite its fundamental importance has, until recently, received little attention. The oligodendrocyte lineage is an ideal system in which to study these processes, as these cells can be easily purified and cultured under conditions promoting either growth or differentiation, and molecular markers are now available for all stages of lineage progression, both in vitro and in vivo. Since a number of putative factors have now been identified from in vitro studies of proliferation, differentiation and survival, this system lends itself to a transgenic approach, where in vivo levels of these molecules can be genetically increased or decreased to ascertain their function and importance in oligodendrocyte population size control.

In this Thesis I have used a transgenic approach to address the role of PDGF in O-2A progenitor cell proliferation and oligodendrocyte survival. I have generated mice which over-express PDGF-A in their nervous system using a neuron-specific promoter, and assessed the effects of this on the population dynamics of both the progenitor cells and their post-mitotic, differentiated progeny, the oligodendrocytes. I have also looked at
mice in which the PDGF-A gene has been disrupted by homologous recombination (PDGF-A knockout mice), to determine whether the functions ascribed to PDGF by in vitro experimentation are relevant to the in vivo development of the oligodendrocyte lineage.

The results obtained from these studies, along with the body of previous work which I have summarized in this introduction, have enabled us to construct a tentative model of the population dynamics of oligodendrocyte development.
2. CHAPTER TWO

MATERIALS AND METHODS
General chemicals and reagents were purchased from Sigma-Aldrich Co Ltd unless otherwise stated, and were Molecular Biology grade where available.

Restriction and DNA/RNA modifying enzymes were all purchased from New England Biolabs (UK) Ltd unless otherwise stated.

Bacterial media components were obtained from Difco Laboratories Ltd, or from the in-house facility.

Sterilization of solutions, where necessary and possible, was performed by autoclaving at 15lb/sq.in. for 15 minutes. Heat-labile solutions were sterilized by filtration through a 0.22µm filter (Millipore). All solutions were stored at room temperature unless otherwise stated.

Bacteriology/molecular biology techniques were based essentially on protocols in Sambrook et al. (1989) unless otherwise stated.

Falcon sterile plasticware was used from Marathon Ltd unless otherwise stated.

2.1 BACTERIOLOGY

2.1.1 BACTERIAL STRAINS, GROWTH AND STORAGE

All general cloning and sub-cloning was carried out using *Escherichia coli* (E. Coli) strain XLI-Blue (*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F' proAB, lacF'ZAM15, Tn10 (TetR*)]). Bacteria were grown at 37°C in Luria Broth (LB, 10g bacteriostone + 5g yeast extract + 10g NaCl per litre) or on LB-agar plates containing
LB+15g/l bacto-agar. If selecting with ampicillin, this was added to the LB or molten LB-agar (after cooling to 55°C) at a final concentration of 50-100µg/ml (100mg/ml stock in H₂O, 0.22µm filter-sterilized and stored in aliquots at -20°C). Liquid cultures were continually agitated in a rotating environmental shaker at 300 rpm.

For long term storage of bacterial strains and clones, glycerol was added to overnight cultures at a final concentration of 15% (v/v), and these stocks were stored in 1ml aliquots at -20°C.

2.1.2 PREPARATION AND TRANSFORMATION OF COMPETENT BACTERIA

E. Coli strain XL1-Blue were made electro-competent, stored and transformed with plasmid DNA as follows. 1ml of an overnight liquid culture of bacteria (grown from a single colony) was used to inoculate 1l of LB. This culture was incubated until it was in mid-log growth phase (an OD₂₆₀ of ~0.6), then chilled on ice and pelleted by centrifugation (3000g, 4°C, 10 minutes). The bacterial pellet was gently resuspended in 1l of ice-cold H₂O, pelleted as before, resuspended in 500ml ice-cold H₂O and pelleted again. This pellet was then resuspended in 20ml ice-cold 10% (v/v) sterile glycerol, centrifuged as before, and finally resuspended in 2.5ml ice-cold 10% (v/v) sterile glycerol. This was stored in 100µl aliquots under liquid nitrogen after snap freezing in liquid nitrogen. The efficiency of competent bacteria produced in this way was normally >10⁸ colonies/µg plasmid DNA.
For transformation of the bacteria aliquots were thawed slowly on ice, and 20μl was transferred to a chilled disposable electroporation cuvette (Bio-Rad Laboratories Ltd) containing ≤2μl of the DNA to be transformed. This was then pulsed using a Gene Pulser electroporator equipped with a Pulse Controller (Bio-Rad Laboratories Ltd) set to 2500V, 200Ω, 25μF. This resulted in a time constant of about 4.7 seconds. The pulsed bacteria were added to 100μl LB and incubated at 37°C for 20 minutes before plating on LB-agar-ampicillin plates.

When cloning into vectors where the multiple cloning site formed part of the β-galactosidase gene, a blue/white colour screen could be used to pick colonies containing vectors with inserts. In this case each plate was spread with 100μl of 1.25% (w/v) X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)/10mM IPTG (Isopropyl β-D-thiogalactopyranoside). The X-gal stock was 2.5% (w/v) in dimethyl formamide; the IPTG stock was 0.1M in H₂O; both were stored at -20°C. Colonies containing religated vector are blue under these conditions, whereas colonies containing vectors with inserts remain white.
2.2 MOLECULAR BIOLOGY

2.2.1 EXTRACTION OF DNA WITH PHENOL/CHLOROFORM/ISO-AMYL ALCOHOL

Samples to be extracted were made 0.25M with respect to NaCl, and an equal volume of 25:24:1 (v/v) phenol/chloroform/iso-amyl alcohol was added. The phenol stock was buffered with Tris-HCl pH 7.5 (Aquaphenol, Appligene Ltd) and stored in aliquots at -20°C. The sample was vortexed and centrifuged at 10000g for 5 minutes. The upper aqueous phase was collected and re-extracted with an equal volume of 24:1 (v/v) chloroform/iso-amyl alcohol; vortexed, centrifuged and collected as before.

2.2.2 PRECIPITATION OF DNA WITH ETHANOL OR ISOPROPANOL

Samples to be precipitated were made 0.25M with respect to NaCl, and 2.5 volumes of ethanol (or 0.6 volumes of isopropanol) were added. The samples were vortexed, incubated at -20°C for 20 minutes and then centrifuged at 10000g for 10 minutes at 4°C to precipitate the DNA. If necessary (i.e. less than 1-2 μg or a low concentration of DNA) 10μg of glycogen (from mussels, molecular biology grade, Boehringer Mannheim) was added to the DNA as a carrier before the precipitation. The DNA pellet was washed with 500μl 70% (v/v) ethanol, centrifuged briefly at 10000g, air dried and resuspended in an appropriate volume of 10mM Tris-HCl pH 8, 1mM EDTA (TE).
2.2.3 AGAROSE GEL ELECTROPHORESIS AND GEL PURIFICATION OF DNA

SeaKem GTG agarose (Flowgen) was used for all agarose gel electrophoresis, at 0.8-2% (w/v) depending on the molecular weight of the DNA of interest. The agarose was dissolved in the appropriate volume of 1xTAE (20xTAE: - 0.8M Tris-acetate pH 7.5, 20mM EDTA) by boiling in a microwave oven, cooled to <60°C, poured into a suitable size Horizon electrophoresis system (Life Technologies Ltd) and left to set. Loading buffer was added to the DNA samples (10x stock: - 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% (w/v) Ficoll (Type 400, Pharmacia)), the gel loaded and run at 5V/cm. The gel was post-stained by soaking in 10μg/ml ethidium bromide for 15 minutes, the DNA visualized on an ultraviolet transilluminator at 302nm and recorded using a CCD camera and thermal printer.

In order to purify DNA bands from agarose gels, the Geneclean II kit (Bio 101) was used. Briefly, the band was cut out of the gel using a sterile razor blade and 3 volumes of 6M sodium iodide was added, assuming a gel density of 1g/ml. This was incubated at 55°C for 5 minutes, or until the gel had melted, then chilled on ice. 5μl of the supplied silica matrix was then added, the suspension vortexed and incubated on ice for 5 minutes. After pelleting in a microcentrifuge, the matrix with bound DNA was washed 3 times in the supplied wash buffer, then resuspended in an appropriate volume of TE. The DNA was eluted from the matrix at 55°C for 5 minutes, then the matrix was again pelleted and the DNA-containing supernatant recovered.
2.2.4 DNA-MODIFYING ENZYME REACTIONS

All DNA-modifying enzyme reactions (restriction, ligation, filling-in recessed ends, removing overhanging ends, de-phosphorylation and phosphorylation) were carried out as described in the enzyme manufacturers protocols (New England Biolabs Ltd) in reaction buffers supplied by the manufacturers. DNA concentration for these reactions was approximately 0.1mg/ml, with the exception of ligations, in which case it was approximately 0.01mg/ml or greater.

2.2.5 DENATURING POLYACRYLAMIDE (SEQUENCING) GEL ELECTROPHORESIS OF DNA/RNA

Sequencing gels were prepared using ready-to-use 6% sequencing gel solution (Sequagel-6, National Diagnostics). For example, for 25ml of gel solution the following reagents were mixed together:

- 20ml Sequagel-6 concentrate
- 5ml 5x Sequagel-6 buffer
- 300μl 10% (w/v) ammonium persulphate

This was quickly poured into an assembled 0.8mm vertical gel apparatus (Model V16, Life Technologies Ltd) and allowed to polymerize for one hour. After denaturing at 75°C for 5 minutes, samples were loaded onto the gel and run at ~20V/cm in 1xTBE
running buffer (10xTBE: 0.9M Tris-borate, 20mM EDTA). After running the gel for the required length of time, the gel plates were separated and (unless the gel was being used for probe purification, see 2.2.6 below) the gel was fixed by soaking in 10% (v/v) methanol/10% (v/v) acetic acid for 30 minutes. The gel was then dried onto 3MM chromatography paper (Whatman) on a commercial gel dryer at 80°C for 1 hour. In order to visualize radiolabeled nucleic acids on the dried gel, it was exposed to autoradiographic film (Hyperfilm-MP, Amersham) in a cassette for the appropriate length of time either at room temperature or at -80°C with intensifying screens (Hyperscreen, Amersham).

2.2.6 PURIFICATION OF ³²P-LABELED RNA PROBES ('RIBOPROBES') FROM SEQUENCING GELS

Riboprobes (see section 2.6.4 below) were run on sequencing gels as described above. However, after running, the gel was exposed to autoradiographic film for 30 seconds without fixing or drying, in order to visualize the full-length transcript. This band was then excised from the gel with a razor blade, and incubated, with shaking, in 400μl elution buffer (2M ammonium acetate, 1% SDS, 25μg/ml tRNA) for 2-4 hours at 37°C. The eluted probe was then removed from the gel slice and precipitated with 1ml ethanol. The resultant pellet was resuspended in 50μl hybridization buffer (80% [v/v] deionized formamide, 40mM PIPES buffer, pH 6.4, 0.4M NaCl, 1mM EDTA) and counted as follows. 1μl of probe was spotted onto filter paper, dried and counted in the ³²P channel.
of a scintillation counter thus giving the 'counts per minute per microlitre' (cpm/µl) of the riboprobe

2.2.7 SMALL SCALE PREPARATION OF PLASMID DNA BY ALKALINE LYSIS('MINIPREPS')

Bacterial colonies to be screened were picked using sterile Gilson pipette tips, which were then ejected into sterile 25ml Universal tubes containing 5ml LB+ampicillin (see 2.1.1 above). Cultures were grown at 37° overnight in a rotary shaker, then 1.5ml was pelleted at 13000rpm for 30 seconds in a microfuge. After removal of the supernatant, the bacterial pellet was resuspended in 100µl of ice-cold 'DNA prep solution 1' (50mM glucose, 25mM Tris-HCl pH 8, 10mM EDTA) and incubated on ice for 5 minutes. 200µl of freshly prepared 'DNA prep solution 2' (1% SDS, 0.2N NaOH) was added and mixed by several gentle inversions of the tube before another 5 minute incubation on ice. 150µl of 'DNA prep solution 3' (3M potassium acetate, 2M acetic acid) was then added and mixed by shaking, the tube again incubated on ice for 5 minutes, and then spun at 13000rpm for 5 minutes in a microfuge. The supernatant was carefully removed and added to 1ml ethanol at -20°C, mixed and then spun at 13000rpm for 1 minute in order to precipitate the plasmid DNA. The resultant pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in 100µl dH₂O, from which 10µl was used per 20µl restriction enzyme digest for diagnostic minipreps.
2.2.8 SCREENING BACTERIAL COLONIES BY FILTER HYBRIDIZATION

('COLONY LIFTS')

Colonies were picked with sterile Gilson tips and streaked first onto a gridded Hybond-N+ membrane (Amersham) laid on a 10cm plastic petri dish containing LB-agar+ampicillin (see 2.1.1 above; the 'test' plate), then replica streaked onto an identically gridded LB-agar+ampicillin 'master' plate without a membrane. Both plates were incubated at 37°C overnight in order for the colonies to grow; the master plate was then sealed with Parafilm and stored at 4°C. The membrane was removed from the test plate using forceps and placed on filter paper soaked in 10% (w/v) SDS for 5 minutes. The membrane was then placed on filter paper soaked in 1.5M NaCl, 0.5N NaOH for 5 minutes, then filter paper soaked in 1.5M NaCl, 50mM Tris-HCl pH 8 for 5 minutes, then rinsed in 2xSSC to remove bacterial debris. After baking for 30 minutes at 80°C in order to fix the DNA to the membrane, it was hybridized with a random-primer labeled DNA probe corresponding to the insert of interest and exposed to autoradiographic film. This was done essentially as for Southern blotting (see sections 2.5.5 and 2.5.6 below), except that the hybridization time was reduced to 2-4 hours, and the exposure time was reduced to 1-2 hours at room temperature. Positive colonies thus identified were then recovered from the 'master' plate.
2.2.9 LARGE-SCALE PREPARATION OF PLASMID DNA BY CESIUM CHLORIDE EQUILIBRIUM CENTRIFUGATION ('MAXIPREPS')

Single bacterial colonies were inoculated into 2ml LB+ampicillin and grown at 37°C in a rotary shaker for 4-6 hours or until in mid-logarithmic growth phase. The growing culture was then added to 250ml LB+ampicillin in a 1 litre flask and grown overnight at 37°C in a rotary shaker. The bacteria were chilled on ice, pelleted at 2500rpm at 4°C, washed by resuspension in 100ml ice-cold STE (0.1M NaCl, 10mM Tris-HCl pH 8, 1mM EDTA) and pelleted again by centrifugation. The pellet was then resuspended in 7ml ‘Solution 1’ (see 2.2.7 above) containing 5mg/ml lysozyme, then 14ml freshly prepared ‘Solution 2’ (see 2.2.7 above) was added and mixed by gentle inversion. After incubation at room temperature for 10 minutes, 7ml ‘Solution 3’ (see 2.2.7 above) was added and mixed first by gentle inversion and then by shaking. After another 10 minute incubation on ice, the solution was centrifuged at 6000rpm (no braking) for 15 minutes and the supernatant recovered. 0.6 volumes isopropanol was added and the solution was centrifuged at 4000rpm for 15 minutes at 4°C. The pellet was resuspended in 6ml TE, then 2ml 10M ammonium acetate was added and the solution was incubated on ice for 20 minutes in order to precipitate proteins. This was then centrifuged at 10000rpm for 10 minutes at 4°C and the supernatant recovered. 16ml ethanol was added and after incubating on ice for 5 minutes the solution was centrifuged at 10000rpm for 10 minutes at 4°C. The pellet was resuspended in 4ml TE, 0.3M sodium acetate and precipitated as before with 8ml ethanol. The resultant DNA/RNA pellet was dried thoroughly in a Savant SpeedVac (~5 minutes), resuspended in 1ml TE containing 1.05g cesium chloride and transferred to a Quick-seal tube (Beckman). 80μl ethidium bromide solution
(10mg/ml) was added, then the tube was filled with 'topping solution' (stock made as follows; 50ml TE, 52.5g CsCl, 4ml ethidium bromide solution (10mg/ml); 0.2μm filtered before use) and sealed. The tube was centrifuged at 100000rpm in a bench-top ultracentrifuge at room temperature for a minimum of 4 hours with no braking. The lower, supercoiled plasmid DNA band (visible as a red band under normal lighting) was collected through the side of the tube using a 2ml syringe and 21-gauge needle and the ethidium bromide removed by repeated extractions with equal volumes of water-saturated 1-butanol (10ml 1-butanol, 0.6ml dH2O) until the (lower) aqueous phase appeared completely clear. The final aqueous phase was diluted to 4 times the original band volume with TE, precipitated with 2 volumes of ethanol and centrifuged at 10000rpm for 15 minutes at 4°C. The pellet was resuspended in 500μl TE, 0.3M sodium acetate and re-precipitated with ethanol. After 2 washes in 70% (v/v) ethanol, the plasmid DNA pellet was resuspended in 500μl TE. The concentration and purity of the plasmid DNA was measured by diluting a sample of the ‘maxiprep’ 1:500 in dH2O and determining its optical density at 260nm and 280nm (OD260, OD280). The concentration can be calculated as [OD260 x 500 (dilution factor) x 50 (DNA multiplication factor)] μg/ml, and the purity assessed by the ratio OD260/OD280, which for pure DNA is 1.7. Lower values indicated contamination of the maxiprep with protein and/or cesium chloride; in which case re-extraction was necessary.
2.3 MAMMALIAN CELL CULTURE

All media, antibiotics and fetal calf serum was obtained from Life Technologies Ltd. Phosphate-buffered saline (PBS) was obtained from the in-house facility, and contained (per litre) 8g NaCl, 0.2g KCl, 0.1g MgCl₂, 1.15g Na₂HPO₄, 0.2g KH₂PO₄. All cells were maintained in culture at 37°C in 5% CO₂/95% air and at 100% humidity.

2.3.1 CULTURE OF COS CELLS

COS-7 is an African green monkey kidney cell line (CV-1) which has been transformed with the SV40 virus and thus constitutively expresses the SV40 large-T antigen. It is an adherent cell line and was cultured in Dulbecco’s Modified Eagle’s medium (DMEM) + 10% FCS. Upon reaching confluence, the cells were passaged (‘split’) by replacing the medium with an equal volume of 0.01% (w/v) trypsin/0.02% (w/v) EDTA, incubating at 37°C for a few minutes until the cells became detached, and then centrifuging the cell suspension at 200g for 5 minutes in order to pellet the cells. The cell pellet was then resuspended in fresh growth medium and replated at low density (5-10% confluent). For storage of frozen stocks, the cells were resuspended after centrifugation at 10⁶ cells/ml in 10% (v/v) dimethyl sulphoxide/90% (v/v) FCS, slowly frozen (0.5°C/minute) to -70°C in cryotubes, then snap frozen and stored in liquid nitrogen.
2.3.2 TRANSFECTION OF COS CELLS

Cells were grown to 70% confluence in 60mm dishes, then washed twice with DMEM before transfection. A total of 3μg of the DNA to be transfected (including "empty" vector DNA as carrier if necessary) in 500μl of diethylaminoethyl (DEAE)-dextran at 1mg/ml in TBS (50mM Tris-HCl pH 8.0, 150mM NaCl) was mixed with 1.5ml of DMEM and incubated on the cells for 30 minutes at 37°C. This was then replaced with 5ml chloroquine solution (7-chloro-4-[4-diethylamino-1-methyl-butylamino]-quinoline, diphosphate salt; 85μg/ml in DMEM), and the incubation continued at 37°C for 3 hours. The chloroquine solution was then replaced with 4ml DMEM+10% FCS and the cells left to recover under normal growth conditions for 24 hours before metabolic labeling (see section 2.3.3 below). Transfection efficiency was determined by placing a couple of small (13mm) cover slips in the 60mm dishes before initially plating the cells. These were then removed prior to metabolic labeling and immunostained to detect the cells expressing the transfected gene (see section 2.7.1.1 below), and these were counted as a proportion of the total cells.

2.3.3 RADIOACTIVE LABELING OF COS CELL PROTEINS

Transfected COS cells (see section 2.3.2 above) were gently washed twice in 5ml cysteine-free DMEM, then incubated for 24 hours under normal growth conditions in 2ml cysteine-free DMEM+10% DMEM containing 50μCi/ml L-[35S]cysteine (10mCi/ml, >1000Ci/mmol, Amersham). The cell supernatant was collected, after cell debris had been removed by centrifugation, and stored at 4°C with 2mM PMSF.
(phenylmethyl-sulphonyl fluoride, 100mM stock solution at -20°C in ethanol) and 0.02% sodium azide to prevent protease activity and bacterial growth respectively. Adherent cells were then washed in PBS and lysed by incubating at 4°C for 15 minutes in 2ml isotonic lysis buffer (150mM NaCl, 10mM Tris-HCl pH 7.5, 0.5% (v/v) Nonidet P-40, 1mM EDTA). The lysate was then collected and stored in the same way as the supernatant.

2.4 PROTEIN ANALYSIS

2.4.1 IMMUNOPRECIPITATION

All incubations were carried out at 4°C for 1 hour on a rotating wheel. 35S-cysteine-labeled Cos cell supernatants and lysates (see 2.3.3 above) were pre-cleared by incubating with 2μl/ml normal rabbit serum. 50μl Immunoprecipitin (10% formalin-fixed, heat-killed Staphylococcus aureus: Life Technologies) was then added and incubated as described. This was precipitated by centrifugation at 13000rpm for 5 minutes, then the supernatant was removed and anti-myc monoclonal antibody 9E10 (mouse IgG1a ascites; (Evan et al., 1985)) was added at 1μl/ml. This was then incubated as described before adding 50μl Immunoprecipitin and incubating again. Proteins conjugated to Immunoprecipitin via the 9E10 antibody were precipitated by centrifugation at 13000rpm for 5 minutes then washed four times by resuspension in 500μl wash buffer (100mM NaCl, 10mM Tris-HCl pH 7.5, 0.05% (v/v) Nonidet P-40, 1mM EDTA) followed by one wash in TE. The pellet was resuspended in 20μl protein sample buffer (62.5mM Tris-HCl pH 6.8, 2% [w/v] SDS, 10% [v/v] glycerol, 0.05%
[w/v] bromophenol blue) and stored at -20°C. If proteins were to be reduced, the sample buffer contained 5% (v/v) 2-mercaptoethanol. Just before SDS-PAGE, samples were vortexed, incubated at 95°C for 3 minutes, the Immunoprecipitin removed by centrifugation at 13000rpm for 5 minutes and the supernatant loaded onto the gel.

2.4.2 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was performed as described previously (Laemmli, 1970) using a Mini-Protean vertical gel system (Bio-Rad). 100μl ammonium persulphate (APS; 10% [w/v]) and 8μl N, N, N’, N’-tetramethylethylenediamine (TEMED) was added to 10ml resolving gel solution (375mM Tris-HCl pH 8.8, 0.1% [w/v] SDS, 0.05% [w/v] bis-acrylamide, 15% [w/v] acrylamide), the resolving gel poured, overlaid with water-saturated butanol, and left to polymerize. The butanol was removed, the resolving gel washed with dH₂O and overlaid with 2.5ml stacking gel solution (125mM Tris-HCl pH 6.8, 0.1% [w/v] SDS, 0.13% [w/v] bis-acrylamide, 5% [w/v] acrylamide) containing 25μl APS and 3μl TEMED, and an appropriate comb inserted. The stacking gel was left to polymerize, the samples loaded and the gel electrophoresed in 1x running buffer (25mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% [w/v] SDS) at 100V through the stack and then 200V until the dye front reached the bottom of the gel.
2.4.3 GEL FLUOROGRAPHY

$^{35}$S-emissions from radioactive SDS-polyacrylamide gels were enhanced by fluorography as described previously (Bonner and Laskey, 1974). Gels were soaked in dimethylsulphoxide (DMSO; 20x gel volume) for 30 minutes, then in 20% (w/v) 2,5-Diphenyloxazole in DMSO (PPO; 4x gel volume) for 1 hour. The PPO was then precipitated within the gel by soaking in dH$_2$O for 15 minutes, the gel dried onto 3MM paper at 80°C under vacuum on a commercial gel drier and exposed to Hyperfilm-MP (Amersham) at -80°C using intensifying screens.

2.5 TRANSGENIC MICE PRODUCTION AND SCREENING

2.5.1 TRANSGENE PLASMID CONSTRUCTION

Construction of the transgene-containing plasmids is described in Chapter 3.

2.5.2 PURIFICATION OF TRANSGENE DNA

Plasmids were initially purified by equilibrium centrifugation in cesium chloride-ethidium bromide gradients as described in section 2.2.9 above. The transgene was released from the plasmid backbone by restriction enzyme digestion with EcoRI and electrophoresed on a 1% (w/v) agarose gel; the transgene band was then excised with a sterile razor blade and the DNA purified using the Geneclean-II kit (see section 2.2.3 above). This gel purification procedure was then repeated before a final purification of the DNA over an Elutip-d affinity column (Schleicher & Schuell). After ethanol
precipitation and two washes with 70% ethanol, the DNA was resuspended in sterile HiPerSolv water (Merck) at a concentration of 100μg/ml (accurately measured by comparison with standards on an ethidium bromide-stained agarose gel) and stored at 4°C.

2.5.3 PRODUCTION OF TRANSGENIC MICE

Transgenic mice were generated commercially at the Transgenic Unit, St Thomas’s Hospital, London according to established methods of pronuclear injection of purified DNA and re-implantation of fertilized oocytes (Hogan et al., 1994). 5mm tail clips were taken from possible founder animals at 1-2 weeks old; the genomic DNA was purified and analyzed for integration of the transgene as described below.

2.5.4 ISOLATION OF GENOMIC DNA

Tail-clips were incubated at 55°C overnight in 600μl ‘tail buffer’ (50mM Tris-HCl pH 8, 100mM EDTA, 100mM sodium chloride, 1% (w/v) SDS) containing 100μg/ml proteinase K (Sigma). Ammonium acetate was added to a final concentration of 1.5M, the sample chilled on ice and centrifuged at 13000g in a microfuge to precipitate proteins. Genomic DNA in the supernatant was precipitated with 0.6 volumes of cold isopropanol and washed with 70% ethanol. The DNA pellet was dissolved in 200μl dH₂O overnight at 4°C, and stored at 4°C.
2.5.5 SOUTHERN BLOT ANALYSIS OF GENOMIC DNA

Genomic DNA samples (10µg) were digested overnight with *Bam*HI restriction enzyme and electrophoresed on TAE-agarose gels. After staining with ethidium bromide and photographing, the gel was partially depurinated for 10 minutes in 0.2N HCl, then soaked for 20 minutes in 0.4N NaOH, and vacuum-blotted for 1 hr in 0.4N NaOH onto Zetaprobe nylon membrane (BioRad). The 'blot' was neutralized in 2xSSC/0.2M Tris-HCl pH 7.4 for 30 seconds, baked at 80°C for 30 minutes and pre-hybridized at 65°C for 30 minutes in hybridization buffer (1.5xSSPE, 10% [w/v] PEG-8000, 7% [w/v] SDS, 100µg/ml denatured, sonicated salmon sperm DNA; 20xSSPE:- 3M NaCl, 0.2M NaH₂PO₄ pH 7.4, 20mM EDTA) in a Hybaid Maxi Oven. The denatured probe (see below) was then added and hybridization continued overnight at 65°C. The blot was then washed in 2xSSC,0.1% (w/v) SDS at room temperature for 10 minutes, then in 0.1xSSC, 0.1% SDS at 65°C for 30 minutes, before being exposed to Hyperfilm-MP (Amersham) overnight at -70°C with intensifying screens.

2.5.6 RANDOM-PRIMER LABELING OF DNA PROBES

The probe used to identify transgenic animals was a full-length human PDGF-A cDNA (Betsholtz et al., 1986) radiolabeled using random primer labeling (Feinberg and Vogelstein, 1984). Briefly, the template DNA was gel purified as described in section 2.2.3 above; 25ng of this DNA was heated to 95°C for 5 minutes in 32µl dH₂O, chilled on ice and the following reagents added in this order:-
10μl 5x labeling buffer (250mM Tris-HCl pH 8, 25mM MgCl₂, 10mM dithiothreitol (DTT), 1M HEPES pH 6.6, 26 OD₂₆₀ units/ml random nonadeoxyribonucleotides)

2μl 25x dNTPs (500μM each of dATP, dTTP, dGTP)

5μl [α-³²P]dCTP (10mCi/ml, ~3000Ci/mmole, Amersham)

1μl Klenow fragment

The reaction was incubated at room temperature for 2-4 hours, then passed over a Micro Bio-Spin 30 column (BioRad) to remove unincorporated radionucleotides, denatured by heating to 95°C for 5 minutes and used immediately.

2.6 ANALYSIS OF TRANSGENIC MICE

2.6.1 STAGING OF EMBRYONIC MICE

Male transgenic mice were caged with wild-type females (C57Bl/6J X CBA/Ca F1 progeny) and the females were checked every morning for vaginal plugs. Fertilization was considered to have occurred at midnight on the night before discovery of the plug. Thus the day of discovery of the plug was termed E₀ (embryonic day zero). Pregnant females were killed by terminal anaesthesia in CO₂ and cervical dislocation at the age required, the embryos removed under sterile conditions and then accurately staged by inspection as described previously (Theiller, 1972).
2.6.2 AVOIDANCE OF RNASE CONTAMINATION

Since the techniques for analyzing mRNA depend on the transcripts being reasonably intact it was imperative that all solutions and equipment used were RNase free. This was achieved by baking all glassware at 200°C, using fresh, sterile plasticware, and treating all solutions with 0.1% (v/v) DEPC before autoclaving. Solutions which could not be DEPC-treated (e.g. Tris) were made up using fresh chemicals in DEPC-treated water. Gel tanks and equipment were soaked in 3% (v/v) H₂O₂ for 30 minutes and then rinsed in DEPC-treated water before use.

2.6.3 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

2.6.3.1 RNA isolation

Total tissue RNA was prepared by an adapted guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). Tissue was freshly dissected and homogenized in GTC solution (4M guanidinium isothiocyanate, 25mM sodium citrate pH 7, 0.5% (v/v) sarkosyl, 0.72% (v/v) 2-mercaptoethanol), using 1ml GTC solution per 100mg of tissue in a suitably sized Dounce homogenizer. 0.1 volumes of 2M sodium acetate pH 4, one volume of phenol and 0.5 volumes of chloroform/isoamyl alcohol (49:1 (v/v)) were then added, shaking the sample vigorously between each addition, and the sample was then incubated on ice for 15 minutes. After centrifugation at 10000rpm for 20 minutes, the upper aqueous phase was removed, mixed with an equal volume of isopropanol, and precipitated on dry ice for 30 minutes. The sample was centrifuged for 20 minutes at
10000rpm and the resulting pellet resuspended in 500μl of GTC solution. This was again phenol extracted, isopropanol precipitated, resuspended in a suitable volume of DEPC-treated water, and incubated with RNase-free DNase (300U/ml; Pharmacia) for 15 minutes at 37°C. After another phenol extraction, a chloroform/isoamyl alcohol extraction and an ethanol precipitation, the pellet was washed in 70% ethanol, air-dried and resuspended in DEPC-treated water. The RNA was stored at -70°C before use.

2.6.3.2 cDNA synthesis

First strand cDNA synthesis was carried out using the Superscript Preamplification kit (Life Technologies). Briefly, 2μg total RNA was incubated at 70°C for 10 minutes with 50ng random hexamer primers in dH2O to 12μl. This was then chilled on ice while the reaction mix was made up:

(per reaction, in this order)

2μl 10x PCR buffer (200mM Tris-HCl pH 8.4, 500mM KCl)
2μl 25mM MgCl2
1μl 10mM each dATP, dGTP, dCTP, dTTP
2μl 0.1M DTT

7μl of reaction mixture was added to each RNA/primer mixture and the reactions incubated at room temperature for 5 minutes. 1μl (200 units) Superscript II reverse transcriptase (RT; Life Technologies) was added to each reaction, and after a 10 minute incubation at room temperature the reactions were continued at 42°C for 50 minutes. The reactions were terminated at 70°C for 15 minutes, chilled on ice, then incubated
with 1μl E. Coli RNase H (2 units/μl; Life Technologies) at 37°C for 20 minutes. 2μl aliquots were stored at -70°C for single use in the PCR, as single-strand cDNA is not resistant to freeze-thawing.

2.6.3.3 Polymerase chain reaction (PCR)

Primers were chosen against regions of identical sequence between mouse and human PDGF-A, in order to amplify both with equal efficiency. They were also chosen to span both the site of insertion of the myc epitope tag in the transgene and the alternatively spliced sixth exon in the mouse cDNA, in order that the PCR products from the human PDGF-A transgene, the mouse PDGF-A short form and the mouse PDGF-A long form could be distinguished from each other by size.

The primers used were:-

Upstream 5'GTCCAGGTGAGGTTAGAGGA3'
Downstream 5'TCACGGAGGAGAACAAAGAC3'

Preliminary PCR using these primers indicated that the optimal Mg$^{2+}$ concentration in the reaction was 3mM. The PCR was set up in the following order:

22μl dH$_2$O
5μl 10x PCR buffer (500mM KCl, 100mM Tris-HCl pH 8.3, 1% (v/v) Triton X-100)
5μl of each primer (100ng/μl)
4μl dNTPs (dATP, dTTP, dCTP, dGTP, each at 2mM pH 7.5)

3mM MgCl₂

2μl cDNA (1/10 of synthesis reaction)

Negative controls were always set up simultaneously either with no cDNA or substituting untranscribed total RNA for the cDNA.

The reactions were then heated to 95°C for 5 minutes, 0.2μl of Taq DNA polymerase (5 units/μl; Promega) was added, they were overlayed with 50μl of paraffin oil and 30 of the following cycles were carried out: 95°C for 30 seconds, 55°C for 2 minutes, 72°C for 2 minutes. Another 0.2μl of Taq polymerase was then added and a further 10 cycles carried out, with the final cycle being: 95°C for 30 seconds, 55°C for 2 minutes, 72°C for 10 minutes. The reactions were then chloroform extracted to remove the oil and ethanol precipitated using 10μg of glycogen as a carrier.

2.6.3.4 Detection of PCR products

The reactions were run on a 2% agarose gel and Southern blotted onto Zetaprobe membrane, before being probed with either an oligonucleotide against the myc epitope tag to specifically display the transgene product, or degenerate oligonucleotides against human and mouse PDGF-A to display both transgenic and endogenous PDGF-A. Another probe used was against the alternatively spliced long form of mouse PDGF-A.

The sequences of the probes used were:
Myc epitope tag:

5’CCTGAGCAAAAGCTCATTTCTGAAGAGGACTTGCTCGAGTAGCC3’

Mouse/human PDGF-A:

5’AAGCACC(A/G)TACATAGTA(T/G)GTTCAGGAATGT(A/C)ACACGCCA3’

Mouse PDGF-A long form:

5’CTGGTGGCCGCTTTAGGTGGTTTTAACCTTTTCC3’

The oligonucleotide probes were end-labeled with [γ-32P]ATP and polynucleotide kinase (PNK) in the following reaction:

1.5μl oligonucleotide (100ng/μl)

2μl 10x PNK buffer (700mM Tris-HCl pH 7.6, 100mM MgCl2, 50mM DTT)

5μl [γ-32P]ATP (10μCi/μl, ~3000Ci/mM; Amersham)

1μl PNK (10 units/μl)

dH2O to 20μl

The reaction was incubated at 37°C for 45 minutes then stopped by heating to 65°C for 15 minutes. The labeled oligonucleotide was purified away from unincorporated nucleotides by passing it over a Biospin-6 column (BioRad). Other protocols used for blotting, hybridizing and washing were identical to those used previously (see section 2.5.5 above) except that hybridization was reduced to 3 hours at 55°C, and wash times were reduced to 15 minutes each.
RNase protection was performed essentially as described previously (Melton et al., 1984). Total RNA was prepared from freshly dissected tissue by the guanidinium isothiocyanate procedure (see section 2.6.3.1 above). \(^{32}\)P-labeled riboprobes were transcribed from restriction enzyme-linearised cDNA clones (Table 2.1). The labeling reaction was set up as follows:

\[
\begin{align*}
4 \mu l & \text{ 5x transcription buffer (200mM Tris-HCl, pH 7.6; 30mM MgCl}_2; 10mM \\
& \text{ spermidine; 50mM NaCl)} \\
2 \mu l & \text{ 100mM DTT} \\
2 \mu l & \text{ 3NTP mix (4mM each ATP, CTP and GTP)} \\
1 \mu l & \text{ RNasin (40 U/\mu l, Promega)} \\
1 \mu g & \text{ linearized template DNA (prepared as for in situ hybridization probes, see} \\
& \text{ section 2.6.5.3 below)} \\
5 \mu l & \text{ [\(\alpha^{32}\)P]UTP (20mCi/ml, 800Ci/mmole, Amersham)} \\
1 \mu l & \text{ bacteriophage RNA polymerase (see table 2.1)}
\end{align*}
\]

The reaction was incubated at 37°C for 45 minutes, then 10 units of RNase-free DNase (Pharmacia) was added and the incubation continued at 37°C for another 15 minutes. The probe was precipitated by adding 2\(\mu\)l tRNA (10mg/ml), 0.25 volumes of 10M ammonium acetate, 3 volumes of ethanol, and incubating at 4°C for 15 minutes. The RNA pellet was recovered by centrifuging at 13000g for 15 minutes at 4°C, resuspended in 10\(\mu\)l of RNA loading buffer (80% (v/v) formamide, 1mM EDTA pH 8, 0.1% [w/v] bromophenol blue, 0.1% [w/v] xylene cyanol) and purified on a denaturing 6% (w/v)
polyacrylamide sequencing gel (see section 2.2.6 above). The hybridization reaction contained 5μg whole tissue RNA and 5 x 10^5 cpm of each probe in a total of 30μl hybridization buffer (80% [v/v] deionized formamide, 40mM PIPES buffer, pH 6.4, 0.4M NaCl, 1mM EDTA). This was incubated overnight at 45°C, then 350μl digest buffer (10mM Tris-HCl pH 7.5, 0.3M NaCl, 5mM EDTA) containing 2μg/ml RNase T1 (Life Technologies Ltd) was added to each reaction and incubation continued at 30°C for 1 hour. The reaction was stopped by incubating at 37°C for 15 minutes with 20μl of 10% (w/v) SDS and 5μl proteinase K (10mg/ml). After phenol/chloroform extraction and ethanol precipitation with 10μg carrier tRNA, the pellets containing the protected RNA fragments were resuspended in 10μl RNA loading buffer (see above), separated on a 6% (w/v) polyacrylamide sequencing gel and subjected to autoradiography. Band intensities were quantified using a phospho-imager and its dedicated software (BioRad).

### 2.6.5 IN SITU HYBRIDIZATION (ISH) USING RADIOLABELED PROBES

#### 2.6.5.1 Preparation of APES-coated slides

Pre-washed microscope slides were coated with 2% (v/v) APES (3-aminopropyltriethoxysilane; Sigma) in acetone for 30 seconds, washed in 9% (v/v) industrial methylated spirits (IMS) for 30 seconds, washed in distilled water for 30 seconds, air dried under sterile conditions, baked at 200°C for 4 hours, and stored at room temperature for up to 3 months.
2.6.5.2 Preparation of tissue sections

The tissue of interest (spinal column, brain, optic nerve) was dissected out under sterile conditions and immersion fixed overnight at 4°C in 4% (w/v) paraformaldehyde in PBS. Older animals were sometimes perfused with PBS pH 7.4 and then perfusion fixed with 4% (w/v) paraformaldehyde in PBS, before tissue was dissected and further fixed overnight at 4°C in 4% (w/v) paraformaldehyde in PBS. The tissue was then cryoprotected by transferring to 20% w/v sucrose in PBS at 4°C for at least 24 hours. Tissue was frozen on dry ice in OCT embedding compound (Miles-Tissuetek) and stored at -70°C until required. Cryosections of 10-15μm thickness were collected on APES-coated microscope slides, air dried at 65°C for 30 minutes, post-fixed in 4% (w/v) paraformaldehyde at room temperature, washed briefly in PBS and then dehydrated through an ascending series of ethanols (30%, 60%, 80%, 95% and absolute) and stored at -70°C under desiccation until required.

2.6.5.3 Preparation of radiolabeled probes.

35S-labeled probes were prepared by in vitro transcription as described previously (Cox et al., 1984). PDGFRα antisense and sense probes were transcribed from a 1636bp EcoRI fragment coding for most of the extracellular domain of mouse PDGFRα (gift from Chiayeng Wang, University of Chicago) cloned into pBluescript-KS (Stratagene). Mouse PDGF-A probes were transcribed from a 907bp EcoRI fragment encompassing the entire mouse PDGF-A cDNA (gift from Chiayeng Wang, University of Chicago) cloned into pGEM-1 (Promega). Human PDGF-A probes were transcribed from a
687bp EcoRI-HinDIII fragment encompassing most of the human PDGF-A cDNA (Betsholtz et al., 1986) cloned into pBluescript-SK (Stratagene). PLP/DM20 probes were transcribed from a 747bp fragment encompassing the entire mouse DM20 cDNA (Timsit et al., 1992) cloned into pBluescript-KS. MBP antisense and sense probes were transcribed from a 750bp BamHI fragment of the rat MBP cDNA (pCJ12, lacks exon 2, a gift from Lynn Hudson) cloned into pBluescript-SK (for more details see Table 2.1). 20μg template DNA in 100μl reaction volume was linearized with the restriction enzyme of choice, then 5μl of 10% (w/v) SDS and 1μl Proteinase K (10mg/ml) was added and incubation continued at 55°C for 15 minutes. The DNA was then extracted twice with phenol/chloroform/isoamyl alcohol, once with chloroform/isoamyl alcohol and precipitated with ethanol. After washing with 70% ethanol, the DNA pellet was resuspended in 50μl DEPC-treated dH₂O and stored at -20°C.

In vitro transcription reactions were set up at room temperature in the following order:

4μl 5x transcription buffer (200mM Tris-HCl, pH 7.6; 30mM MgCl₂; 10mM spermidine; 50mM NaCl)
2μl 100mM DTT
0.5μl (20 units) of RNasin (ribonuclease inhibitor; Promega)
4μl of ATP, GTP, CTP (each at 2.5mM; Pharmacia)
2.4μl 100μM UTP
2.5μl linearized template DNA
5μl [α-35S]UTP (10mCi/ml; >1000Ci/m mole; Amersham)

20 units T3, T7 or SP6 RNA polymerase (Pharmacia; see Table 2.1)

dH2O to 20μl

The reaction was incubated at 37°C for 45 minutes. Then, in order to remove the DNA template, 1μl of RNase-free DNase (7.5 unit/μl, Pharmacia) was added, with 20 units of RNASin and 1μl of carrier tRNA (25mg/ml; Sigma), and the reaction incubated at 37°C for a further 15 minutes. The reaction mixture was then diluted in 200μl 10mM DTT, and 1μl was removed to run on a formaldehyde/agarose gel in order to confirm full length transcription. The remaining probe solution was then precipitated with ethanol, and the resulting pellet briefly air dried and resuspended in 50μl 10mM DTT. For optimal penetration of tissue sections, the 35S-labeled probe was reduced to ~100-150bp fragments by limited alkaline hydrolysis with an equal volume of 100mM carbonate buffer, pH 10.2 (65mM Na2CO3; 35mM NaHCO3) at 60°C for 30 minutes. After terminating the reaction by adding 100μl of neutralizing buffer (0.2mM sodium acetate, 1% (v/v) glacial acetic acid, 10mM DTT), 1μl of the neutralized sample was removed for analysis by agarose gel electrophoresis, in order to determine the extent of the hydrolysis. The remainder of the digested probe was then precipitated with ethanol and the pellet dissolved in 50μl 10mM DTT/50% (v/v) deionized formamide and stored at -70°C until use (usually the same or the following day).
### Table 1(a) Riboprobes used for RNase protection assays

<table>
<thead>
<tr>
<th>Probe</th>
<th>Plasmid</th>
<th>Restriction enzyme used</th>
<th>RNA polymerase used</th>
<th>Probe length (bp)</th>
<th>Protected probe length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human PDGF-A</td>
<td>pSKAH</td>
<td>AvrII</td>
<td>T7</td>
<td>332</td>
<td>286</td>
</tr>
<tr>
<td>Mouse PDGF-A</td>
<td>pMACH</td>
<td>Smal</td>
<td>T7</td>
<td>245</td>
<td>230</td>
</tr>
<tr>
<td>Rat GAPDH</td>
<td>pSK-GAPDH</td>
<td>BstXI</td>
<td>T7</td>
<td>171</td>
<td>157</td>
</tr>
<tr>
<td>Mouse PDGFαR</td>
<td>pKS+/6-1</td>
<td>SspI</td>
<td>T7</td>
<td>408</td>
<td>346</td>
</tr>
</tbody>
</table>

### Table 1(b) Riboprobes used for in situ hybridisation.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sense/Antisense</th>
<th>Plasmid</th>
<th>Restriction enzyme used</th>
<th>RNA polymerase used</th>
<th>Probe length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse PDGFαR</td>
<td>S</td>
<td>pKS+/6-1</td>
<td>BamHI</td>
<td>T3</td>
<td>1637</td>
</tr>
<tr>
<td>Mouse PDGFαR</td>
<td>A/S</td>
<td>pKS+/6-1</td>
<td>HinDIII</td>
<td>T7</td>
<td>1637</td>
</tr>
<tr>
<td>Rat MBP</td>
<td>S</td>
<td>pCJ12</td>
<td>NotI</td>
<td>T7</td>
<td>750</td>
</tr>
<tr>
<td>Rat MBP</td>
<td>A/S</td>
<td>pCJ12</td>
<td>SmaI</td>
<td>T3</td>
<td>750</td>
</tr>
<tr>
<td>Mouse PLP</td>
<td>S</td>
<td>pBS-DM20</td>
<td>EcoRI</td>
<td>T7</td>
<td>747</td>
</tr>
<tr>
<td>Mouse PLP</td>
<td>A/S</td>
<td>pBS-DM20</td>
<td>BamHI</td>
<td>T3</td>
<td>747</td>
</tr>
<tr>
<td>Mouse PDGF-A</td>
<td>S</td>
<td>pMACH</td>
<td>PvulI</td>
<td>SP6</td>
<td>907</td>
</tr>
<tr>
<td>Mouse PDGF-A</td>
<td>A/S</td>
<td>pMACH</td>
<td>BamHI</td>
<td>T7</td>
<td>907</td>
</tr>
<tr>
<td>Human PDGF-A</td>
<td>S</td>
<td>pSKAH</td>
<td>HinDIII</td>
<td>T3</td>
<td>687</td>
</tr>
<tr>
<td>Human PDGF-A</td>
<td>A/S</td>
<td>pSKAH</td>
<td>EcoRI</td>
<td>T7</td>
<td>687</td>
</tr>
<tr>
<td>SV40 poly-A signal</td>
<td>S</td>
<td>pSKpolyA</td>
<td>BamHI</td>
<td>T7</td>
<td>450</td>
</tr>
<tr>
<td>SV40 poly-A signal</td>
<td>A/S</td>
<td>pSKpolyA</td>
<td>EcoRI</td>
<td>T3</td>
<td>450</td>
</tr>
</tbody>
</table>
2.6.5.4 Pretreatment of sections

For tissue sections, the ISH protocol was based on that of (Lawrence and Singer, 1985), with the modifications described (Pringle et al., 1992). All the incubations in this section were done at room temperature. Tissue sections at -70°C were allowed to come up to room temperature under desiccation, rehydrated through a descending series of alcohols (absolute, 95%, 80%, 60%, 30%) and then briefly rinsed in PBS. After a 5 minute incubation in proteinase K buffer (50mM Tris-HCl, 5mM EDTA pH 7.5), the sections were incubated in the same buffer with proteinase K (20μg/ml) for 7.5 minutes. The reaction was stopped with a 0.2% w/v glycine solution in PBS, the sections briefly washed in PBS and then fixed in 4% w/v paraformaldehyde in PBS for 15 minutes. After washing in PBS, the sections were incubated in 0.1M triethanolamine (pH 8) for 5 minutes, then twice in acetic anhydride in 0.1M triethanolamine (1 in 400 (v/v)) for 10 minutes each, the acetic anhydride having been added to the triethanolamine and shaken vigorously just before use. This acetylation step is designed to reduce non-specific binding of the probe to the sections. After a 3 minute wash in PBS, the sections were dehydrated through an ascending series of alcohols as before and allowed to dry thoroughly in a sterile environment.

2.6.5.5 Hybridization of sections with radioactive probes.

The longer the 35S-labeled probe is stored at -70°C, the greater the extent of probe degradation and the lower the signal to noise ratio. Therefore, in general the probe was used on the day it was transcribed, or at least no more than a 1-2 days later. Probes were
diluted (usually no more than tenfold) in hybridization buffer (see below), denatured at 80°C for 5 minutes and ~75µl of diluted probe was placed on each slide. A baked glass coverslip (22x60mm) was placed gently on each slide and the probe allowed to spread evenly across the sections, taking care to avoid bubbles. The slides were placed in a sealed box on blotting paper soaked in 50% (v/v) deionized formamide/0.3M NaCl and incubated overnight at 55°C.

**Hybridization buffer**

- 0.3M NaCl
- 10mM Tris-HCl pH 6.8
- 5mM EDTA
- 10% (w/v) dextran sulphate
- 0.1mg/ml yeast tRNA
- 1x Denhardt's solution
- 10mM DTT
- 50% (v/v) deionized formamide

**100x Denhardt's solution**

- 2% (w/v) Ficoll
- 2% (w/v) polyvinylpyrrolidone
- 2% (w/v) BSA (Fraction V)

---

### 2.6.5.6 Post-hybridization procedure

After the overnight hybridization, coverslips were removed by sliding them off carefully with a gloved finger, and the slides were incubated in 4xSSC for 1 hour at room temperature. Slides were then incubated in wash buffer (2xSSC, 2mM DTT, 50% (v/v) deionized formamide) at 65°C for 30 minutes followed by two 5 minute preincubations.
at room temperature in RNase A buffer (0.5M NaCl, 10mM Tris-HCl pH 7.5, 0.1mM EDTA). The sections were then RNase A treated (20μg/ml RNase A in RNase A buffer) for 30 minutes at 37°C followed by a further 15 minute incubation at 37°C in RNase A buffer. Next, the slides were incubated in wash buffer at 65°C for 30 minutes and were then finally washed in 2xSSC and 0.1xSSC at 45°C for 30 minutes each. Sections were dehydrated through an ascending series of alcohols as before and air dried.

2.6.5.7 Autoradiography and developing

The slides were dipped in Ilford K5 photographic emulsion under a safelight and left to air dry in total darkness for 2 hours before being placed in a light-tight box containing silica gel desiccant and exposed for up to 30 days at 4°C. Slides were developed under a safelight in Kodak D-19 developer for 2 minutes at 20°C, stopped with 1% (v/v) acetic acid, and fixed for 5 minutes in 30% (w/v) sodium thiosulphate. The slides were then washed in distilled water for 30 minutes, dehydrated through an ascending series of alcohols (30% to absolute), cleared in xylene and mounted under cover slips in XAM mountant (BDH).
2.6.6 ISH USING DIGOXGENIN-LABELED PROBES

2.6.6.1 Preparation of tissue sections.

Tissue for analysis was prepared, sectioned and the sections collected as described in section 2.6.5 above. However, after collection and air drying at room temperature for 2 hours, the sections were not treated further before hybridization.

2.6.6.2 Preparation of digoxigenin-labeled probes

Template DNA was prepared as described in section 2.6.5.3. In vitro transcription reactions were set up at room temperature in the following order:

- 2.5μl linearised template DNA
- 4μl 5x transcription buffer (section 2.6.5.3)
- 6μl 100mM DTT
- 2μl 10xDIG RNA labeling mix (10mM each of ATP, CTP, GTP, 6.5mM UTP, 3.5mM DIG-UTP; Boehringer Mannheim)
- 1μl RNasin (Promega)
- 20 units T3, T7 or SP6 RNA polymerase (see Table 2.1)
- dH2O to 20μl

The reaction was incubated at 37°C for 2 hours, precipitated with ethanol, washed with 70% ethanol, resuspended in 100μl 10mM DTT and stored at -20°C for up to 1 year.
The optimal dilution of each probe was determined by titration of the probe on control sections to achieve the maximum signal to noise ratio, and was usually between 1:500 and 1:2000.

2.6.6.3 Hybridization of sections with digoxigenin-labeled probes.

Probes were diluted immediately before use in hybridization buffer (see below), denatured at 75°C for 5 minutes and ~75μl of diluted probe was placed on each slide. The slides were coverslipped and hybridized as described in section 2.6.5.5, except that the hybridization temperature was 65°C.

<table>
<thead>
<tr>
<th>Hybridization buffer</th>
<th>10x salts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x salts</td>
<td>2M NaCl</td>
</tr>
<tr>
<td>50% (v/v) deionized formamide</td>
<td>100mM Tris-HCl pH 7.2</td>
</tr>
<tr>
<td>10% (w/v) dextran sulphate</td>
<td>50mM NaH₂PO₄</td>
</tr>
<tr>
<td>1mg/ml yeast RNA</td>
<td>50mM Na₂HPO₄</td>
</tr>
<tr>
<td>1x Denhardt’s solution</td>
<td>50mM EDTA</td>
</tr>
</tbody>
</table>

2.6.6.4 Post-hybridization washes.

All incubations were carried out in coplin jars. After overnight hybridization, slides were incubated in wash buffer (1xSSC, 50% (v/v) deionized formamide, 0.1% (v/v) Tween-20) at 65°C for at least 15 minutes or until the cover slips became detached, then washed
twice further in wash buffer at 65°C for 30 minutes each. The slides were then incubated 2x30 minutes at room temperature in MABT (100mM maleic acid pH 7.5, 150mM NaCl, 0.1% [v/v] Tween-20).

2.6.6.5 Blocking and antibody staining.

The slides were dried off around the sections using tissue paper, the sections circled with a Pap Pen (Agar Scientific), transferred to a humidified chamber and incubated in blocking solution (MABT containing 2% blocking reagent (Boehringer Mannheim), 10% [v/v] heat-inactivated sheep serum) for 1 hour at room temperature without a coverslip. The blocking solution was then replaced with alkaline phosphatase-conjugated antidigoxigenin antibody (Fab fragments; Boehringer Mannheim) diluted 1:1500 in blocking solution, and the incubation continued overnight at 4°C.

2.6.6.6 Post-antibody washes and colour reaction.

The slides were transferred to coplin jars and washed 5x20 minutes in MABT; then 2x10 minutes in prestaining buffer (100mM Tris-HCl pH 9, 100mM NaCl, 5mM MgCl₂). The prestaining buffer was then replaced with staining buffer (100mM Tris-HCl pH 9, 100mM NaCl, 5mM MgCl₂, 5% polyvinyl alcohol (Mw 70-100000, Merck), 0.2mM 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Boehringer Mannheim), 0.2mM nitroblue tetrazolium salt (NBT, Boehringer Mannheim) and incubated in the dark at 37°C until the signal reached a satisfactory intensity (usually overnight). The slides were
HC1 pH 9, 100mM NaCl, 5mM MgCl₂, 5% polyvinyl alcohol (Mₙ 70-100000, Merck), 0.2mM 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Boehringer Mannheim), 0.2mM nitroblue tetrazolium salt (NBT, Boehringer Mannheim) and incubated in the dark at 37°C until the signal reached a satisfactory intensity (usually overnight). The slides were then washed in distilled water for 30 minutes, dehydrated through an ascending series of alcohols (30% to absolute), cleared in xylene and mounted under cover slips in XAM mountant (BDH). Sections were photographed within a few days as the colour reaction faded with time.

2.6.7 IN VIVO BrdU LABELING AND SPINAL CORD CULTURE

Embryonic dividing cells in S-phase were labeled by intra-peritoneal injection of the pregnant mouse with 5-bromo-2'-deoxyuridine (BrdU, Boehringer Mannheim). The amount given was 50μg per gram maternal body weight, which has been shown to be non-toxic to dividing neural cells in vivo (Nowakowski et al., 1989), and was injected at 10mg/ml in sterile PBS. Numbers of injections and length of pulse given was as described in the text.

Embryos were isolated as in section 2.6.1, and spinal cords were dissected away from surrounding tissue in HEPES-buffered minimal essential medium (MEM-H). The meningeal membranes were removed with watchmakers’ forceps and the spinal cord tissue transferred to 2ml Earle’s balanced salt solution without calcium or magnesium (EBSS; Life Technologies) containing 0.0125% (w/v) trypsin (Boehringer Mannheim).
trituration with a Pasteur pipette. The resulting cell suspension was filtered through a 20μm pore-diameter mesh and washed by centrifugation and re-suspension in DMEM containing 10% FCS. The number of live cells in a sample of the suspension was counted in a hemocytometer, using trypan blue (0.4% [w/v] in PBS) exclusion as the criterion of viability. 50000 cells were then plated on poly-D-lysine-coated 13mm diameter glass coverslips in a 50μl droplet. The cells were allowed to attach for 30 minutes at 37°C. 350μl of modified Bottenstein and Sato’s (BS) medium (Hall et al., 1996) containing 10ng/ml PDGF-AA (Peprotech) was added and incubation continued overnight at 37°C in 5% CO₂.

The procedure for culturing postnatal spinal cords was the same as that described above for embryonic tissue except that, following trituration, the cell suspension was washed twice in DMEM containing 10% FCS to remove much of the cell and myelin debris that is generated when postnatal neural tissue is dissociated.

2.7 HISTOCHEMISTRY, CYTOCHEMISTRY AND IMAGING

2.7.1 TISSUE CULTURE CELLS

Coverslips were washed by dipping 3 times in PBS between each step below. All incubations were carried out in a humidified chamber at room temperature for 1 hour unless otherwise stated.
2.7.1.1 Immunolabeling of Cos cells

Transfected Cos cells on glass coverslips were fixed for 5 minutes in 4% (w/v) paraformaldehyde, then permeabilized for 10 minutes in 0.5% (v/v) NP40. The primary antibody, 9E10 (Evan et al., 1985), was a mouse IgG1a ascites diluted 1:1000 in DMEM, 10% FCS. The secondary antibody was an FITC-conjugated goat-anti-mouse IgG (Amersham) diluted 1:100 in DMEM, 10% FCS. Coverslips were then mounted onto microscopes slides in Citifluor anti-fade reagent (UKC) and sealed at the edges with translucent nail varnish.

2.7.1.2 Immunolabeling of primary spinal cord cultures

Cells on coverslips were lightly fixed in 2% (w/v) paraformaldehyde in PBS for 5 minutes, then incubated in neat anti-NG2 supernatant (mouse monoclonal N11.4 raised against mouse and rat NG2 proteoglycan core protein (Nishiyama et al., 1996; Stallcup and Beasley, 1987)) for 30 minutes, followed by FITC-conjugated sheep-anti-mouse IgG (1:100 in PBS; Jackson Immunoresearch) for 30 minutes, then post-fixed in acid-alcohol (70% v/v ethanol, 20% v/v glacialacetic acid in water) for 5 minutes to prevent the bound antibodies being removed during the following steps. The cells were then incubated in 70% (v/v) ethanol for 20 minutes at -20°C, permeabilised in 1% (v/v) Triton X-100 in PBS for 20 minutes, and then incubated in 6M HCl, 1% (v/v) Triton X-100 in PBS for 15 minutes followed by 0.1M Na$_2$B$_4$O$_7$ (pH 8.5) for 10 minutes. The cells were then blocked with 50% normal goat serum in 1% Triton X-100 for 15 minutes, incubated overnight at 4°C in anti-BrdU (monoclonal BU209 (Magaud et al.,
1989)), 1:5 in 1% (v/v) Triton X-100 in PBS), then incubated in TRITC-conjugated
goat-anti-mouse IgG (1:100 in 1% Triton X-100 in PBS; Pierce). Cells were post-fixed
in 4% paraformaldehyde in PBS for five minutes at room temperature and then mounted
in Citifluor as above.

2.7.2 TISSUE SECTIONS

2.7.2.1 Tissue preparation

Tissue was prepared, sectioned and collected on APES-coated slides in the same way as
for in situ hybridization, except that after collection the sections were air dried then
fixed for 10 minutes at room temperature in 4% (w/v) paraformaldehyde.

2.7.2.2 Immunocytochemistry

For intracellular epitopes 0.1% (v/v) Triton X-100 (BDH) was added to all solutions.
All incubations were done in humidified chambers at room temperature, or 4°C for
overnight incubations, unless otherwise stated.

Sections were blocked in 50% (v/v) normal goat serum (NGS) in PBS for 1 hour. If
immunoperoxidase staining was to be used, the sections were then incubated in 0.6%
(v/v) H₂O₂/80% (v/v) methanol in PBS for 30 minutes to block endogenous peroxidase
activity. After rinsing in PBS, the sections were incubated overnight with the primary
antibody diluted in blocking solution. The dilutions of primary antibody depended on
which antibody was used and are indicated in the text. After overnight incubation the sections were washed three times in PBS before being incubated for 1 hour with a biotinylated species-specific secondary antibody (1:100 dilution in blocking buffer; Amersham). After another three washes in PBS, the sections were then processed either for immunofluorescence or immunoperoxidase staining.

i) Immunofluorescence

Sections were incubated for 1 hour in streptavidin-fluorescein conjugate (1:100 dilution in blocking buffer; Amersham), washed three times in PBS, and mounted under glass coverslips in Citifluor anti-fade reagent (UKC).

ii) Immunoperoxidase staining

The Vectastain ABC kit and the DAB substrate kit for horseradish peroxidase (both from Vector Labs) were used to visualize the biotinylated secondary antibody. Briefly, the sections were incubated for 1 hour with the Vectastain AB (Avidin-Biotin) reagent, made up in PBS at least 30 minutes earlier; then washed three times in PBS (without Triton X-100), and incubated in DAB (diaminobenzidine) substrate solution until a sufficiently strong precipitate could be visualized under a microscope. The DAB substrate was made up just before use and consisted of 2 drops of buffer stock solution, 4 drops of DAB stock solution and 2 drops of hydrogen peroxide solution in 5ml dH₂O.
2.7.2.3 Propidium iodide staining

Propidium iodide (PI) was used to identify dead and dying cells in sections, both on its own and in conjunction with immunofluorescence. Just before mounting, sections were fixed and permeabilised in 70% ethanol at -20°C for 10 minutes, then stained with propidium iodide (4µg/ml) and DNase-free RNase (100µg/ml) solution in PBS for 30 minutes at 37°C. They were then washed three times in PBS and mounted. All apparently pyknotic nuclei were confirmed under phase microscopy.

2.7.3 MICROSCOPY

ISH was viewed under bright and dark field using a Wild dissecting microscope. Immunofluorescence was viewed under a Zeiss Axioskop microscope equipped with fluorescence optics, which could also be used for viewing nuclei stained with propidium iodide using either rhodamine or ultraviolet filters. The same microscope was used with bright field optics to view immunoperoxidase-stained and DIG-labeled sections. In addition, a BioRad MRC-1024 confocal microscope was used to view immunofluorescence and propidium iodide staining.

2.7.4 PHOTOGRAPHY

Black-and-white photographs of ISH, immunofluorescence and immunoperoxidase staining were taken using either Kodak T-Max 400ASA Professional film, sometimes pushed to 1600ASA in the case of dim immunofluorescence, or Kodak Technical Pan
25ASA Professional film. Colour images were taken on Kodak Ektachrome 400ASA Professional slide film. In the case of confocal images, image files were manipulated (assigned false colours, merged etc.) on an Apple Macintosh computer using Adobe Photoshop software, and then recorded on an Agfa slide maker.
3. CHAPTER THREE

A TRANSGENIC APPROACH TO MODULATE PDGF ACTIVITY IN THE CNS
3.1 INTRODUCTION

A wealth of circumstantial evidence that PDGF-AA may be important for the development of the oligodendrocyte lineage has accumulated over the past several years. First, PDGF-AA has been shown in vitro to be a potent mitogen (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988) and survival factor (Barres et al., 1992a) for oligodendrocyte progenitors; second, when added to serum-free defined medium, it can recapitulate the normal in vivo time course of differentiation into oligodendrocytes (Raff et al., 1985; Raff et al., 1988) Third, PDGF-AA is expressed at appropriate times during development of the CNS by both astrocytes (Pringle et al., 1989; Richardson et al., 1988; Yeh et al., 1991) and by neurons (Yeh et al., 1991), while PDGFRα is expressed by oligodendrocyte progenitors (Hart et al., 1989b; McKinnon et al., 1990); finally, injection of PDGF-secreting Cos cells into the sub-arachnoid space of the newborn rat brain can influence the normal development of the oligodendrocyte lineage in the optic nerve (Barres et al., 1992a). In the latter study, Barres et al showed that a transient increase in the supply of PDGF-AA to the developing optic nerve caused an increase in survival of both oligodendrocyte progenitors and newly formed oligodendrocytes in the short term, although there was no apparent change in the mitotic index of the oligodendrocyte progenitors.

Nevertheless, despite all this evidence, it was not known whether PDGF is important for normal oligodendrocyte development in vivo. We set out to test this using a transgenic approach; by both over-expressing and inhibiting neuronal PDGF-AA in the developing CNS.
We adopted a dominant-negative approach to the inhibition of gene function, by neuronal expression of an endoplasmic reticulum (ER)-retained form of PDGF-A, which could dimerise with endogenous PDGF monomers and sequester them in the ER, thus preventing secretion and paracrine stimulation of PDGF receptors.

In this chapter I describe the testing of this approach in vitro, and my attempts to employ the dominant-negative approach to inhibiting PDGF signaling in transgenic mice.

3.2 RESULTS

3.2.1 Construction of in vitro expression vectors

In order to address the function of PDGF-AA on the developing oligodendrocyte lineage, we first needed to construct expression vectors to over-express and inhibit PDGF signaling in vivo. For initial in vitro “proof of principle” studies, we decided to use a Cos cell expression system, as we could generate high levels of expression using vectors containing the SV40 origin of replication. As Cos cells are transformed with the SV40 large T antigen, such vectors can replicate in these cells and build up to a high copy number. In fact, ultimately the cells are killed, as the gene of interest becomes the major cellular gene product.

For over-expression, a human PDGF-A cDNA (Betsholtz et al., 1986) was inserted as a HindIII-EcoRI fragment into a vector containing the adenovirus major late promoter (Ad
pro), the herpes simplex virus thymidine kinase intron and polyadenylation signal (HSV-TK poly-A) and the SV40 origin of replication (SV40 ori). In addition an oligonucleotide coding for a short epitope tag recognized by the anti-c-Myc monoclonal antibody 9E10 (Evan et al., 1985) was inserted into a StuI site near the 3'-end of the cDNA. The oligonucleotide also contained a stop codon at its 3' end. The resulting transcript coded for the 'short' form of PDGF-A, lacking the extracellular matrix-binding motif coded for by the alternatively spliced exon 6 (Khachigian et al., 1992; LaRochelle et al., 1991; Östman et al., 1991; Pollock and Richardson, 1992; Raines and Ross, 1992). This construct (PDGF-AS+TAG; Pollock, 1992) is referred to in this Thesis as pHYKA<sub>myc</sub>, and its gene product PDGF-A<sub>myc</sub>.

In order to make a dominant negative mutant, an oligonucleotide coding for the ER retention signal ‘lysine-aspartate-glutamate-leucine’ (KDEL) (Munro and Pelham, 1987) was inserted into pHYKA<sub>myc</sub> just downstream of the epitope tag; this construct was named pHYKA<sub>KDEL</sub> and its gene product PDGF-A<sub>KDEL</sub>. PDGF-A monomers containing this KDEL signal should dimerise with the normally secreted PDGF-A monomers and re-route them from the Golgi back into the ER, thus decreasing PDGF-AA secretion.

3.2.2 Expression of pHYKA<sub>myc</sub> and pHYKA<sub>KDEL</sub> in Cos cells

The two constructs were transiently transfected separately into Cos cells in order to confirm that the wild-type and ER-retained forms of PDGF-A could be correctly transcribed and translated. After incubating the cells overnight the cells were fixed with paraformaldehyde and stained with the 9E10 antibody. In a proportion of the Cos cells
(2-5%, depending on the experiment), intense perinuclear staining of the Golgi was seen with pHYKA<sub>myc</sub>. With pHYKA<sub>KDEL</sub> the endoplasmic reticulum was strongly labeled as well, suggesting a sequestering of PDGF-A<sub>KDEL</sub> within this structure (Figure 3.1).

For the dominant negative approach to work the PDGF-A<sub>KDEL</sub> must be capable of forming dimers with wild-type PDGF-A when they are both expressed in the same cell. To confirm that this is the case, pHYKA<sub>KDEL</sub> and pHYKA<sub>myc</sub> were transfected into Cos cells either separately or together, and the cells were metabolically labeled overnight with <sup>35</sup>S-cysteine (PDGF-A contains no methionine residues). In this experiment, a pHYKA<sub>KDEL</sub> construct was used in which the c-Myc epitope tag had been replaced by a nucleoplasm epitope tag (Colin Dingwall, personal communication) recognized by the monoclonal antibody PA3C5. This enabled us to specifically immunoprecipitate either PDGF<sub>myc</sub> or PDGF<sub>KDEL</sub> isoforms and look for co-precipitation of the other isoform, in order to demonstrate heterodimerisation. This experiment is illustrated in Figure 3.2.

The crucial test for the dominant negative PDGF-A<sub>KDEL</sub> is that when heterodimers form between itself and wild-type PDGF-A, the resultant dimers are retained inside the cell. This was tested by co-transfecting into Cos cells increasing amounts of pHYKA<sub>KDEL</sub> plasmid with a fixed amount of pHYKA<sub>myc</sub> plasmid, then metabolically labeling and immunoprecipitating as before, using an antibody which specifically recognized PDGF-A<sub>myc</sub>. Subsequent analysis by SDS-PAGE showed decreasing amounts of PDGF-A<sub>myc</sub> in the cell supernatants with increasing ratios of pHYKA<sub>KDEL</sub> to pHYKA<sub>myc</sub>. The retained
heterodimers are not detected in the cell lysates however, presumably due to masking of the antibody epitope by the PDGF-A_{KDEL} isoform (Figure 3.3A). In the control transfections, the pHYKA_{KDEL} was replaced by a form of pHYKA_{myc} lacking the epitope tag, and under these conditions no decrease was seen in secreted PDGF-A_{myc} as the ratio was increased (Figure 3.3B).

Overall these data demonstrate that the mutant PDGF-A isoform, PDGF-A_{KDEL}, can be expressed in mammalian cells, can form heterodimers with endogenous PDGF-A monomers, and that the resultant heterodimers are retained inside the cell. It should therefore be able to function as a dominant-negative in transgenic mice.
Figure 3.1 - PDGF-A$_{KDEL}$ protein expressed in Cos cells is retained in the endoplasmic reticulum. Immunofluorescent staining using the 9E10 antibody on permeabilized Cos cells transfected with pHYK$_{A_{myc}}$ (A) or pHYK$_{A_{KDEL}}$ (B). Intense staining of the perinuclear region is visible in (A), corresponding to the Golgi apparatus, since PDGF-A$_{A_{myc}}$ is a secreted protein. However in (B) the endoplasmic reticulum is also strongly labeled, suggesting that PDGF-A$_{A_{KDEL}}$ is retained within this structure.
Figure 3.2 - PDGF-A_{KDEL} can form dimers with wild-type PDGF-A. Cos cells were transfected with pHYKAm_{yc} or pHYKAK_{KDEL}, either alone or as a co-transfection with equal amounts of DNA, and the cells were metabolically labeled with $^{35}$S-cysteine. Supernatants (SUP) and cell lysates (LYS) were collected, immunoprecipitated with the antibodies shown, and analyzed by SDS-PAGE under reducing conditions. anti-myc recognizes only PDGF-A_{myc} and anti-NP recognizes only PDGF-A_{KDEL}. In the cell lysate of the co-transfection, anti-NP immunoprecipitates PDGF-A_{myc} in addition to PDGF-A_{KDEL}, showing that the two subunits are present as heterodimers. Arrowhead: intracellular PDGF-A_{KDEL}, Black arrow: unprocessed PDGF-A_{myc}, Grey arrow: mature PDGF-A_{myc}. The band above intracellular PDGF-A_{KDEL} in the cell lysate is presumably unprocessed PDGF-A_{KDEL}.

103
Figure 3.3 - PDGF-A_{KDEL} acts as a dominant negative to sequester PDGF-A inside the cell. (A) Cos cells were transfected with pHYKA_{myc} either alone or together with increasing amounts of pHYKA_{KDEL}. The cells were then metabolically labeled with $^{35}$S-cysteine, supernatants (S) and cell lysates (L) collected, immunoprecipitated with the 9E10 antibody and analyzed by non-reducing SDS-PAGE. The amount of PDGF-AA dimers (arrow) present in the cell supernatants decreases with increasing ratios of pHYKA_{KDEL} to pHYKA_{myc}. The lower one of the two intense bands in the cell supernatants is non-specific, as it can be detected in untransfected Cos cells (not shown). The strong, lower band in the cell lysates is due to an irrelevant c-Myc-tagged expression plasmid used to normalize the amount of transfected DNA. $M_w$: molecular weight markers. (B) The experiment was repeated replacing pHYKA_{KDEL} with an equivalent construct containing wild-type PDGF-A cDNA (lacking a c-Myc-tag). In this case there is no reduction in PDGF-AA dimers detected in the cell supernatants with increasing ratio of PDGF-A to PDGF-A_{myc} demonstrating that the reduction seen in (A) is caused specifically by PDGF-A_{KDEL} and represents a dominant-negative effect.
3.2.3 Construction of PDGF-Expression Vectors

For transgenic mice production, the cytomegalovirus PDGF-A cDNA and...
3.2.3 Construction of PDGF-A transgene vectors

For transgenic mice production, the myc-tagged, wild-type human PDGF-A cDNA and its ER-retained derivative (PDGF-A\textsubscript{myc} and PDGF-A\textsubscript{KDEL} respectively) were sub-cloned into a vector containing the rat neuron-specific enolase promoter (NSE-pro) and the SV40 polyadenylation sequence (poly A). This vector has been described elsewhere (Forss-Petter et al., 1990), where it was shown to express a heterologous transgene (β-galactosidase) in a large subset of post-mitotic neurons in transgenic mice. This vector was digested with HindIII and partially with EcoRI, in order to isolate a HindIII-EcoRI fragment containing the NSE promoter and the SV40 poly A. These were then ligated with the HindIII-EcoRI fragments PDGF-A\textsubscript{myc} or PDGF-A\textsubscript{KDEL} in order to generate NSE-PDGF-A and NSE-PDGF-A\textsubscript{KDEL} respectively. Again using partial EcoRI digestion followed by filling in of recessed 3’ ends and re-ligation, the EcoRI site used in the cloning was ‘killed’, enabling the entire transgene to be isolated from vector sequences by EcoRI digestion and gel purification (Figure 3.4). Purified transgene DNA was then injected into fertilized mouse oocytes in order to generate transgenic mice. This was carried out by Alex Harper at the Transgenic Unit, St Thomas’ Hospital, London.
Figure 3.4 Structure of the NSE-PDGF-A transgenes

The ‘killed’ EcoRI site is shown crossed out, and the position of the KDEL sequence in NSE-PDGF-A_{KDEL} is indicated.

NSE pro - rat neuron-specific enolase promoter

hPDGFA - human PDGF-A cDNA

myc - epitope tag recognized by the 9E10 antibody

poly A - SV40 polyadenylation sequence

3.2.4 Results of oocyte injections

20 litters of possible NSE-PDGF-A transgenic mice were analyzed by Southern blots of tail-clip DNA; a total of 101 mice. From this analysis, 10 were shown to have the transgene integrated into the genome. Three of these died before they were sexually mature, leaving seven whose offspring were analyzed. Of these, three apparently did not exhibit germline transmission, so four independent mouse lines were generated, termed #64, 72, 75 and 82. It was possible to ascertain from the band patterns and intensity on Southern blots the approximate transgene copy numbers for each line, with #64 having a single copy, #72 having 5-10 copies, #75 having 3 copies and #82 having 5-10 copies (Figure 3.5).
Figure 3.5 - Transgene copy numbers among the four independent NSE-PDGF-A lines generated. A Southern blot of BamHI-digested genomic DNA isolated from the four NSE-PDGF-A founder mice (64, 72, 75, 82), probed with $^{32}$P-labeled human PDGF-A cDNA. The ~4kb band present in lanes 72, 75, and 82 (arrow) indicates tandem repeats of the transgene, and its absence in lane 64 indicates integration of a single transgene copy. The different sized bands above and below that of the transgene represent flanking sequences on either side of the site of integration. Densitometry analysis of the ~4kb band in lanes 72, 75, and 82 gives an indication of the approximate copy numbers (#72 having 5-10 copies, #75 having 3 copies and #82 having 5-10 copies).
4 litters of possible NSE-PDGF-A<sub>kdel</sub> transgenic mice were analyzed by Southern blotting of tail-clip DNA; a total of 27 mice. In this case, 4 were shown to have the transgene integrated into their genome. All of these founder animals demonstrated germline transmission with Mendelian inheritance, so again four independent mouse lines were generated, termed #3, 21, 23 and 26. Interestingly, subsequent breeding demonstrated that in line #26 the transgene had integrated into the X chromosome, as when a male transgenic was mated with a wild-type female, all the male offspring were wild-type, while all the female offspring were transgenic. If a female transgenic was mated with a wild-type male however, the transgenic status of the offspring was not related to its sex.

3.2.5 PDGF expression in the normal developing CNS

Before analyzing transgene expression in the NSE-PDGF-A transgenic mice, expression of PDGF-A was first examined in the normal developing spinal cord and brain by in situ hybridization, focusing on mid-to-late embryonic and early postnatal stages (Fig. 3.6). In the spinal cord, PDGF-A mRNA was first detected in the floor plate at E11, as previously reported (Orr-Urtreger and Lonai, 1992), and persisted there until after E12 (Fig. 3.6A). This is before the appearance of any PDGFRα-expressing cells in the ventral cord; PDGFRα<sup>+</sup> oligodendrocyte precursors first appear around E12.5 in the mouse, at the luminal surface near the floor plate (this Thesis; Hardy, 1997; Pringle et al., 1996). By E13, PDGF-A transcripts were also detected in the cell bodies of motor neurons in the ventral horns of the cord (not shown). By E15, near the end of neuronogenesis but before astrocyte or oligodendrocyte production, PDGF-A was
expressed by the majority of neurons throughout the grey matter of the cord, most strongly by motor neurons (Fig. 3.6B; Yeh et al., 1991). PDGF-A continued to be expressed by many neurons throughout embryonic development into postnatal life (Fig. 3.6C-E). By postnatal day 9 (P9), PDGF-A was also expressed by numerous small cells, presumably astrocytes, in the developing white matter (Fig. 3.6E). Astrocytes from spinal cord (Yeh et al., 1991), optic nerve (Mudhar et al., 1993; Pringle et al., 1989) and cortex (Richardson et al., 1988) have previously been shown to express PDGF-A. PDGF-A transcripts are also present in many neuronal cell bodies in the embryonic and postnatal brain (data not shown; Yeh et al., 1991).

It was previously shown that PDGF-B is expressed in capillary blood vessels of the embryonic spinal cord and brain (Lindahl et al., 1997a). PDGF-B mRNA is not expressed at detectable levels by either neurons or glia before birth (not shown). However, after birth PDGF-B begins to be expressed at low levels by many cells, presumably neurons, throughout the CNS (Sasahara et al., 1991; Sasahara et al., 1995).

### 3.2.6 Expression of the transgenes

As described elsewhere (Fruttiger et al., 1996), a retinal phenotype was observed in three out of our four lines of NSE-PDGF-A transgenic mice (#64, #75 and #82). Briefly, a hyperplasia of PDGFRα+ retinal astrocytes occurs postnatally, with a secondary over-production of the associated retinal vasculature. We therefore chose to analyze expression of the transgene in the line with the strongest phenotype in the eye, this being #75.
Figure 3.6 - *PDGF-A* transcripts in the developing mouse spinal cord. Sections from the upper thoracic cord were subjected to in situ hybridization with a DIG-labeled probe for mouse *PDGF-A*. Panel A, E12.5 cord. *PDGF-A* is expressed in the floor plate and the ventral ventricular zone (VZ). Panel B, E15 cord. *PDGF-A* is expressed at a low level in neurons throughout the cord, but at higher levels by motor neurons (mn) and cells in the dorsal root ganglia (drg). This expression pattern persists until birth (panel C). Panel D, P7 cord. Expression is up-regulated in many neurons throughout the cord and also by astrocytes in the circumferential white matter. Panel E, higher magnification micrograph of a P9 cord. Note the large strongly positive neurons (arrows, N) and the many small white matter astrocytes (arrows, A). Scale bars, 100 μm.
I first performed reverse transcriptase-PCR (RT-PCR) on total RNA prepared from P3 retina, at which age the phenotype is most striking in the eye. PCR primers were chosen to amplify both endogenous (mPDGF-A) or transgene-derived (hPDGF-A) PDGF-A with equal efficiency (Figure 3.7A), and the PCR products were then detected on a Southern blot with radioactive probes against either mouse/human PDGF-A or the c-Myc tag contained in the transgene (see Materials & Methods). Robust expression of the transgene was detected in transgenic retina, but not in wild-type or in any of the controls (Figure 3.7A). The primers were also designed to span the alternatively spliced sixth exon, contained in the extracellular matrix-binding form of PDGF-A (Khachigian et al., 1992; LaRochelle et al., 1991; Östman et al., 1991; Pollock and Richardson, 1992; Raines and Ross, 1992). I was unable to detect expression of this splice variant in the mouse retina, even when the blot was probed with the sixth exon itself (not shown), indicating that only the 'short' form of PDGF-A is expressed in the P3 retina.

I next tried to demonstrate expression of the transgene at the protein level in NSE-PDGF-A transgenic mice. However I was unable to detect the protein at any age, either by immunocytochemistry or by Western blotting; I will discuss later why I think I was unable to do this and why I believe nonetheless that the protein is translated, secreted and functional. I attempted to detect the transgene product in NSE-PDGF-A_{KDEL} transgenic mice instead, as the protein should theoretically build up to a higher level in the cell bodies of their neurons than with the secreted, NSE-PDGF-A gene product. I analyzed line #26, as this transgene is X-linked and thus genotyping the animals was simple. Using immunofluorescence with the 9E10 antibody against the c-Myc tag I
found strong expression of the NSE-PDGF-A\textsubscript{KDEL} protein in retinal ganglion cells (RGCs) and, to a lesser extent, in the photoreceptor cells and cells of the inner nuclear layer (Figure 3.7B). This expression pattern was reproduced in another NSE-PDGF-A\textsubscript{KDEL} line (#21), and was also very similar to the expression in NSE-lacZ transgenic mice (Forss-Petter et al., 1990; Seiler and Aramant, 1995), and that of an NSE-Bcl-2 transgene (Martinou et al., 1994). I was therefore confident that expression of our transgene had not been greatly affected by cis-acting regulatory elements near the site of integration.

I was interested in using these mice for studies of the spinal cord oligodendrocyte lineage; it was therefore necessary to analyze expression of the transgenes in the developing spinal cord. I first prepared spinal cord total RNA from various ages from E11.5 onwards and performed RT-PCR as before, using a mouse/human probe to detect both endogenous and transgene mRNA (Figure 3.8). This demonstrated transgene expression at all ages studied; again I did not observe expression of the ‘long’ form of PDGF-A at any of these ages. However the RT-PCR technique is only semi-quantitative for comparison of expression levels, so I repeated the analysis using the RNase protection assay (RPA). This again showed transgene expression from at least as early as E11.5 in the spinal cord, with expression staying relatively constant between E15 and P6, and continuing at a lower level into adulthood. The endogenous PDGF-A level, on the other hand, continued to rise between E15 and P6, presumably due to astrocytic PDGF expression starting around birth (Figure 3.9A; compare Figure 3.6).
Figure 3.7 - Expression of the human PDGF-A transgenes in the retina. (A) Expression of NSE-PDGF-A transgene-derived mRNA was detected by RT-PCR. Left, diagram showing the predicted structures of the transgenic (hPDGF-A) and endogenous (mPDGF-A) mRNAs, and the relative positions of oligonucleotide PCR primers (arrows) and hybridization probes (P1, P2) used for detection. The position of exon 6 (69 bp), which encodes an extracellular matrix binding motif that can be inserted by alternative splicing, is indicated. Right, agarose gel electrophoresis of RT-PCR products generated from line #75 transgenic (tg) or wild-type (wt) P3 retinae and a control reaction (-RT) in which reverse transcriptase was omitted from the PCR reaction, Southern blotted and probed with 32P-labeled probes P1 (detects all PDGF-A mRNA species) or P2 (detects only transgene-derived mRNA). The predicted sizes of the PCR products are 211 bp ("short" mPDGF-A mRNA lacking exon 6), 280 bp ("long" mPDGF-A mRNA including exon 6) or 318 bp (transgenic hPDGF-A mRNA). The band intensities indicate that the transgene is moderately over-expressed relative to the endogenous gene in line #75, and also that no "long" form mPDGF-A mRNA can be detected in wild-type or transgenic retinae. (B) Immunofluorescence localization of transgene-derived hPDGF-A KDEL in the retina of a P14 transgenic mouse from line #26. Monoclonal 9E10 (anti-c-Myc) was the primary antibody, FITC-conjugated rabbit-anti-mouse IgG was the secondary antibody. The transgenic retina is on the right, a wild-type littermate on the left. The strongest signal is detected in the cell bodies of retinal ganglion cells (RGC); a weaker signal is detected in the cell bodies of photoreceptor cells in the outer nuclear layer (ONL, arrowheads), and in the inner nuclear layer (INL, arrows). The strongest signal in the pigment epithelium (PE) is background autofluorescence. IPL, inner plexiform layer; OPL, outer plexiform layer; PRL, photoreceptor layer. Scale bar, 50μm.
A.

Expected PCR product size

<table>
<thead>
<tr>
<th>Probe P1</th>
<th>Probe P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>tg</td>
<td>wt</td>
</tr>
<tr>
<td>318 bp</td>
<td></td>
</tr>
<tr>
<td>211 bp</td>
<td></td>
</tr>
</tbody>
</table>

B.

Figure 3.7 - Expression of the human PDGF-A transgenes in the retina.
Figure 3.8 - Analysis of expression of NSE-PDGF-A transcripts in the spinal cord by RT-PCR. RT-PCR was carried out on wild-type (wt) and transgenic (tg) spinal cord RNA at the ages shown; the resultant cDNA was Southern blotted and hybridized with Probe P1 (see Figure 3.7A) to detect both endogenous (lower band) and transgene-derived (upper band) PDGF-A mRNA. Both transgene and endogenous PDGF-A is expressed at all ages studied, although the relative levels cannot be accurately quantified by this method. -ve: Reverse transcriptase enzyme omitted from RT reaction.
Figure 3.9 - Expression of PDGF-A in wild-type and NSE-PDGF-A transgenic spinal cords. (A) RNase protection assays for PDGF-A transcript abundance in NSE-PDGF-A transgenic and wild-type spinal cords. Hybridization reactions contained either a mixture of probes for endogenous (mouse) and transgenic (human) PDGF-A or a probe for GAPDH as a control for RNA amounts. The undigested PDGF probes are shown on the left. Protected fragments corresponding to endogenous and transgene-derived PDGF-A transcripts can be distinguished (mPDGF-A and hPDGF-A, respectively). The human PDGF-A probe did not cross-hybridize to endogenous mouse PDGF-A mRNA in a wild-type embryo (P6 wt); comparison of lanes P6 and P6wt also demonstrates that the presence of the transgene did not affect expression of the endogenous PDGF-A gene. Both endogenous and transgene-derived transcripts are expressed from before E11 until adulthood. The transgene was expressed at a roughly constant level from E15-P6, whereas endogenous gene transcription increased between E15 and P0 but did not change much after that. The transgene was expressed at a similar level to endogenous transcripts. (B) Comparison of the spatial distributions of endogenous and transgene-derived PDGF-A transcripts. Sections through the spinal cords of E15 wild-type (wt) or hemizygous NSE-PDGF-Atransgenic (tg) mice were subjected to in situ hybridization with DIG-labeled probes for mouse or human PDGF-A. Cross-hybridization of the human probe to endogenous mouse transcripts was not a problem under our assay conditions (compare center and right panels). Both endogenous and transgene-derived PDGF-A transcripts are expressed widely throughout the spinal cord in many neurons. Endogenous PDGF-A is most strongly expressed in motor neuron pools in the ventral horns, while the transgene is expressed more strongly in the dorsal cord. Scale bar, 100 µm. (C) Distribution of PDGF-AKDEL protein in transgenic spinal cord. Spinal cords of NSE-PDGF-AKDEL transgenic mice (two independent lines; #26 and #21) were sectioned and immunolabeled with monoclonal 9E10 (anti-c-Myc), followed by peroxidase-coupled goat-anti-mouse IgG secondary antibodies. Both transgenic lines gave results similar to the one shown. A wild-type cord was labeled in parallel. The distribution of transgene-encoded PDGF-AKDEL protein resembles that of the mRNA. Magnification as in Fig. 3.9B.
A

probes
human mouse
E11 E15 P6 Adult P6 wt

hPDGF-A
mPDGF-A
GAPDH

B

wt
tg
wt

mPDGF-A
hPDGF-A
hPDGF-A

in situ probes

C
In order to analyze the spatial distribution of transgene expression, I performed in situ hybridization on sections of E15 wild-type and NSE-PDG-F-A spinal cord, with probes specific for either mouse or human PDGF-A (Figure 3.9B). This showed that transgene-derived PDGF-A mRNA, like endogenous PDGF-A mRNA (Yeh et al., 1991), is expressed widely throughout the developing spinal cord by neurons. The overall distributions of transgene-derived and endogenous PDGF-A transcripts were therefore similar. Endogenous PDGF-A is also expressed in white matter astrocytes (Pringle et al., 1989; Richardson et al., 1988; Yeh et al., 1991; this Thesis, Figure 3.6), but these cells are not generated in large numbers until after birth. I was again unable to demonstrate the presence of the NSE-PDG-F-A gene product by any method, and I therefore again turned to the NSE-PDG-F-AKDEL transgenic mouse to see if I could detect transgene-derived protein in these animals. Using immunocytochemistry with the 9E10 antibody, I was able to demonstrate expression of the NSE-PDG-F-AKDEL protein in many spinal cord neurons at P0, with the exception of most ventral motor neurons (Figure 3.9C).

3.2.7 Analysis of oligodendrocyte progenitor cell numbers in NSE-PDG transgenic mice

Since it was known from previous work that oligodendrocyte progenitors in vitro express the PDGFRα (Hart et al., 1989b; McKinnon et al., 1990) and can be stimulated to divide by PDGF-AA (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988), we reasoned that the over-expression or inhibition of PDGF-A in vivo might have an effect on the size of the progenitor cell population. I therefore performed in situ hybridization with a probe to PDGFRα on sections of both NSE-PDG-F-A and NSE-
PDGF-A<sub>KDEL</sub> spinal cords in order to analyze the effect of transgene expression on the number of oligodendrocyte progenitors in vivo.

At E15, NSE-PDGF-A spinal cords from line #75 showed an approximately three-fold increase in the number of oligodendrocyte progenitors over the wild-type cords (Fig. 3.10). This apparent over-proliferation of progenitors was not uniform across the spinal cord, presumably reflecting differences in the spatial distribution of transgene expression (see Fig. 3.9C). The phenotype was maintained throughout development, with a similar increase in progenitor numbers being observed at P6 (Fig. 3.10) and in the adult (not shown), although absolute numbers of progenitors were reduced in the adult, both in wild-type and NSE-PDGF-A spinal cords. This phenotype was confirmed in line #82 (not shown), and will be described in greater detail in the following chapter.

If the NSE-PDGF-A<sub>KDEL</sub> transgene product acted to inhibit the secretion of PDGF from neurons, as it does in vitro from Cos cells, we might have expected to observe a decrease in the proliferation of O-2A progenitors, resulting in a reduction in the number of cells present during development. However I was unable to detect any significant difference in the number of cells at either E15 or P6 (Figure 3.11), suggesting that expression of the NSE-PDGF-A<sub>KDEL</sub> transgene had little or no effect on the proliferation of O-2A progenitors in the spinal cord.
Figure 3.10 - Numbers of oligodendrocyte progenitors are increased in the spinal cords of NSE-PDGF-A transgenic mice. Oligodendrocyte progenitors in wild-type (wt) or transgenic (tg) spinal cords from NSE-PDGF-A line #75 were visualized by in situ hybridization with a $^{35}$S-labeled probe against PDGFRα, autoradiographed and photographed under dark-field illumination. Each white “dot” within the spinal cord represents a cluster of silver grains overlying a single progenitor cell. Sections of equal thickness were taken from equivalent rostro-caudal levels of wild-type and transgenic mice (at the level of the forelimb) by aligning the embryos side-by-side in the same block and sectioning both animals simultaneously. At E15 and P6 there are approximately three times as many progenitors in the transgenic as in the wild-type embryonic spinal cord and this increase is maintained throughout the postnatal period. Analysis of NSE-PDGF-A line #82 gave a similar but more pronounced effect (not shown). Scale bar, 100 μm.
Figure 3.11 - Numbers of oligodendrocyte progenitors are not greatly affected in the spinal cords of NSE-PDGF-A<sub>KDEL</sub> transgenic mice. Oligodendrocyte progenitors in wild-type (wt) or transgenic (tg) spinal cords from NSE-PDGF<sub>KDEL</sub> line #26 were visualized by in situ hybridization with a probe against PDGFRα as in Figure 3.10, except that the probe was labeled with digoxygenin which was then detected by immunocytochemistry and photographed under bright-field illumination. Therefore in this figure each black dot in the cord represents a single oligodendrocyte progenitor. There was no apparent difference in the number of oligodendrocyte progenitors either at E15 or at P6. Scale bar, 100 μm.
3.3 DISCUSSION

3.3.1 Transgenic mice which over-express and inhibit PDGF in the nervous system

I set out to over-express and inhibit PDGF-A in the CNS of transgenic mice. In order to achieve this, I generated expression vectors for wild-type PDGF-A and an ER-retained isoform which I have shown to function as a dominant negative in a Cos cell expression system, by demonstrating that this mutant isoform can dimerise with endogenously produced PDGF-A and sequester it within the lumen of the ER. I also placed these two forms of PDGF-A under the control of a neuron-specific promoter (NSE), and generated four lines of transgenic mice with each of these constructs.

I have demonstrated expression of both of these transgenes at the mRNA level in many spinal cord neurons, in a spatial and temporal pattern broadly similar to that of endogenous PDGF-A, and I therefore believed that these transgenic mice would be useful in providing insights into the effect of both over- and under-expressing PDGF-A in areas of the CNS and at times when it is normally present during development. I also detected expression of PDGF-A_\text{kdel} protein, although I was unable to demonstrate the presence of PDGF-A gene product. However, the identical retinal phenotype observed in three of the four lines of NSE-PDGF-A transgenic mice is one which would be predicted to be associated with the presence of excess PDGF in the extracellular space, and the fact that expression of the very closely related transgene NSE-PDGF-A_\text{kdel}, which accumulated to a high level in RGCs of these mice, did not elicit any phenotype in the retina provides strong genetic evidence that the phenotype is dependent on the
production and secretion of the PDGF-A transgene product. I therefore believe, as discussed in Chapter 4, that PDGF-A is expressed and secreted by neurons in the CNS, but that it does not accumulate to a detectable degree either inside cells or in the extracellular space following secretion.

3.3.2 Over-expression of PDGF-A by neurons results in an increase in the number of oligodendrocyte progenitors

I examined the number of oligodendrocyte progenitors in the spinal cords of NSE-PDGF-A transgenic mice and found there to by an increase of around three-fold over the wild-type cords. This 'over-proliferation' starts before E15 and continues throughout embryonic and postnatal life, although it seems to have no effect on the viability or lifespan of the animals, which continue to thrive and breed normally.

There are a number of reasons for this increase in the number of oligodendrocyte progenitors in the transgenic spinal cord. An increase in the level of PDGF-A in the spinal cord may cause the oligodendrocyte lineage to be specified at an earlier embryonic age (possibly by up-regulation of the PDGFRα), or alternatively it may result in an increase in either the division rate or survival rate of oligodendrocyte progenitors. In order to investigate this further, I decided to extend the in situ hybridization studies to earlier embryonic ages, and in addition to study the changes in cell cycle times of progenitors throughout development, both in wild-type and NSE-PDGF-A mice; this analysis is described in the following Chapter.
3.3.3 Neuronal expression of the dominant negative PDGF isoform PDGF-A_{KDEL} has no effect on the number of oligodendrocyte progenitors

I examined oligodendrocyte progenitor numbers in the spinal cord, both embryonic and postnatal, and found no difference between NSE-PDGF-A_{KDEL} and wild-type animals. The reason for this could be that PDGF is present in the developing spinal cord in saturating amounts for oligodendrocyte lineage development, even after expression of the dominant-negative. Alternatively, the level of expression of the transgene might be too low to elicit an effect, as discussed below.

In order for the ER-retained form of PDGF-A to function efficiently as a dominant-negative PDGF subunit, it would be necessary to generate transgenic mice which express PDGF-A_{KDEL} at a level several-fold higher than, and in the same cells as its endogenous counterpart. This was clear from the in vitro studies; assuming that the ratio of transfected plasmid DNA accurately reflected the ratio of expression levels, the mutant form only caused a significant drop in PDGF secretion if over-expressed four-fold with respect to the wild type form. From our mRNA studies, it was clear that the level of expression of the transgene was approximately equal to that of the endogenous gene, and there may also have been a number of cells where the two were not co-expressed. Therefore it seems quite likely that the dominant-negative effect of PDGF_{KDEL} was insufficient to significantly affect extracellular PDGF levels.
We had originally decided to use a dominant-negative approach to inhibit PDGF activity in transgenic mice as we reasoned that ‘knocking out’ the PDGF-A gene by homologous recombination would prove to be lethal to the early embryo, as PDGF and its receptors are normally expressed during early embryogenesis in the mouse (Mercola et al., 1990a). As it turned out however, PDGF-A knockouts are not necessarily embryonic-lethal, although a proportion do die at around E10, so we obtained these mice in order to look at the development of the oligodendrocyte lineage. We reasoned that not only would these animals enable us to study oligodendrocyte development in the absence of PDGF-A, but we could also analyze heterozygous knockout mice which would presumably express only half the normal amount of PDGF-A. The phenotypes of these mice, together with the NSE-PDGF-A transgenic mice are described in the following two chapters.
4. CHAPTER FOUR

CONTROL OF OLIGODENDROCYTE PROGENITOR CELL NUMBER BY COMPETITION FOR LIMITING AMOUNTS OF PDGF
4.1 INTRODUCTION

In the previous Chapter I described the transgenic approach we took in order to over-express and inhibit PDGF signaling in the developing nervous system. I generated transgenic mice that express PDGF-A under the control of the neuron-specific enolase (NSE) gene promoter. I analyzed development of the oligodendrocyte lineage in two independent lines of NSE-PDGF-A mice, showing that over-expression of PDGF-A in neurons causes increased numbers of oligodendrocyte progenitors in the CNS. One possible explanation for this is that the level of expression of PDGF influences the rate of division of oligodendrocyte progenitors. I therefore decided to carry out an extensive study of progenitor cell proliferation through embryonic and postnatal development in the wild-type spinal cord, both by in situ hybridization and BrdU incorporation, and see what effect over-expression of PDGF-A had on this proliferation. This study has provided insights into the role of PDGF in controlling the proliferation rate and final number of oligodendrocyte precursors during central nervous system (CNS) development.

Unfortunately I failed to see a phenotype in the NSE-PDGF A<sub>KDEL</sub> transgenic mice, probably due to insufficient expression of the transgene. I took this approach because I reasoned that PDGF-A-knockout mice would be unlikely to survive long enough to study the development of the nervous system, since PDGF-A is expressed in the embryo as early as the blastula stage (Mercola et al., 1990b). However, this turns out not to be the case; PDGF-A-knockout mice survive into postnatal life, although they
generally do not survive more than a few weeks before dying from pulmonary failure (Boström et al., 1996). PDGF-B knockout animals are haemorrhagic and die at birth (Lindahl et al., 1997a). Therefore it was possible to study pre-natal oligodendrocyte development in animals lacking either PDGF-A or PDGF-B. This work was done by Anita Hall in the Richardson lab, but is described briefly in this Chapter as it forms necessary background for my own studies of transgenic mice that over-express PDGF-A in the CNS.

4.2 RESULTS

4.2.1 Oligodendrocyte lineage development depends on PDGF-AA, but not -AB or -BB

PDGF is a homo- or heterodimer of A and B chains, the products of separate genes. All three dimeric forms - AA, AB and BB - are capable of binding to and activating PDGFRα, the PDGF receptor subunit present on O-2A progenitors. Although much is known about the effects of PDGF on O-2A progenitors in vitro, the relevance of this in the developing animal was unknown. We therefore obtained mice in which the PDGF-A gene had been disrupted by homologous recombination, such that no functional PDGF-AA or -AB is present during development. These animals are born relatively normal, but fail to thrive and subsequently die within a few weeks probably from pulmonary failure (Boström et al., 1996). However, much of oligodendrocyte development occurs in the prenatal and early postnatal period, allowing us to study some aspects of oligodendrocyte development in the knockout mice.
In situ hybridization with a probe against the PDGFRα to visualize O-2A progenitors demonstrated that PDGF-A was not required for their normal specification at E12.5 (not shown). However subsequent proliferation was very much reduced such that by E17 there was only about 5% of the normal number of O-2A progenitors in the spinal cord (Figure 4.1; 236±35 progenitors/10μm section in wild-type cords and 12±8 in PDGF-A-null cords, means ± s.d. of three sections from each of two embryos of each genotype), and similarly at P9 it has been shown that there are virtually no O-2A progenitors in the spinal cord of PDGF-A knockout mice (Fruttiger et al., 1999). The low number of progenitors does not seem to be the result of premature differentiation, because in situ hybridization with probes specific for oligodendrocytes (PLP or MBP) does not show a difference between the wild-type and the transgenic at E17 (Figure 4.1, arrows). In addition, migration of O-2A progenitors seems to be unaffected by the lack of PDGF-A, as the few progenitors which are present in the knockout are widely dispersed from their ventral site of origin (Figure 4.1, arrowheads).

We have performed a similar analysis on PDGF-B knockout mice, which showed no abnormalities in O-2A progenitor numbers at all ages studied (not shown). We therefore conclude from these studies that PDGF-AA, but not PDGF-AB or PDGF-BB, is crucial for the normal embryonic development of the oligodendrocyte lineage. The role of the PDGF-B isoform in postnatal oligodendrocyte development remains unclear because the PDGF-B knockout mice die at birth. A more complete account of
oligodendrogenesis in the CNS of PDGF-A and PDGF-B knockout mice has been presented elsewhere (Fruttiger et al., 1999).

4.2.2 Proliferation of O-2A progenitors in the spinal cord ceases before birth

In order to quantify O-2A progenitor cell proliferation in the normal mouse spinal cord, I performed in situ hybridization on a developmental series of spinal cord sections from wild-type mice, using PDGFRα as a marker for O-2A progenitors, and then counted the number of PDGFRα+ cells per 15μm section. After specification of the first O-2A progenitors at E12, the number of cells increased rapidly over the next 3-4 days. Surprisingly, however, this proliferation soon slowed down and then apparently stopped as the number of O-2A progenitors reached steady state before E17. At steady state there were approximately 350 cells per section, this level being maintained until at least P3 (Figures 4.2 and 4.3). Differentiated oligodendrocytes did not appear in the spinal cord until after E17, thus this ‘leveling off’ of O-2A progenitor numbers must reflect either a cessation of cell division, an increase in cell death, or a combination of the two.
Figure 4.1 - PDGF-A is required for normal proliferation of oligodendrocyte progenitors in the spinal cord. Sections were cut through the upper thoracic spinal cords of E17 homozygous PDGF-A knockout mice or wild-type littermates. These were hybridized with a $^{35}$S-labeled RNA probe against PDGFRα, autoradiographed and photographed under dark-field illumination. In PDGF-A null cords (top, right) there were less than 5% of the normal number of progenitors compared to wild-type (top, left). The lack of progenitor cells in PDGF-A null mice was not caused by premature differentiation into oligodendrocytes, because there were very few cells positive for the myelin proteolipid protein (*PLP/DM-20*) mRNA in either PDGF-A null (bottom, right) or wild-type cords (bottom, left) at this age. Scale bar, 100 µm.
Figure 4.2 - Proliferation of oligodendrocyte progenitors in normal and transgenic spinal cords ceases after E15. Sections through the upper thoracic spinal cord were subjected to in situ hybridization with a probe to PDGFRα as in Fig. 4.1. In wild-type cords (left panels), the number of progenitor cells increased rapidly between E12.5 (arrow) and E15, but the rate of increase slowed down substantially after that, arresting between E15 and E17 (see Fig. 4.3). In hemizygous NSE-PDGF-A transgenic cords (right panels), progenitor cell number was normal up until E13.5, but approximately three times the normal number of progenitors developed between E13.5 and E15, and this increase persisted until later times. Scale bar, 100 μ.m.
Figure 4.3 - Numbers of PDGFRα⁺ progenitors in sections of spinal cords of hemizygous transgenic NSE-PDGF-A mice (squares) and their wild-type littermates (triangles). Data is plotted as mean ± SD of counts of three or four sections from each of two animals of each age and genotype from separate litters. Error bars are not shown when the SD is less than the size of the symbol. Proliferation ceases after E15 in both wild-type and transgenic cords.
4.2.3 O-2A progenitor proliferation arrest reflects both a slowing of the cell cycle and an increase in cell death

We used BrdU incorporation in vivo to estimate the cell cycle times of O-2A progenitors during embryonic and early postnatal development. We gave a single pulse of BrdU by intraperitoneal injection of either pregnant females or postnatal animals, then 2 hours later dissociated and cultured spinal cords from the embryos or pups, and subsequently counted the number of BrdU\(^+\) O-2A progenitors as a percentage of the total number of O-2A progenitors. As explained below, this “BrdU labeling index” can be used to estimate the cell cycle times. In these and all subsequent experiments in culture, O-2A progenitors were identified using immunofluorescence with an antibody against the NG2 chondroitin sulphate proteoglycan (see Chapter 2; Nishiyama et al., 1996; Stallcup and Beasley, 1987).

At E13, when in situ hybridization indicates that the number of O-2A progenitors present in the cord is increasing rapidly, 80% of these cells were labeled with a single 2 hour pulse of BrdU. However, when a similar experiment was performed one day later, at E14, the number of cells labeled by a 2 hour pulse of BrdU dropped to around 30%, and by E17 through to P3 this number stabilized at \(\sim 17\%\) (Figure 4.4). These results indicate that there is a substantial increase in the cell cycle time of O-2A progenitors between E13 and E17.
Figure 4.4 - The progenitor cell division cycle slows down markedly before birth. Pregnant female mice or young postnatal animals were given a single intra-peritoneal injection of BrdU. The embryos or pre-weanlings were killed two hours later and dissociated spinal cord cells were cultured on glass coverslips before immunolabeling with anti-NG2 proteoglycan (a marker of oligodendrocyte progenitors) and anti-BrdU (upper and lower micrographs, respectively). BrdU\(^+\), NG2\(^+\) double-positive progenitor cells (e.g. arrows) were counted and plotted as a proportion of the total NG2\(^+\) progenitor population. The BrdU labeling index fell from around 80% to around 20% between E13 and E17 in both wild-type (triangles) and hemizygous transgenic NSE-PDGF-A animals (squares), corresponding to an increase in cell cycle time from about 6 to 22 hours (see Fig 4.5, 4.6, 4.7). Each data point represents the mean ± s.d. of at least two and up to six animals (each in triplicate) from two litters.
Figure 4.5 - The entire progenitor cell population is cycling as a single population. Pregnant females were given sequential injections of BrdU at four-hour intervals (vertical arrows), starting at E14 (left) or E17 (right), and the BrdU labeling indices of NG2-positive progenitor cells determined as for Fig. 4.4. Close to 100% of progenitor cells can be labeled with BrdU both in wild-type (triangles) and hemizygous transgenic NSE-PDGF-A embryos (squares) at both E14 and E17, indicating that all of the progenitors were actively cycling at these ages. The time taken for 100% of progenitors to label with BrdU was longer in wild-type embryos than transgenic embryos at E14 (left panel), indicating that the cell cycle is shorter in the transgenics at this age. The time taken for 100% of progenitors to label at E17 is longer than at E14 (right panel), indicating that the cell cycle slows down after E14 in both wild-type and transgenics. Moreover, the rate of BrdU incorporation (and hence the rate of cell division) is the same in wild-types and transgenics at E17 (~22 hours, right panel). Each data point represents the mean ± s.d. from at least two and up to ten animals (each in triplicate) from one or two litters. Error bars are not shown where the s.d. is less than the size of the symbol.
To confirm that the BrdU labeling index in these single injection experiments reflects the rate of division of the O-2A progenitor population as a whole, we performed serial BrdU injections into pregnant females at 4 hour intervals, starting at E14 and at E17. The number of BrdU⁺/NG2⁺ progenitors was determined at various times after the first injection. Essentially all of the progenitors could be labeled by sequential BrdU injections both at E14 and E17, demonstrating that all of the progenitors were actively engaged in the cell cycle (Figure 4.5). The BrdU labeling index increased in a linear fashion with time after first injection, providing evidence that the whole population of progenitors was cycling together at the same rate. The fact that it took longer to cumulatively label 100% of the progenitors at E17 than at E14 confirmed that the cell cycle had indeed slowed down between these ages.

From the single and multiple BrdU injection experiments, we are able to calculate cell cycle times at various ages from three independent sets of data: i) the single injection experiments (Figure 4.6) ii) the multiple injection experiments (Figure 4.7) and iii) counting cells in sections at various ages by in situ hybridization with PDGFRα. All these calculations are consistent with O-2A progenitor cell cycle times of ~6-8 hours at E13, ~14 hours at E14 and ~ 24 hours at E17.

I ignored the small correction needed for the fact that the BrdU pulse in the single injection experiments is not instantaneous, as when we repeated these experiments with a 15 minute BrdU pulse there was no significant change in the number of cells labeled (A. Hall, personal communication). I also assumed that the length of S phase is invariant.
at about 4 hours. This is the case for mouse embryonic cortical precursor cells (Takahashi et al., 1995), and although the data from the cumulative labeling experiments are not sufficient to obtain an accurate estimate of $T_s$, back-extrapolation (Figure 4.7) is consistent with a value for $T_s$ of around 4 hours.

From these calculations we can therefore be fairly confident that after O-2A progenitors are initially specified at E12.5 in the spinal cord, they start to rapidly divide about once every 6-8 hours; however this rate quickly drops off and then stabilizes after E17 at about one division per day.

Absolute O-2A progenitor numbers reach a plateau by E17, even though they are still dividing once a day. It seems likely that an increase in cell differentiation or death must account for this stabilization in cell number. I looked for dying progenitor cells in spinal cord sections by in situ hybridization with PDGFRα combined with propidium iodide (PI) to visualize cell nuclei. At E15 I was unable to detect any PDGFRα+ cells with pyknotic nuclei (i.e., undergoing apoptosis), whereas at E17 I did observe dying, PDGFRα+ cells (not shown). I also found small numbers of pyknotic GC+ oligodendrocytes at E17 (see Chapter 5), and although these dying cells were present in very small numbers (~1-2 cells per 15μm section), I presume that stabilization of O-2A progenitor cell number in the embryonic spinal cord is a result of dramatic slowing of the cell cycle coupled with an increase in cell death.
With an instantaneous BrdU pulse, only the cells in S phase will be labeled.

=> The proportion of cells labeled, \( L = \frac{T_S}{T_c} \) where \( T_S \) is the length of S phase and \( T_c \) is the cell cycle time.

=> Cell cycle time,
\[
T_c = \frac{T_S}{L} \approx \frac{4}{0.77} \approx 5\text{ hours at E13}
\]
\[
T_c = \frac{T_S}{L} \approx \frac{4}{0.28} \approx 14\text{ hours at E14}
\]
\[
T_c = \frac{T_S}{L} \approx \frac{4}{0.17} \approx 24\text{ hours at E17} \quad \text{(see Figure 4.4)}
\]

This assumes that S phase is invariant at about 4 hours (see Takahashi et al (1995))

Figure 4.6 - Labeling of a dividing cell population by BrdU incorporation.
Figure 4.7 - Cumulative labeling of a dividing cell population by BrdU incorporation

A. Instantaneous pulse of BrdU labels all cells in S phase

B. Time taken to cumulatively label all cells, \( t = T_c - T_s \)  (Nowakowski et al (1989))

For an idealized, linear cumulative labeling experiment:

Labeling index \( L = mt + I \)  (where \( m = \) gradient
\( I = \) intercept
\( t = \) BrdU exposure time)

and at \( t = 0 \) (i.e. instantaneous BrdU pulse) \( L = T_s / T_c = I \)  (see Figure 4.6)

=> At \( L = 1 \) (i.e. all cells just labeled) \( 1 = mt + T_s / T_c \)

\[ t = T_c - T_s \]  (see above)

=> Gradient of cumulative labeling, \( m = (1 - T_s / T_c) / (T_c - T_s) \)

\[ = 1 / T_c \]

The first pulse of BrdU labels all the cells in S phase at that time. If multiple pulses are given, as long as the interval between pulses is less than the length of S phase, the time taken to label all cells in a homogeneously dividing population will be \( T_c - T_s \).

Simple algebra then shows that since the intercept in such an experiment is equal to \( T_s / T_c \) (Figure 4.6), the gradient will be \( 1 / T_c \). Therefore dividing the intercept by the gradient will give us the length of S phase (\( T_s \)).
=> Intercept / gradient = $T_S$
4.2.4 PDGF becomes limiting in the embryonic spinal cord and causes slowing of the O-2A progenitor cell cycle

Why does the O-2A progenitor cell cycle slow down so markedly and over such a short period of time? A possible explanation might be that the concentrations of extracellular mitogens such as PDGF become limiting for proliferation of these cells. If so, increasing the supply of PDGF in NSE-PDGFA transgenic mice might be expected to overcome this slowing of the cycle. These mice express human PDGF-A transgene mRNA in neurons from before E11, which results in an increase in the number of oligodendrocyte progenitors at E15 (see Chapter 3). I have been unable to demonstrate expression of the PDGF-A transgene at the protein level by Western blotting or immunofluorescence; however we are confident that transgene-derived PDGF-A peptide is produced and secreted from neurons because we have observed a phenotype consistent with neuronal expression of the transgene in the retina of three independent transgenic lines (#64, #75 and #82) (Fruttiger et al., 1996). In addition, we had already demonstrated protein expression of the closely related transgene NSE-PDGF-AKDEL (Chapter 3), and the observation that this transgene, which encodes a cell retained protein, does not elicit a phenotype provides a strong genetic argument that the NSE-PDGF-A phenotype is dependent on the secretion and function of the transgene product.

In situ hybridization with a probe against PDGFRα demonstrated that until E13.5 there was no significant difference between wild-type and NSE-PDGFA spinal cords with respect to O-2A progenitor cell development. However, by E15 there were more than three times the number of O-2A progenitors in the cords of NSE-PDGF-A mice
compared to their wild-type littermates (805 ± 71 compared to 246 ± 18, data from four sections from each of two animals of each genotype) (Chapter 3; Figure 4.2 and 4.3). The number of O-2A progenitors reached a plateau before birth, just like wild-types, except that there were approximately three times more cells at steady state in the transgenic.

When cell cycle analysis was performed on the transgenic spinal cords, the results reflected the data obtained from the in situ hybridization study. Initially, at E13, O-2A progenitors in the transgenic were cycling at the same rapid rate (~6-8 hours) as in the wild-type. However, by E14 the transgenic cell cycle slowed down, although not as much as the wild-type, so that at this age the progenitors were cycling faster in the transgenic than in the wild-type. Similarly, at E17, progenitors from transgenic mice were still cycling slightly faster than their wild-type counterparts. By P3 however, O-2A progenitors from both transgenic and wild-type were dividing at the same low rate, about once a day (Figure 4.4) Therefore there is a window of time, between E13.5 and birth, when progenitors are cycling slightly faster in the transgenic; this allows them to undergo more divisions in the transgenic within the same period of time and their numbers build to greater than normal levels.

At E13, therefore, just after the PDGFRα⁺ cells are first specified in the ventral ventricular zone, these cells are dividing as fast as they can. PDGF is presumably present in saturating amounts, because artificially raising its level in transgenic mice has no effect on O-2A progenitor cell cycle time. By E14 and later the concentration of
PDGF in the wild-type spinal cord becomes limiting for O-2A progenitor cell division, because increasing the PDGF supply causes the cells to cycle faster than in the wild-type. However, PDGF eventually becomes limiting even in the hemizygous transgenic spinal cord, because increasing PDGF expression further still in homozygous transgenic animals increases O-2A proliferation further and generates yet more cells at steady-state (approximately seven times normal in homozygotes compared to three times in hemizygotes) (Figure 4.8).

4.2.5 O-2A progenitor cell number is proportional to the level of expression of PDGF-A

The experiments with NSE-PDGF-A transgenic mice show that the number of O-2A progenitor cells at steady-state (E17 and after) is determined by the level of expression of PDGF-A, and suggest that the number of progenitors in the spinal cord will always reach a steady state, whatever the level of expression of PDGF, and that this steady state number will be proportional to PDGF expression. To test this, I examined five different genotypes of mice with respect to PDGF-A: PDGF homozygous knockouts (no functional PDGF), PDGF heterozygous knockouts (presumably 50% of wild-type expression), wild-type mice, hemizygous NSE-PDGF-A mice and homozygous NSE-PDGF-A mice (twice the transgene copy number of hemizygous animals). I performed in situ hybridization with a PDGFRα probe at E15 in order to count the number of O-2A progenitors, and measured PDGF-A mRNA expression by RNase protection assay (RPA) followed by densitometry of the resultant autoradiographs.
Figure 4.8 - PDGF dose dependency of oligodendrocyte progenitor proliferation in vivo. Oligodendrocyte progenitors in P11 wild-type (top), hemizygous (middle) and homozygous (bottom) NSE-PDGF-A transgenic spinal cords were visualized by in situ hybridization with a DIG-labeled probe for PDGFRα. There are approximately three times the normal number of progenitors in hemizygotes and seven times the normal number in homozygotes. Scale bar, 100μm.
When the number of progenitors in the various genotypes was plotted against the total level of PDGF-A mRNA expression (the sum of endogenous and transgene-derived mRNA), there was an approximately linear relationship between the two variables (Figure 4.9).

These data suggested that the stimulation of progenitor cell proliferation seen in the transgenics might be essentially unsaturable; raising PDGF expression to higher and higher levels might continue to increase steady-state progenitor cell numbers, the only limit being the capacity of the spinal cord to accommodate more cells. To attempt to test this, I crossed two independent lines of NSE-PDGF-A mice to step up PDGF expression still more. I was no longer able to count 0-2A progenitors in sections at these higher PDGF expression levels because of the extremely high progenitor cell densities, however it is clear by inspection of the sections that even at the highest level of expression (75, 82, + / 75, 82, +) the number of progenitors is still rising in response to an increase in PDGF supply (Figure 4.10). In fact, this level of PDGF expression in the nervous system is lethal to postnatal animals for reasons we have not yet ascertained. We have yet to find a newborn mouse born of (75, 82, + / +) x (75, 82, + / +) parents with a genotype consisting of more than two transgenes. The 'highest' genotype which survives to adulthood (75 +/-, 82 +/-) develops spinal curvature and possibly also motor and sensory defects. We therefore decided not to cross in any further copies of the PDGF transgene from other lines, but it is interesting to note that simply overexpressing a paracrine growth factor, without mutating or otherwise
interfering with intracellular cell cycle controls, can cause a cell population to grow to such an extent that it can be lethal to the host.
Figure 4.9 - Progenitor cell number is proportional to the PDGF supply in a range of transgenic backgrounds. (A) Progenitor cells, visualized by in situ hybridization with a DIG-labelled probe to PDGFRα, in E15 spinal cords of homozygous PDGF-A knockout (-/-), heterozygous knockout (-/+), wild-type (+/+), hemizygous NSE-PDGF-A<sub>S</sub> (+,tg/+ ) and homozygous NSE-PDGF-A<sub>S</sub> transgenic mice (+,tg/+,tg). Scale bar, 100 μm. (B) RNase protection assay for PDGF-A transcripts in E15 spinal cords of transgenic and wild-type mice. Hybridization reactions contained a mixture of probes for endogenous (mPDGF-A), transgenic (hPDGF-A) PDGF-A or a probe for GAPDH as a control for RNA amounts. Note that our probe for mPDGF-A does not distinguish between wild-type transcripts and non-functional hybrid PDGF-neo transcripts in knockout mice. (C) Plot of progenitor cell number versus functional PDGF-A transcript abundance at E15. We assumed no functional PDGF-A mRNA in the homozygous knockout and half the wild-type amount of functional mRNA in the heterozygous knockout. The total amounts of mRNA in the transgenics were measured with a phospho-imager from the data in Fig. 4.9B. The dose-response is approximately linear, arguing that PDGF-A directly controls oligodendrocyte progenitor cell number in the spinal cord.
Figure 4.10 - Numbers of oligodendrocyte progenitors continue to rise in response to a step-wise increase in the supply of PDGF. Different transgenic backgrounds (NSE-PDGF-A lines #75 and #82) of mice were interbred to obtain mice containing increasing numbers of copies of the PDGF-A transgene. Sections of E15 spinal cords from these mice were then subjected to in situ hybridization either with a probe against PDGF-A to visualize expression of the transgene, or PDGFRα to visualize progenitors. The number of progenitors continued to rise even at the highest level of PDGF supply, showing that even at this level the PDGF concentration was not saturating for progenitor proliferation.
4.3 DISCUSSION

4.3.1 PDGF-AA is necessary for proliferation of O-2A progenitors in vivo

I have used a transgenic approach to analyze the role of PDGF in the development of O-2A progenitors in the mammalian spinal cord. O-2A progenitors first appear in PDGF-A knockout mice as in the wild-type at E12.5, but they fail to proliferate normally and there is a dramatic reduction in the number of O-2A progenitors throughout embryonic and early postnatal development. However the deletion of PDGF-B has no discernable effect, indicating that PDGF-AA, not -AB or -BB, is crucial for proliferation, but not initial specification, of these cells during normal development. PDGF-B is known to be produced by capillary endothelial cells in the CNS from early embryogenesis onwards (Lindahl et al., 1997a; Mudhar et al., 1993), but it is presumably unavailable to O-2A progenitors in the developing spinal cord. Although spinal cord neurons express PDGF-B from around birth (Sasahara et al., 1991), it proved impossible to assess the importance of this neuron-derived isoform as PDGF-B knockout mice die at birth.

The number of mature oligodendrocytes in the postnatal spinal cord is also greatly reduced in PDGF-A knockout mice, although it is still at least 10% of the number seen in the wild-type (Fruttiger et al., 1999). It is not yet known whether this small but significant number of oligodendrocytes in the knockout spinal cord are derived from the residual O-2A progenitor population, or whether they represent a separate PDGF-independent, PDGFRα oligodendrocyte lineage, the existence of which has been suggested (Peyron et al., 1997; Timsit et al., 1995). However there is other evidence that the PDGFRα+, PDGF-dependent O-2A progenitors are the only class of oligodendrocyte precursors in the spinal cord (Hall et al., 1996; Pringle et al., 1998). There is also evidence (Calver et al., 1998; Chapter 5, this Thesis) that the final number of oligodendrocytes in the spinal cord is independent of progenitor cell number, but depends solely on selective cell survival. Indeed, it has been shown that 50% of all newly formed oligodendrocytes die in the developing optic nerve, presumably as a
result of competition for limited quantities of survival factors (Barres et al., 1992a), so it is quite possible that a decrease in naturally occurring cell death of oligodendrocyte lineage cells could account for all the oligodendrocytes seen in the knockout animal, without the need to postulate a second lineage. If this is correct one might expect that eventually the number of oligodendrocytes and level of myelination in the PDGF-A knockout would reach wild-type levels. Unfortunately the PDGF-A null animals do not survive to adulthood, so this hypothesis remains to be tested.

It is not known what drives the low level of proliferation of O-2A progenitors that we see in the PDGF-A knockout. It is possible that PDGF-B could partially substitute for PDGF-A; this could possibly be tested by studying PDGF-A/B double knockout mice, as long as this genotype is not lethal to the early embryo. Alternatively, another growth factor such as NT-3 may have some measure of redundancy with PDGF-A, as might be the case for the many other PDGFRα+ lineages that are relatively unaffected in the PDGF-A knockout embryo. Another explanation might be that maternal PDGF-AA crosses the placenta and partially complements the loss of embryonic PDGF. This might explain why the several developmental defects of PDGF-A knockout mice seem to be exacerbated after birth.

4.3.2 O-2A progenitor proliferation and final cell number is controlled by the supply of PDGF

In normal mouse spinal cords, O-2A progenitors proliferate rapidly after their initial specification at E12.5, before reaching a steady state number between E15 and E17. In contrast, O-2A progenitors in NSE-PDGF-A spinal cords go through one or two extra divisions before reaching steady state, giving rise to three times the normal number of cells at E17. This demonstrates that the rate of PDGF supply is limiting for O-2A progenitor proliferation in the normal developing spinal cord. In fact it seems possible that PDGF can never be anything but limiting, because the number of progenitors continues to increase as the transgene copy number and level of expression is increased.
by interbreeding with another transgenic strain. The ultimate limit on progenitor cell number seems not to be the capacity of the cells to proliferate, but the capacity of the spinal cord to contain them, because the CNS phenotype is lethal to postnatal animals before the level of PDGF expression is reached at which O-2A proliferation is saturated. This is in some ways surprising, as it would be expected that an organism would have some fail-safe mechanism to check proliferation at the cell cycle control level, as exemplified by the accepted view that carcinogenesis is a multi-factorial process. However, there is no evidence of malignancy in these PDGF-induced hyperplasias.

4.3.3 Why does the progenitor cell cycle slow down?

The surprising finding that O-2A progenitor cell proliferation in the spinal cord ceases before birth prompted us to compare cell cycle times by BrdU incorporation in utero. This showed that after their initial appearance in very small numbers at E12, O-2A progenitors proliferate rapidly ($T_c=6-8$ hours) as they migrate away from the ventricular zone. This proliferation soon slows down as the cells increase in number, before stabilizing ($T_c=24$ hours) at around E17, at which point the population size also reaches a plateau. O-2A progenitor numbers reach a steady state, even though the cells are still cycling, because the rate of cell death increases until it equals the rate of generation of new progenitors. This is presumably due to an increased demand for survival factors as the cell population increases, one of which could be PDGF itself (Barres et al., 1992a).

The increase in the number of O-2A progenitors at steady state in hemizygous NSE-PDGF-A transgenic mice is due to a transient change in the dynamics of the cell cycle. Although the cells start off cycling at the same fast rate in wild-type and transgenic spinal cords, and end up cycling at the same, much slower rate, the period over which this occurs is extended in the transgenic, resulting in more cells being generated over the same period of time.
So why does the cell cycle slow down, both in the wild-type and the transgenic spinal cord? The simplest explanation would be that PDGF expression declines between E13 and E17, yet there is no evidence that this is so, at least at the mRNA level (Chapter 3, Figure 3.9). It seems likely that the reason the cell cycle slows down is more interesting than this. Bearing in mind that a requirement of any model is that final cell number is roughly proportional to PDGF expression level, several possibilities suggest themselves, three of which are described in the following sections.

4.3.4 Autocrine inhibition of O-2A progenitor proliferation

It has been shown that members of the TGF-β superfamily are anti-proliferative for O-2A progenitors, and that progenitors synthesize and secrete TGF-β in vitro (McKinnon et al., 1993). It is possible, therefore, that as the size of the O-2A progenitor cell population increases in vivo, the extracellular concentration of growth inhibitors might accumulate to such a level that PDGF-dependent proliferation was inhibited. Increasing the supply of PDGF might delay this inhibition by requiring a correspondingly higher concentration of growth inhibitors to neutralize PDGF-driven mitogenesis, and generate a greater number of O-2A progenitors. This model seems unlikely as the concentration of the anti-mitogen would be highest at the surface of the cell which was producing it, implying that its effect would likely be relatively independent of the population as a whole. In addition, preliminary results in our laboratory suggest that the O-2A progenitor population is unaffected in TGF-β1 knockout mice (P. van Heyningen and W.D. Richardson, unpublished observations).

Secreted in vivo antagonists of receptor tyrosine kinases (RTKs) have also recently been described, both in Drosophila and in mice. Argos, a secreted Drosophila protein
containing a single EGF motif, has been shown to restrict the level and duration of
Drosophila EGF receptor signaling by a novel inhibitory feedback loop. Activation of
the EGF receptor by its ligand, Spitz, results in up-regulation and secretion of Argos,
which subsequently antagonizes EGF receptor signaling in nearby cells, thus restricting
the actions of Spitz to cells close to its site of production (Golembo et al., 1996;
Schweitzer et al., 1995). Interestingly, Argos acts in a paracrine fashion, such that EGF
receptor signaling is not inhibited in cells in which Argos is induced. It is not known
why this happens, but it may be due to the fact that cells that go on to express Argos
have been exposed to relatively higher levels of Spitz than their neighbors, thus causing
an intrinsic change rendering them unresponsive to the inhibitory effects of Argos.

The most convincing example of a naturally occurring vertebrate RTK antagonist is
angiopoietin-2 (Ang-2). This protein appears to compete with angiopoietin-1 (Ang-1),
both in vitro and in vivo, for binding to the Tie-2 RTK (Maisonpierre et al., 1997), and
appears to act as a competitive antagonist of Ang-1 in endothelial cells. Interestingly,
Ang-2 can act as an agonist of Tie-2 in some non-endothelial cells, suggesting that
endothelial cells express accessory components required for antagonist activity. Ang-1
has been shown to be crucial for normal angiogenesis in the mouse (Suri et al., 1996),
and a transgenic study has shown that overexpression of Ang-2 disrupts blood vessel
formation in the mouse embryo presumably by antagonizing signaling of Ang-1 via the
Tie-2 RTK (Maisonpierre et al., 1997).
These antagonists all appear to be involved in differentiation and/or maturation pathways mediated by RTKs; an anti-proliferative effect by such a molecule has yet to be demonstrated in vivo. Although no such molecule has yet been identified for the PDGF family of receptors, we cannot rule out that such a molecule is expressed by oligodendrocyte progenitors in vivo and inhibits their proliferation in response to PDGF, and one could postulate that such a mechanism would result in the self-limiting proliferation of the progenitor cell population. However, as with an anti-mitogen, an argument against this model is that a progenitor-derived antagonist would necessarily function in an autocrine manner and would therefore be unlikely to influence the population as a whole, as its concentration would be highest at its site of production.

4.3.5 Decreasing cellular response to PDGF stimulation

Another explanation for the slow-down of progenitor cell division is that the sensitivity of O-2A progenitors to the mitogenic effect of PDGF decreases as they proliferate, in other words their PDGF dose-response shifts to the right (higher PDGF concentrations). In this case a constant supply of PDGF could initially be saturating for proliferation but subsequently become limiting, thus causing the cell-cycle to slow down. Increasing the rate of PDGF supply in this model would enable the growth factor to remain saturating for a longer time, thus delaying deceleration of the cell-cycle and resulting in an increase in progenitor cell number (Figure 4.11A).
Figure 4.11 - (A) Decreasing cellular response to PDGF stimulation versus (B) decreasing extracellular concentration or activity of PDGF; two models to explain the slowing-down of the cell cycle.
4.3.6 Decreasing extra-cellular concentration or activity of PDGF

A simple, attractive explanation that accounts for all our observations is that proliferation of O-2A progenitors is a self-limiting process - the number of progenitors rises until the rate of consumption of PDGF, by receptor binding and internalization, is equal to the rate of supply of PDGF by other cells. Since the rate of supply of PDGF is higher in NSE-PDGF-A mice, the number of O-2A progenitors can increase to a higher level than wild-type before their rate of consumption of PDGF matches the rate of supply and the extracellular concentration of PDGF drops to the point where it becomes limiting. This would also explain why the final cell cycle rates are the same in transgenic and wild-type mice. Although the higher rate of supply of PDGF in the transgenic mice can support a higher number of proliferating cells, in both cases the ultimate rate of PDGF consumption is equal to the rate of supply, therefore the supply of PDGF to each individual proliferating cell will be the same in the transgenic spinal cord as in the wild type (Figure 4.11B).

This model of competition between progenitor cells for fixed supplies of mitogenic factors is analogous to the competition that occurs between post-mitotic cells (e.g. neurons) for limited supplies of survival factors.

This model might also explain why we have been unable to demonstrate the presence of the transgene-derived PDGF-A protein either by Western blotting or by immunofluorescence. If PDGF is being consumed at the same rate as it is being
produced, its effective extracellular concentration will be very small and possibly beyond the limits of detection of conventional techniques.

4.3.7 Competition for limiting mitogens in vivo - a calculation

That competition for mitogens is the driving force behind supposed ‘contact-mediated’ or ‘density-dependent’ inhibition of growth in vitro has been suspected for many years, as demonstrated by the observation that the maximum density of 3T3 cell monolayers is directly proportional to the concentration of serum in the medium (Holley and Kiernan, 1968). Some simple arithmetic demonstrates that the concept of competition for mitogens is entirely feasible.

At a PDGF-AA concentration of 10ng/ml (3x10^{-10}M, saturating for mitogenic activity on O-2A progenitors; Richardson et al., 1988, Pringle et al., 1989), there are approximately 2x10^{11} molecules of PDGF/ml. There are approximately 2x10^{5} PDGF receptors on an O-2A progenitor cell (Hart et al., 1989b), and so in a typical culture of 10^{6} cells in 2-3ml of medium (for example in a 60mm dish), numbers of ligand molecules outnumber numbers of receptors by only a few fold. We estimate that a perinatal mouse spinal cord contains about 10^{7} O-2A progenitors (10^{12} receptors) in 1ml of tissue, although the extracellular space must be much less than this. Therefore it is highly likely that there are more receptors than there are ligand molecules, and that competition for these ligand molecules must be severe.
4.3.8 Controls on cell cycle time are a general feature of embryonic development

Slowing of the cell cycle is a common feature of proliferating cell populations in the mammalian CNS. For example, Caviness and colleagues have carried out a systematic study of the cell cycle time of neural precursors in the mouse cerebral cortex between E11 and E17 (Takahashi et al., 1994; Takahashi et al., 1995). They showed that the length of the cell cycle for these cells increases from around 8 to 24 hours during this period, with most of the variation occurring in G1. The reason for this slow-down is not known, but it is quite possible that it is the result of competition for limiting mitogens.

Another striking example of the use of the cell cycle to control cell population size is with so-called 'aggregation chimeras' of early mouse embryos. In these experiments up to four 8-cell embryos can be mechanically aggregated and then re-implanted into the oviduct as a larger-than-normal embryo; these chimeras often are born as normal sized animals (Petters and Markert, 1980). Size regulation occurs shortly after implantation when cells in the aggregation chimeras divide less rapidly than in normal embryos, enabling cell numbers in the normal embryos to 'catch up' with the chimeras (Lewis and Rossant, 1982). Although the underlying mechanism is not understood, it is possible that the size of the embryo is controlled at the level of cell number by limiting amounts of a growth factor supplied by a fixed number of cells in the uterine wall. These examples raise the possibility that competition for limited supplies of mitogenic factors might be a common way of controlling cell numbers during development.
5. CHAPTER FIVE

OLIGODENDROCYTE NUMBER AND DISTRIBUTION IS
CONTROLLED ENTIRELY BY SELECTIVE CELL SURVIVAL
5.1 INTRODUCTION

In Chapter 4 I described the effect of varying the levels of PDGF-A on the proliferation rate and final steady-state numbers of O-2A progenitors in the mouse spinal cord, and concluded that the cells limit their own growth by consuming growth factors, with the result that the final number of progenitors is directly proportional to the level of expression of PDGF-A. I next decided to investigate the effect of this over-production of O-2A progenitors on the differentiation and final number of post-mitotic oligodendrocytes in the spinal cord.

Oligodendrocytes first appear in the mouse spinal cord in the ventral marginal zone at around E17/18 (Yu et al., 1994). Their appearance subsequently spreads to the dorsal and lateral funiculi about a day later, and the number of oligodendrocytes in these regions rises rapidly after birth as myelination proceeds (Jordan et al., 1989; Yu et al., 1994). It is not known what causes the oligodendrocytes to differentiate, neither is it known what causes the accumulation of oligodendrocytes specifically in the marginal axon tracts, when their precursors, the O-2A progenitors, are distributed more-or-less evenly throughout the cord. It is reasonable to suppose that signaling between axons and oligodendrocytes or their progenitors is important for oligodendrocyte development, and there is some evidence to suggest that this is the case (reviewed in Chapter 1, and Barres and Raff, 1994). In particular, neurons have been shown to express several survival factors for O-2A progenitors and oligodendrocytes in vitro and in vivo, including PDGF (Mudhar et al., 1993; Yeh et al., 1991), NT-3 (Schecterson and Bothwell, 1992), IGF-1 (Bondy et al., 1992) and GGF-2/neuregulin (Canoll et al.,
It has also been reported that the presence of axons is necessary for oligodendrocyte survival in the optic nerve (Barres et al., 1993a), although this remains controversial as another group have more recently reported that oligodendrocyte survival is unaffected following Wallerian degeneration (Butt and Kirvell, 1996). It is not known whether neuron-derived growth factors are normally released from or associated with axons.

In the normal developing optic nerve, 50% of oligodendrocytes die by PCD soon after they differentiate (Barres et al., 1992a). This is presumably as a result of competition for limiting amounts of survival factors, as exogenous delivery of PDGF (Barres et al., 1992a), CNTF (Barres et al., 1993a), IGF-1 (Barres et al., 1993a) or NT-3 (B. Barres, personal communication) can prevent this death in the short term. All of these factors have been shown to be survival factors for O-2A lineage cells in vitro, although oligodendrocytes down-regulate PDGF receptors and become unresponsive to the ligand soon after they differentiate (Hart et al., 1989b; McKinnon et al., 1990). It has been suggested that competition for neuron-derived survival factors could be a way of matching the number of oligodendrocytes to the number of axons they have to myelinate, analogous to the neurotrophin-dependent number matching between neurons and their targets (Barres and Raff, 1994).

I therefore reasoned that I might obtain some insights into oligodendrocyte survival by studying the development of oligodendrocytes in the NSE-PDGF-A transgenic mice, in
which the number of O-2A progenitors has been greatly increased by over-expressing PDGF-A in neurons.

5.2 RESULTS

5.2.1 Overexpression of PDGF causes a sustained increase in steady-state numbers of O-2A progenitors

As I have described in previous chapters, NSE-PDGF-A transgenic mice overexpress PDGF-A in many mature neurons in the CNS from before E11. This does not have an effect on development of O-2A progenitors up to E13.5, presumably because PDGF is present in saturating amounts at the early times. After E13.5 however, PDGF becomes limiting in the wild-type spinal cord and the O-2A progenitor cell cycle begins to slow down. The slow-down is delayed in NSE-PDGF-A mice, allowing completion of more divisions than in wild-type mice over the same period of time, and a consequent increase in the steady-state number of progenitors. Since the transgene is expressed in neurons through to adulthood, this phenotype (more progenitors) is maintained throughout postnatal life (see Chapters 3 and 4).

5.2.2 Excessive and ectopic production of oligodendrocytes in NSE-PDGF-A transgenic mice

Even though the number of spinal cord O-2A progenitors reaches a steady state in both normal and NSE-PDGF-A transgenic mice by E16/17, these cells still divide once every day (Chapter 4). Therefore to maintain a steady state the rate of division must be
matched by the rate of differentiation and/or death. In order to test whether the increased proliferation of O-2A progenitors observed in NSE-PDGF-A transgenic mice was matched by an increase in oligodendrocyte differentiation, I performed in situ hybridization on spinal cords of wild-type and transgenic animals with a probe against the myelin-specific transcript of proteolipid protein and its alternatively spliced form DM-20 (PLP/DM-20). Before E17 there was no obvious differentiation of O-2A progenitors in either the wild-type or the transgenic spinal cord. At E17 small numbers of oligodendrocytes were present in the ventral part of the wild-type cord, whereas in the NSE-PDGF-A cord at E17 there were many more PLP/DM-20-positive cells, not only in ventral areas but also in the dorsal and lateral funiculi and some in the central grey matter. In the wild-type, very few oligodendrocytes ever appear in the central grey matter, which mainly contains neuronal cell bodies. By E19 the transgenic phenotype had become much more pronounced, with a large increase in the number of ectopic oligodendrocytes differentiating in both the grey and white matter of the transgenic spinal cord. The phenotype was confirmed with a probe against another major myelin transcript, myelin basic protein (MBP). When in situ hybridization was performed a week later at P6, however, the situation seemed to have resolved and there appeared to be no difference between the wild-type and the transgenic animals with respect to oligodendrocyte number and location (Figure 5.1).
Figure 5.1 - Excessive and ectopic production of oligodendrocytes in NSE-PDGF-A spinal cords. Oligodendrocytes were visualized in sections of spinal cord from wild-type (wt) and hemizygous transgenic (tg) mice by in situ hybridization with probes against MBP or PLP/DM-20, and photographed under dark-field illumination. At E19, there are many more MBP-positive, PLP/DM-20-positive oligodendrocytes present in the transgenic spinal cord than in the wild-type cord. Many of the extra oligodendrocytes in the transgenic are found ectopically in the central gray matter of the cord. However, within one week (P6) this situation resolves and the number and distribution of oligodendrocytes in the transgenic cord appears normal. Scale bar, 100 μm.
Figure 5.2 - The number of oligodendrocytes surviving postnatally is independent of the number of oligodendrocyte progenitor cells. Numbers of PDGFRα⁺ progenitor cells in sections of wild-type (wt), hemizygous transgenic (tg) and homozygous transgenic (tg/tg) E15 spinal cords are displayed in comparison with the numbers of PLP/DM-20-positive oligodendrocytes in sections of P6 cords (means ± SD of a total of eight to twelve sections from two or three animals of each genotype). Despite the approximately seven-fold increase in the number of oligodendrocyte progenitor cells in homozygous transgenic animals, the number of surviving oligodendrocytes is completely normal.
I then counted the number of PLP/DM-20-positive cells at P6 in the wild-type, hemizygous and homozygous (where the phenotype is even more pronounced) transgenic spinal cords, and compared them to the number of O-2A progenitors at steady state in these animals. Even with a seven-fold increase in the number of O-2A progenitors in the homozygous mouse, the number of oligodendrocytes at P6 is the same as in the wild-type (Figure 5.2). Thus, progenitor cell number and oligodendrocyte number are controlled independently of each other.

5.2.3 Elimination of excess oligodendrocytes by programmed cell death

To discover whether the excess oligodendrocytes in the NSE-PDGF-A mice at E19 were eliminated by PCD, I labeled sections of E19 wild-type and transgenic spinal cords with propidium iodide (PI) to visualize pyknotic cells, and anti-galactocerebroside (GC) to identify oligodendrocytes (Figure 5.3). There was a small number of GC-negative dying cells (presumably neurons) in both wild-type and transgenic cords, approximately 25 per section. In the wild-type, there were also one or two dying GC-positive cells per section. However, there were many more dying GC-positive cells in the hemizygous transgenic, and still more in the homozygous transgenic (Figure 5.4). In fact, the vast majority of the excess oligodendrocytes in the transgenic turned out to be pyknotic, and many of these were in the central grey matter, presumably corresponding to the ectopic PLP/DM-20-positive oligodendrocytes that disappear between E19 and P6 (see Figure 5.1).
Figure 5.3 - Superfluous oligodendrocytes in NSE-PDGF-A transgenic spinal cords are eliminated by programmed cell death. Sections of wild-type (wt), hemizygous transgenic (tg) and homozygous transgenic (tg/tg) E19 spinal cords were immunolabeled with monoclonal anti-GC to visualize oligodendrocytes and counter-stained with propidium iodide to reveal pyknotic nuclei characteristic of apoptotic cells. The lower two micrographs show a higher magnification view (left, anti-GC; right, PI) of the region indicated above by a single arrow. Double-labeled cells (dying oligodendrocytes) are indicated (arrows). Scale bars; 100 μm (above) and 20 μm (below).
Figure 5.4 - Many more dying oligodendrocytes can be detected in NSE-PDGF-A spinal cords than in wild-type. The total numbers of cells with pyknotic nuclei, as well as the numbers of GC$^+$ oligodendrocytes with pyknotic nuclei, were counted in 15 μm sections. There were many more GC$^+$ pyknotic cells (dying oligodendrocytes) in hemizygous transgenic cords (tg) than in wild-type cords, and even more in homozygous transgenics (tg/tg). There were, however, no significant differences in the numbers of GC$^-$ pyknotic cells in the transgenics compared to wild-type.
5.2.4 Superfluous oligodendrocytes are eliminated at a distinct, immature developmental stage

It became clear under further examination that there were in fact two categories of PLP/DM-20-expressing cells in the spinal cord, distinguished by their level of expression of PLP/DM-20. This is most easily seen when sections of wild-type and transgenic spinal cord from E19 and P6 were processed for in situ hybridization on the same slide. Thus all the incubation and development times were identical, and this demonstrated that the majority of the ectopic, "premature" oligodendrocytes present in the E19 transgenic spinal cord were expressing much lower levels of PLP/DM-20 than the mature, myelinating oligodendrocytes at P6 in both wild-type and transgenic (Figure 5.5). It seems likely that these E19 cells are immature oligodendrocytes, which have not yet reached the level of PLP/DM-20 expression of their mature, axon-associated counterparts. It is these faintly PLP/DM-20-positive oligodendrocytes that are preferentially eliminated by PCD in the transgenic spinal cords. Using an appropriate development time for the in situ hybridization reaction product, it was also possible to demonstrate the presence of these cells in the central grey matter of the P6 NSE-PDGF-A transgenic cord (Figure 5.5). They are not visible in Figure 5.1 due to the short exposure time used for dark field viewing of the silver grains produced with radioactive riboprobes. It therefore seems that in PDGF-overexpressing mice with increased numbers of O-2A progenitors, oligodendrocytes are continually being over-produced and 'trimmed back' throughout late embryonic and early postnatal life.
Figure 5.5 - Excess oligodendrocytes die at an early stage of differentiation. Sections of wild-type (wt), hemizygous transgenic (tg) and homozygous transgenic spinal cords (tg/tg) were subjected to in situ hybridization with a probe against PLP/DM-20 and visualized by autoradiography (three days exposure time) and bright-field microscopy. Sections from E19 (left column) and P6 (right column) spinal cords were processed and exposed in parallel, under identical conditions, so that the in situ signal intensities are directly comparable in all sections. There are two distinct populations of oligodendrocytes that can be distinguished on the basis of their PLP/DM-20 signal intensity. This is most obvious in the transgenic spinal cords; oligodendrocytes in the ventral and dorsal white matter of the P6 cords express PLP/DM-20 transcripts strongly (large arrowhead), whereas many oligodendrocytes in the central grey matter of the P6 cords as well as the majority of oligodendrocytes in the E19 cords express PLP/DM-20 at a much lower level (small arrows). The latter faint cells probably represent young (i.e. recently formed) oligodendrocytes that have not yet accumulated maximal levels of myelin gene products. It is these faint oligodendrocytes - mainly in the central regions of the transgenic cords - that are eliminated by PCD (compare Figures 5.1 and 5.3). Small numbers of “faint”, newly-formed oligodendrocytes can also be observed in wild-type spinal cords at both E19 and P6, arguing that continuous production and elimination of superfluous and ectopic oligodendrocytes is a normal feature of late embryonic and early postnatal development that is greatly exaggerated in the transgenic animals.
Therefore when the PDGF supply is increased in the developing spinal cord, leading to an increase in the steady state level of O-2A progenitors, this in turn leads to an increase in the rate of differentiation of oligodendrocytes, and an increase in the level of PCD to eliminate the excess newly-formed oligodendrocytes. The final outcome is that the number and distribution of mature, myelinating oligodendrocytes is unchanged. This is a happy result, as the number and arrangement of axons which need to be myelinated is the same in the transgenic as it is in the wild-type.

5.2.5 Elimination of excess, ectopic, newly-formed oligodendrocytes is a normal developmental process

I theorized that the phenotype which I saw in the NSE-PDGF-A transgenic mouse was in fact just an exaggeration of normal oligodendrocyte development in the spinal cord, only visible due to a saturation of the normal PCD clearance machinery. In other words, it could be that oligodendrocytes normally differentiate stochastically anywhere in the spinal cord, and at any time, but only survive when and where there are survival factors to support them. This model predicts that if it was possible to slow down or stop the clearance of dying oligodendrocytes in the normal spinal cord, this would qualitatively recreate the phenotype I see in the NSE-PDGF-A mice.

In order to achieve this I obtained mice which carried a targeted deletion in the gene coding for the ‘ets’ transcription factor PU-1. Mice homozygous for this mutation are completely devoid of all macrophage lineage cells, including microglia (McKercher et al.,
Since microglia are the ‘professional phagocytes’ of the CNS, we reasoned that in their absence the clearance time for pyknotic cells in the spinal cord might increase, allowing dying oligodendrocytes to survive long enough for us to detect them by PI staining. When sections of E18 spinal cord from these mice were stained with anti-GC, to visualize oligodendrocytes, and anti-caspase, to visualize cells undergoing PCD, a phenotype very similar to that of NSE-PDGF-A mice was observed (Figure 5.6). A large number of dying, GC-positive oligodendrocytes were present in the PU-1−/− spinal cord, many of which were in the central grey matter where few mature oligodendrocytes ever appear.

These results argue strongly that the differentiation of spinal cord oligodendrocytes is a stochastic process, and that the final oligodendrocyte population size and distribution is in fact controlled by selective cell survival rather than differentiation.
Figure 5.6 - Death of excess spinal cord oligodendrocytes is a normal developmental process. Sections of wild-type (wt) and PU-1<sup>−/−</sup> (PU-1 ko) E18 spinal cord were subjected to immunocytochemistry either with an antibody to galactocerebroside (α-GC) to visualize oligodendrocytes, or an antibody to the caspase family of proteases (α-caspase) to visualize cells undergoing programmed cell death. Many more dying oligodendrocytes can be seen in the spinal cords lacking microglia (PU-1 ko, arrows) than in the wild-type.
5.3 DISCUSSION

5.3.1 Overproduction of oligodendrocyte progenitors in NSE-PDGF-A transgenic mice

The primary effect of over-expression of PDGF-A by neurons in transgenic mice was to stimulate proliferation and increase the O-2A progenitor population size at steady state. I described this phenotype in greater detail in the last chapter. This is due to the fact that the size of the population is determined by the level of PDGF available during development, probably by a process of self-limiting proliferation. In this model, the size of the progenitor population rises in response to the saturating levels of growth factor until at some point this growth factor becomes limiting, due to the consumption of PDGF (by receptor binding and internalization) overtaking its rate of supply. This causes the average length of time the cells spend in G1 to increase, thus increasing the average cell cycle time. Subsequently the cell cycle time lengthens to the point at which the rate of division is equal to the combined rate of differentiation and death, and proliferation ceases.

5.3.2 Secondary over-production of oligodendrocytes in NSE-PDGF-A spinal cords

PLP/DM-20-positive oligodendrocytes were generated prematurely, in excess and at ectopic sites in the spinal cords of NSE-PDGF-A transgenic mice. In wild-type mice most oligodendrocytes arise in the marginal zone of the cord, with very few in the central grey matter. This is due to the fact that the axons which need to be myelinated
are mostly in the marginal zone, whereas the grey matter contains predominantly neuronal cell bodies. However in the transgenic many oligodendrocytes differentiate in the central grey matter, as well as an excess occurring in the developing white matter. It is not known what cause this premature, ectopic differentiation, although it presumably is a secondary effect of the over-production of O-2A progenitors observed in these mice. In vitro at least, optic nerve O-2A progenitors have a cell intrinsic ‘clock’ which causes them to differentiate after about eight divisions. I have shown (Chapter 4) that the increase in O-2A progenitor numbers seen in NSE-PDGF-A transgenic mice is caused by a transient increase in the average division rate of these cells between E13.5 and E17, so the premature differentiation could be caused by the clock being ‘speeded up’ in these mice. However there is recent evidence that the clock in fact measures elapsed time, not cell divisions (Gao et al., 1997). As discussed below, it is likely that the differentiation is neither premature nor ectopic, but instead is only an exaggeration of a normal stochastic process of temporal and spatial oligodendrocyte differentiation.

5.3.3 Oligodendrocyte number and distribution is controlled by programmed cell death

The most striking aspect of the phenotype in NSE-PDGF-A transgenic mice was that the excess, ectopic oligodendrocytes were all cleared soon after differentiation by PCD, with the result that the final number of mature, myelinating oligodendrocytes was completely normal. The cells seemed to be removed at an early, immature developmental stage, when they were already expressing GC but only low levels of mRNA for PLP/DM-20 and MBP. This is probably due to the fact that when O-2A
progenitors differentiate to form oligodendrocytes they lose their receptors for PDGF, and therefore must become dependent on other factors for survival. These factors are presumably present in limiting amounts, or possibly absent at the time and in the regions where I see large scale death in the transgenic.

Small numbers of dying oligodendrocytes were also observed in the wild-type spinal cord from E17 onwards, and these pyknotic cells were also distributed randomly across the cord. This suggested to us that the normal differentiation of oligodendrocytes could in fact be a stochastic process, both temporally and spatially, and that the accumulation of mature myelinating oligodendrocytes specifically in the marginal axon tracts was not due to preferential differentiation in axon tracts, as recently suggested (Hardy and Friedrich, 1996), but instead was due to the presence of axon-derived long-term survival signals. Consistent with this is the fact that axons produce long-term survival factors for oligodendrocytes in vitro (Barres and Raff, 1994).

It has recently been shown that many oligodendrocytes die in the developing rodent CNS fibre tracts at an immature stage when they express DM-20 but not yet PLP (Trapp et al., 1997), and it is also known that 50% of oligodendrocytes die in another developing fibre tract, the optic nerve (Barres et al., 1992a), again at an immature developmental stage. I do not know whether the 'faint' PLP/DM-20-positive cells I observe in the spinal cord correspond to the PLP/DM-20\(^+\) class of pre-myelinating oligodendrocytes, but it seems likely that this is the case.
5.3.4 Extending the clearance time of apoptotic cells displays the true extent and distribution of oligodendrocyte death during normal development

It is important to remember that the number of pyknotic cells seen at any time is not only dependent on the rate of cell death, but also on the rate at which these dead cells are phagocytosed and removed (the ‘clearance time’). We believe that the clearance time, as well as the rate of death, is increased in our transgenic mice, because although there are only three times as many O-2A progenitors in the hemizygous spinal cord, and seven times as many in the homozygous, there are ten times and thirty times as many dead oligodendrocytes in the hemizygous and homozygous cords respectively. This suggests that in the transgenic mice the clearance machinery (presumably microglia) has become saturated, and maybe other, less efficient, cells are recruited to carry out the phagocytosis. This could also explain why I see a non-uniform pattern of dying oligodendrocytes in the transgenic spinal cord, without the need for a non-uniform pattern of differentiation/death, if the clearance time itself is non-uniform throughout the cord due to different cells having different phagocytic efficiencies.

To confirm these ideas, I analyzed mice in which the gene for the ets transcription factor PU-1 had been deleted. This transcription factor is hematopoietic lineage-specific, and knockout animals have a complete lack of macrophages or microglia(McKercher et al., 1996). As expected from our model, a similar phenotype with respect to oligodendrocytes as that of NSE-PDGF-A transgenic mice was seen in these mice, either using PI or an antibody against the cell death specific enzyme family of caspases. Many of the dead oligodendrocytes detected in these mice were again in the developing grey
matter, where mature oligodendrocytes are normally very rare, strongly suggesting that oligodendrocytes do in fact differentiate randomly across the entire cord, then specifically accumulate in areas where, and at times when, specific long-term survival factors are present. Why oligodendrocyte survival factors should suddenly specifically appear in axon tracts just before birth is a mystery, but perhaps is related to the making or maturing of synaptic contact with axonal targets.

The fact that the number of O-2A progenitors reaches a steady state of over 200 cells/15μm section several days before the first oligodendrocytes are seen, even though they continue to divide once a day (Chapter 4) also indicates that 200 cells/section/day must be dying, either as O-2A progenitors or, more likely, after initiating their differentiation pathway. I can only detect a tiny proportion of these cells, presumably due to a very rapid clearance time. So it seems likely that oligodendrocyte differentiation is going on for a number of days before they start to survive and myelinate axons.

This model seems to go against the idea of a cell intrinsic ‘clock’ which controls the timing of oligodendrocyte differentiation (Gao et al., 1997; Raff et al., 1985; Raff et al., 1988; Temple and Raff, 1986), which has been shown to exist for optic nerve O-2A progenitors, at least in vitro. One aspect of this clock is that clonal progeny of a single progenitor cell, after a fixed number of divisions in vitro, differentiate synchronously into oligodendrocytes (Temple and Raff, 1986). This has been shown not to be the case with spinal cord O-2A progenitors, where an O-2A lineage clone in culture generally contains both O-2A progenitors and differentiated oligodendrocytes (Ibarrola et al.,
Moreover, all spinal cord O-2A progenitors seem to descend from a small number of cells which are initially specified during a narrow window of time around E12.5. If the clonal progeny of these cells were all to subsequently differentiate synchronously, then all spinal cord O-2A progenitors would become oligodendrocytes at approximately the same time in vivo. This is obviously not the case, as O-2A progenitors and oligodendrocytes co-exist in the spinal cord of the animal throughout life (compare Figures 3.10 and 5.2). Therefore oligodendrocyte differentiation and survival must be largely controlled by environmental factors, not cell intrinsic mechanisms.
6. CHAPTER SIX

GENERAL DISCUSSION
In this Thesis I have investigated the role of PDGF in the population dynamics of the oligodendrocyte lineage, both at the level of proliferation of progenitor cells and survival of post-mitotic oligodendrocytes. I have used a transgenic approach to alter the levels of PDGF in the developing nervous system, enabling me to examine in a semi-quantitative way how varying the PDGF-A supply affects oligodendrocyte development. Many studies of PDGF in oligodendrocyte development have been carried out in vitro (see Chapter 1); the work described in this Thesis both complements and extends these studies. We are now able to propose a tentative model of the in vivo development of the murine spinal cord oligodendrocyte lineage (Figure 6.1). The key elements of this model are:

1) proliferation of oligodendrocyte progenitor cells is limited by the PDGF supply
2) progenitor cell differentiation is stochastic
3) oligodendrocyte number and distribution is controlled by selective cell survival.

### 6.1 POPULATION DYNAMICS OF OLIGODENDROCYTE DEVELOPMENT: A MODEL

#### 6.1.1 Lineage specification

The first sign of oligodendrocyte lineage specification in the spinal cord is the expression of the PDGF alpha receptor by a discrete population of neuroepithelial cells at the ventricular surface at around E12.5. This specification is directly or indirectly dependent on signals emanating from the notochord and/or floor plate, including the protein Sonic hedgehog (Poncet, 1996; for review see Miller, 1996; Orentas and Miller, 179
1996; Pringle et al., 1996; Richardson et al., 1997; Trousse et al., 1995) and the neuregulin family of growth factors (Vartanian et al., 1999).

### 6.1.2 Progenitor cell proliferation and migration

As a result of their expression of PDGFRα, the progenitor cells begin to divide rapidly in response to PDGF, mRNA for which is present in the spinal cord for at least 1-2 days prior to receptor expression (see Chapter 3, this Thesis; Orr-Urtreger and Lonai, 1992). The highly migratory progenitor cells also migrate away from the ventricular surface into the parenchyma of the cord. At these early stages of development, while the numbers of PDGFRα⁺ cells are still low and the level of PDGF is relatively high, the oligodendrocyte progenitors are dividing very rapidly, with a cell cycle time of around 6-8 hours. However, as the number of progenitors increases with successive divisions - with a concomitant increase in the consumption of PDGF - the PDGF supply becomes limiting and the progenitor cell-division cycle begins to slow down from around E13.5 onwards. The cell cycle continues to slow until a steady-state ensues, when the rate of consumption of PDGF by receptor binding and internalization is equal to the rate of PDGF release from neurons (demand equals the supply). At this point (sometime after E15) the slowing down of the progenitor cell cycle ceases and cell cycle time stabilizes at about once a day. The consequence of this is that the progenitor cell population is self-limiting regardless of the rate of supply of PDGF, and the final number of progenitor cells, but not their ultimate cell division rate, is controlled by the rate of supply of PDGF.
6.1.3 Stochastic differentiation of oligodendrocyte progenitors

Even though the rate of division of oligodendrocyte progenitors slows down dramatically between E12 and E17, at steady-state they are all still dividing about once a day. However, the increase in cell cycle time causes each successive generation of cells to spend longer in G1, increasing the probability of cell cycle arrest and exit into G0 (i.e. differentiation), relative to the probability of entering the next division cycle (Brooks and Riddle, 1988). When these two probabilities become equal, sometime between E15 and E17, net proliferation of progenitor cells ceases - the same number of cells differentiate each cycle as re-enter S-phase. Thus the onset and subsequent rate of oligodendrocyte differentiation in the spinal cord is determined predominantly by the kinetics of progenitor cell division, which is in turn controlled by the level of extracellular growth factors.

6.1.4 Abortive oligodendrocyte differentiation

As oligodendrocyte progenitors exit the cell cycle and start to differentiate, they rapidly down-regulate expression of PDGFRα and become unresponsive to PDGF (Hall et al., 1996; Hart et al., 1989b). Their survival now becomes dependent on other factors. When the first progenitors leave the cell cycle, oligodendrocyte survival factors are not available in the cord; thus these ‘early’ oligodendrocytes all die at a stage before they express high levels of myelin markers such as PLP and MBP. These dying oligodendrocytes are not readily observed, presumably because the clearance time of
dead cells is rapid. However, artificially increasing the number of progenitors, and hence the number that exit the cycle (and die) in a given time leads to the accumulation of observable numbers of dying immature oligodendrocytes, possibly helped because the clearance mechanisms become saturated and therefore delayed. Progenitors are distributed, and thus differentiate, ubiquitously throughout the cord, so the pattern of premature, dying oligodendrocytes must reflect differences in the local clearance times of these pyknotic cells, possibly due to differences in the phagocytic potential of their neighbours or the availability of dedicated phagocytes. It is not known what cells normally clear dead or dying oligodendrocytes in the spinal cord, but evidence from PU-1 knockout mice, which are devoid of macrophage lineage cells, suggests strongly that many are removed by the resident microglia (Chapter 5, this Thesis).

6.1.5 Long term oligodendrocyte survival and myelination

The final aspect of our model is how the long-term survival and maturation of oligodendrocytes is mediated. It seems likely that, shortly before birth, long-term oligodendrocyte survival signals begin to be expressed in association with axons in the spinal cord, initially in the ventral axon tracts but subsequently in the more lateral and dorsal white matter. This results in an accumulation of maturing oligodendrocytes in these areas, which begin to express high levels of the various myelin components such as MBP and PLP. We presume that levels of the relevant survival signals remain low in the grey matter throughout and beyond this time, with the result that oligodendrocytes do not accumulate in the grey matter, and indeed are continually observed in NSE-PDGF-A transgenic mice as small numbers of immature, pyknotic cells.
Oligodendrocyte accumulation continues in the white matter until axon myelination is complete, at which point the number of mature oligodendrocytes reaches a plateau due to competition for the (now limiting) axon-derived survival signals. Thus the final distribution of mature, myelinating oligodendrocytes in the adult spinal cord is dependent on the availability of long-term survival signals for these cells, not on the number, distribution or differentiation of their progenitors.
Figure 6.1 - A tentative model of spinal cord oligodendrocyte development. Time runs from top to bottom (not to scale). Oligodendrocyte progenitors are specified in the ventral ventricular zone of the spinal cord and are first recognized by virtue of their expression of PDGFRα at E12.5 in the mouse (E14 in rat). This is a direct or indirect effect of signals, including Sonic hedgehog, from the notochord and/or floorplate. O-2A progenitors proliferate rapidly at first (6 hour cell cycle) in response to PDGF-AA and migrate throughout the spinal cord to become more or less evenly distributed in both gray and future white matter. As they increase in number, their division rate slows down (denoted by longer time lines), so that by E17 their division cycle stabilizes at around 24 hr. With the lengthening cell cycle, cells spend longer in G1 and the probability of their dropping out of cycle into G0 and differentiating into oligodendrocytes increases relative to the probability of their entering the next division cycle. When the number of progenitors that drops out of division each cycle matches the number that reenters S-phase, progenitor cell number reaches steady-state (i.e. proliferation ceases); this happens between E15 and E17 in wild-type mice. At first, the newly differentiating oligodendrocytes die for lack of survival factors. Starting around birth, they start to survive and accumulate, mainly in developing white matter. Numbers of progenitor cells (blue circles) and surviving oligodendrocytes (red circles) are illustrative only. Dying, immature oligodendrocytes are denoted by a cross over the symbol.
The ideas discussed above are derived from the work carried out in the fifteen years since the oligodendrocyte progenitor was first described (Raff et al., 1983) in the light of the experiments described in this Thesis. A number of issues are raised by this hypothesis which need to be addressed by further studies. One intriguing question is that of the intrinsic changes in the oligodendrocyte progenitor through developmental time. I have shown that the cycle time of these cells increases in the days after their initial specification and proliferation regardless of the in vivo supply of growth factors. It has already been shown that in ‘saturating’ concentrations of PDGF in vitro, perinatally-derived progenitors only divide once a day (Gao and Raff, 1997). Does a progressive decrease in the available PDGF in vivo, suggested by the self-limiting proliferation hypothesis described above, cause this apparent change in sensitivity to PDGF, or is this change totally cell autonomous? One way to address this question might be to culture progenitors in saturating PDGF at a time when they are dividing rapidly (i.e. E13) and subsequently maintain a saturating PDGF concentration by frequent addition of the growth factor to the tissue culture medium. The cell cycle time could then be measured a few days later and compared to control cultures. If deceleration of the cell cycle in vitro can be prevented by supplying an adequate level of PDGF, this would then indicate that the slowing-down observed in vivo is caused by environmental changes, not an intrinsic cellular program. Intrinsic versus environmental effects could also be assessed by specifically labeling dissociated cells from older embryos, for example with lipophilic dyes, then mixing and culturing these cells with cells from younger embryos to see if their cell cycle times remain different in co-culture,
or alternatively if their cell cycles converge, either by the “old” cells speeding up or the “young” cells slowing down.

An approach to test the hypothesis in vivo would be to use transgenic technology to “knock-in” the PDGF-A gene into the PDGFRα gene locus by homologous recombination, thus placing PDGF-A expression under the control of the PDGFRα gene promoter. This should have the effect of creating an autocrine loop in all cells that express the receptor, including oligodendrocyte progenitors, and we could then determine whether progenitor cell division continues at its initial rate in these transgenic animals, or still slows down during development. These experiments would again test whether the change in progenitor cell sensitivity to PDGF was cell-intrinsic or caused by a change in the availability of the mitogen.

One limitation of the experiments described in this Thesis is that they have only involved the manipulation of the in vivo supply of PDGF to the dividing cell population. A complementary approach would be to manipulate the number of PDGF receptors on cells, and hence, presumably, the rate of removal of PDGF from the system. A similar analysis of population dynamics under these conditions would further test the hypothesis that matching the demand to the supply of growth factors ultimately regulates progenitor population size.

In addition, as I described in Chapter 1, a number of other growth and survival factors have been implicated in the proliferation, differentiation and survival of the
oligodendrocyte lineage, both in vitro and in vivo. These include members of the FGF, IGF, TGF-β and neurotrophin families, and similar genetic studies with these molecules would further elucidate how the interplay of such factors controls the process of myelination in the CNS.

Our model is based on a vast body of work accumulated over the years by many workers including ourselves, which this Thesis both complements and extends. It is intended as a starting point for further work and discussion - clearly much remains to unravel in the complex area of oligodendrocyte population dynamics in vivo. However the one message that stands out from this work is the extreme degree of flexibility built into the mechanisms for regulating differentiated cell populations, as one can artificially increase the numbers of CNS oligodendrocyte progenitors by at least an order of magnitude in the multiple NSE-PDGF-A transgene animals, without overwhelming the controls on oligodendrocyte number and distribution, which operate exclusively at the level of selective cell survival.
7. REFERENCES


development and dispersal of oligodendrocyte precursors in the embryonic chick spinal


oligodendrocytes is dependent on local influences from the notochord. *Dev Biol*,
**177**:43-53.

Developmental expression of the α receptor for platelet-derived growth factor, which is

receptor are expressed in separate, but adjacent cell layers of the mouse embryo.
*Development*, **115**:1045-58.


