Functional Analysis of Gene Regulation in Oligodendrocyte Progenitor Cells

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Contribution of Authors

The results of PLP/DM20 studies have been published in 2008 (Tuason et al., 2008). Maria Clarita Tuason designed experiment and built constructs used to evaluate regulatory function. I designed part of the experiment and built construct to evaluate regulatory function of wmN2(313). Tanja Kuhlmann analyzed CNS expression programs. Cécile Goujet-Zalc designed initial constructs. Bernard Zalc participated in analysis of fetal expression programs and manuscript preparation. Samar Dib performed the peripheral nerve regeneration studies. Hana Friedman participated in construct design and manuscript preparation. Alan Peterson participated in experimental design, expression program analysis, and manuscript preparation.

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Abstract

Myelin is elaborated in the Central Nervous System (CNS) by oligodendrocytes and is essential for rapid action potential conduction. During embryogenesis and early postnatal stages of CNS development, oligodendrocyte progenitor cells (OPCs) proliferate and migrate in response to platelet derived growth factor-A (PDGF-A), the ligand for PDGFR*a*. This study describes the identification of regulatory elements and potential corresponding transcription factors controlling *PDGFRa* expression in OPCs, which will contribute to a better understanding of oligodendroglial lineage specification including OPC proliferation, migration and final maturation. Based on previous studies, the enhancer(s) driving expression in oligodendrocytes was only known to be located somewhere within a 380kb yeast artificial chromosome (YAC). To refine the search, a transgenic mouse line containing a bacterial artificial chromosome (BAC), containing a *lacZ* reporter gene, was generated. The lacZ reporter gene was inserted into the BAC, using a recombineering strategy. This line contains an 87kb BAC (CTD-2243G22) that starts –16kb upstream of the transcription start site, and ends +2kb downstream of the 3'UTR. It demonstrates robust lacZ expression in brain regions where OPCs originate. Based on interspecies sequence comparison and the presence of relevant transcription factor binding sites, such as for Sox and oligs, four candidate non-protein coding modules were identified within the 87kb defined by the BAC. These modules are being evaluated for *in vivo* function in the context of *lacZ* reporter genes docked at the hypoxanthine phosphoribosyl transferase (*HPRT*) locus. One of these constructs, which contains

600bp of intron 21 of the *PDGFRa* gene, shows a similar pattern of expression to the BAC, suggesting that the sequence containing the OPC enhancer may have been captured out of a 380kb region. Deletions have been made in this 600bp, to discover which elements are necessary and sufficient for the activity of this enhancer.

In addition, complementary studies on the regulation of *PLP/DM20* expression in OPCs were extended. PLP/DM20 is the most abundant structural protein in the CNS myelin and is also present in the peripheral nervous system (PNS) myelin. In the CNS, as OPCs differentiate into mature oligodendrocytes, the expression of the DM20 isoform decreases while the PLP isoform expression increases. A 300bp enhancer, out of a predefined 1200bp region, was located. This enhancer drives expression of the *PLP/DM20* gene in several cell types of the nervous system, including OPCs during embryonic development. Sequence comparison among different species has revealed four highly conserved regions in this enhancer. These conserved sequences are now under analysis *in vivo*, individually and in combinations with each other, by generating constructs docked at the mouse *HPRT* locus, to reveal their role in *PLP/DM20* expression. These experiments taken together, will, in the future, complete the objective of finding unique enhancers that drive expression in OPCs during embryonic and postnatal life.

Abrégé

La myéline est élaborée dans le système nerveux central (CNS) par les oligodendrocytes et elle est essentielle pour la conduction rapide du potentiel d'action. Pendant l'embryogénèse et les premières étapes du développement postnatal du CNS, les progéniteurs d'oligodendrocytes (OPCs) prolifèrent et émigrent en réponse au *Platelet derived growth factor-A* (PDGF-A), le ligand pour PDGFR*a*. Cette étude décrit l'identification des éléments régulateurs et les facteurs de transcription qui contrôlent l'expression de *PDGFRa* dans les OPCs, ce qui contribuera à faciliter la compréhension de la spécification du lignage des oligodendrocytes, ainsi que de la prolifération, la migration et la maturation finale des OPCs. Basé sur des études précédentes, nous savons que l'élément *enhancer* responsable de l'expression dans les oligodendrocytes est situé quelque part à l'intérieur d'un chromosome de levure artificiel (YAC) de 380 kb. Afin de peaufiner la recherche, une lignée transgénique de souris contenant un chromosome bactérien artificiel (BAC) et un gène rapporteur *lacZ* a été générée. Le gène rapporteur *lacZ* a été inséré dans le BAC en utilisant une stratégie de *recombineering*. Cette lignée contient un BAC de 87 kb (CTD-2243G22) qui commence -16 kb en amont du site d'initiation de la transcription, et finit +2 kb en aval des séquences non-traduites en 3' (3'-UTR). Elle démontre une expression robuste de *lacZ* dans les régions du cerveau où les OPCs ont leur origine. Basé sur la comparaison génomique entre espèces et la présence de sites de liaison de facteurs de transcription pertinents, tel que Sox et olig, quatre modules non codants candidats ont été identifiés à l'intérieur des 87 kb définits par le BAC.

Ceux-ci ont été ou seront évalués pour leur fonction *in vivo* dans le contexte du gène rapporteur *lacZ* arrimé au locus d'*hypoxanthine phosphoribosyl transferase* (HPRT). Une de ces constructions qui contient 600 bp de l'intron 21 du gène *PDGFRa* montre un profil d'expression similaire au BAC, ce qui suggère que la séquence qui contient l'*enhancer* de l'OPC a probablement été capturée à partir de la région de 380 kb. Des délétions seront faites dans cette séquence de 600 bp pour découvrir les éléments qui sont nécessaires et suffisants pour l'activité de cet *enhancer*.

En parallèle, des études complémentaires sur la régulation de l'expression génique de *PLP/DM20* ont été conduites. PLP/DM20 est la protéine de structure la plus abondante dans la myéline du CNS. Cette protéine est aussi présente dans la myéline du système nerveux périphérique (PNS). Dans le CNS, pendant que les OPCs différencient en oligodendrocytes matures, l'expression de l'isoforme DM20 diminue alors que l'expression de l'isoforme PLP augmente. Un *enhancer* de 300 bp a été situé dans une région de 1200 bp prédéfinie. Cet *enhancer* contrôle l'expression du gène *PLP/DM20* dans plusieurs types de cellules du système nerveux, y compris les OPCs pendant le développement embryonnaire. La comparaison génomique entre espèces différentes révèle quatre régions extrêmement conservées dans cet *enhancer*. Ces séquences conservées sont présentement sous analyse *in vivo*, individuellement et en combinaison, en générant des constructions arrimées au locus HPRT de la souris, afin de révéler leur rôle dans l'expression de *PLP/DM20*. Ces expériences complèteront, dans le

futur, l'objectif de trouver des *enhancers* uniques qui contrôlent l'expression dans les OPCs pendant la vie embryonnaire et postnatale.

Introduction

I. Myelin

Invertebrates conduct action potentials along axons at a maximum speed of 1 meter per second, sufficient for their small size. However, in larger animals, more rapid conduction of action potentials is critical. Cephalopods solved this problem by making axons with calibers up to a few millimeters while jawed vertebrates adapted a more efficient strategy elaborating an electrically inert lipo-protein membrane, named myelin, around axons. Myelin acts as an insulator and results in action potential conduction at speeds of 50 to 100 meters per second through axons with calibers of only 10 to 40 micrometers (Colman et al., 2001; Zalc and Colman, 2000).

In the central nervous system, myelin sheaths are the extension of plasma membranes of cells called oligodendrocytes. Each axonal segment ensheathed by myelin is called an internode. Between internodes, the small gap of bare axon is called a node of Ranvier and this axonal segment is enriched in ATP-dependent Na+/K+ channels. Because of the insulating effects of myelin, the action potential passes along the axon jumping between nodes in a manner referred to as saltatory conduction. Because only nodal axon membrane is depolarized, the ATP requirement for repolarization is reduced. Thus, myelin contributes to both faster conduction and reduced ATP expense (Martini and Schachner, 1997; Quarles, 2002; Squire et al., 2003).

II. Oligodendrocyte Progenitors

Oligodendrocytes develop from oligodendrocyte progenitor cells (OPCs), that originate mainly from the ventral part of the CNS (Hall et al., 1996). Several markers such as Olig1, Olig2, NG2 and PDGFR*a* have been used to detect OPCs in the CNS. Among these, only PDGFR*a* is specific to OPCs and is not expressed by other cell types in the CNS. PDGFR*a* is one of the earliest markers of OPCs and in the mouse spinal cord, PDGFRa^+ cells first appear on embryonic day 12.5 (E12.5) as they differentiate from neuro-epithelial stem cells (Ligon et al., 2006; Liu and Rao, 2004; Polito and Reynolds, 2005; Pringle and Richardson, 1993; Pringle et al., 1996). In response to PDGF-A, the ligand for PDGFR*a*, they proliferate and migrate dorsally and by E15.5 they are present in most parts of the spinal cord. In the absence of PDGF-A, PDGFRa^+ OPCs fail to proliferate and migrate. This results in the absence of oligodendrocytes and myelin in the CNS (Fruttiger et al., 1999; Hoch and Soriano, 2003; Woodruff et al., 2004). Upon differentiation of OPCs into mature oligodendrocytes PDGFR*a* is downregulated, making it a unique marker for OPCs (Woodruff et al., 2001). Some OPCs do not differentiate into oligodendrocytes but rather, persist as progenitors in the adult CNS (Reynolds and Hardy, 1997; Wolswijk and Noble, 1989). After demyelinating insults, OPCs in the adult CNS can proliferate and differentiate into new oligodendrocytes capable of restoring normal myelin. However, in progressive demyelinating diseases like multiple sclerosis (MS), this repair process eventually fails leading to permanently demyelinated lesions and axonal loss. This leaves the patient in a progressively debilitated state (Chang et al.,

2000; Franklin, 2002; Nait-Oumesmar et al., 2007; Wilson et al., 2006; Wolswijk, 1998).

In addition to the ventral spinal cord, other sources of OPCs including the dorsal spinal cord have been identified (Fogarty et al., 2005; Spassky et al., 1998). A recent experiment has shown that OPCs in the brain originate from three different regions at three different ages during primary development; the first OPCs originate in the ventral forebrain at E12.5. The second origin is more lateral and becomes active at E15.5. Finally, the third wave of OPCs originates from more dorsal regions at postnatal day 0 (P0) (Kessaris et al., 2006).

III. PDGFR*a*

III.1. Gene

PDGFRa is a 60kb gene located on chromosome 4 of human and a 42kb gene located on chromosome 5 of mouse. It contains 23 exons and the translation start site is located on exon 2. The largest intron is the first intron which is almost 30kb in human and 6kb in mouse. The first neighbor upstream gene is GSX2, which is located 130kb upstream from the human gene, and the first neighbor downstream gene is KIT, which is located 360kb downstream from the human *PDGFRa* gene (Bioinformatics).

III.2. Protein

Platelet derived growth factor receptor alpha (PDGFR*a*) is a membrane receptor tyrosine kinase. It contains five immunoglobulin domains in the extracellular side and a tyrosine kinase domain in the cytoplasmic region. Upon binding to its ligand, PDGFR*a* dimerizes, and phosphorylates the intracellular tyrosine kinase domains. This phosphorylation is the start of downstream intracellular pathways (Hoch and Soriano, 2003; Lee et al., 1990).

III.3. Expression

PDGFRa is expressed by a wide variety of cells during development including lung alveoli, intestinal villi, mesenchymal dermis and hair follicles, OPCs, neural crest derived cells, leydig cells, somites and the skeletal system (Betsholtz, 2004; Hoch and Soriano, 2003; Mercola et al., 1990). Mice lacking *PDGFRa* have abnormal embryonic development, mainly midline defects, absence of oligodendrocyte progenitor cells (OPCs), abnormal lung, gastrointestinal and testicular development and they usually die before birth (Hoch and Soriano, 2003; Soriano, 1997; Sun et al., 2000). Also, abnormal over-expression of *PDGFRa* is observed in many proliferative diseases such as glioma, embryonal carcinoma and breast cancer (Carvalho et al., 2005; Kraft et al., 1996; Nister et al., 1991).

III.4. Regulation of *PDGFRa* gene expression

Previous studies have attempted to locate the regulatory sequence controlling *PDGFRa* expression. Consistent with its wide range of activities, the transcriptional control of *PDGFRa* expression appears to involve numerous tissue specific regulatory elements (Reinertsen et al., 1997; Sun et al., 2000; Zhang et al., 1998). Transgenic mice harboring reporter genes with different lengths of the promoter in front of the bacterial *lacZ* reporter gene indicate that the –2.2kb sequence upstream of the transcription start site contains most of the *cis*-regulatory elements responsible for tissue specific expression of *PDGFRa*. However, this sequence does not drive expression in lung smooth muscles or in OPCs. In contrast the sequence extending to –6kb upstream of the transcription start site drives expression in lung smooth muscles. However, this extended sequence remains unable to drive expression in OPCs (Reinertsen et al., 1997; Zhang et al., 1998). In another study, a Yeast Artificial Chromosome (YAC) containing the human *PDGFRa* gene from -3.5kb upstream of the transcription start site to +300kb downstream of the 3' UTR, was used to make transgenic mice. Expression of the human *PDGFRa* by OPCs was evaluated by in situ hybridization with probes specific for human *PDGFRa* and, with the exception of lung, was found to parallel that of the endogenous locus. Further, except for the persistence of abnormal lung development, the YAC rescued the *PDGFRa*-KO phenotype in mice (Sun et al., 2000). Therefore, it seems that the *cis*-acting regulatory elements essential for expression in the smooth muscles of the lung lie between –3.5kb to –6kb upstream of the transcription start site while the essential

regulatory elements driving *PDGFRa* expression in OPCs are located between –3.5kb upstream of the transcription start site and +300kb downstream of the 3' UTR.

IV. PLP/DM20

PLP/DM20 is a 17kb gene located on chromosome X of human. In mouse, it is also located on chromosome X but with the size of 15kb. It consists of seven exons and six introns. Intron one makes about half of the gene and contains important regulatory elements for *PLP/DM20* expression. Transgenic mice studies have shown that constructs containing -2.4kb upstream sequence, exon 1, intron 1, and part of exon 2 express in a similar pattern to that of the *PLP/DM20* gene. Lack of intron 1 in the context of the construct failed to reveal expression (Fuss et al., 2000; Li et al., 2002b; Spassky et al., 1998; Wight et al., 1993). PLP/DM20 is the most abundant structural protein in the CNS myelin and is also present in peripheral nervous system (PNS) myelin (Eng et al., 1968). It contains two isoforms; PLP and DM20. The DM20 isoform is produced when an alternative 5' splice site, located in exon 3, is utilized in precursor mRNA splicing (Nave et al., 1987). In the CNS, the DM20 isoform is expressed by OPCs (Dickinson et al., 1996; Timsit et al., 1992). As OPCs differentiate into mature oligodendrocytes, the expression of the DM20 isoform decreases while the PLP isoform expression increases (LeVine et al., 1990; Verity and Campagnoni, 1988). PLP/DM20 is also expressed by Schwann cells which are the myelin forming cells in the PNS (Griffiths et al., 1995; Ikenaka et al., 1992).

V. Gene regulation

Close to 95% of the human genome is composed of non-protein-coding sequence within which a small fraction confers regulatory activity controlling the level, location and timing of gene expression. Such regulatory elements can be upstream, within introns, within exons or downstream of genes at distances of <1kb to >100kb. Therefore, locating regulatory elements can be a major challenge (Khandekar et al., 2004; Pennacchio and Rubin, 2001).

One powerful approach to locating functional non-protein-coding regions is to check for sequence conservation between different species (Hardison, 2000). This approach made it possible to locate the regulatory modules of genes such as β-globin, some interleukins and myelin basic protein (Farhadi et al., 2003; Hardison et al., 1997; Loots et al., 2000).

VI. Transcriptional control

The enzyme that transcribes protein-coding genes in eukaryotes is RNA polymerase II. To initiate transcription, RNA polymerase II plus a set of general transcription factors and co-activators make the transcription initiation complex, which binds to the promoter of the gene. First, the general transcription factor TFIID binds to the TATA box of the promoter. The binding of TFIID then facilitates recruitment of other factors and RNA polymerase II (Alberts et al., 2002; Carey, 1998).

In Eukaryotes the transcriptional machinery has to overcome the complex DNA structure. Therefore, eukaryotic gene expression also needs regulatory proteins that bind to regulatory sequences and control the rate of transcription initiation. Transcriptional activator proteins bind to enhancers (binding site for transcriptional activator proteins) and recruit chromatin remodeling complexes and histone acetylases to facilitate the formation of the transcription initiation complex. On the other hand, transcriptional repressor proteins act in the opposite direction with mechanisms such as recruitment of repressive chromatin remodeling complexes and histone deacetylases. There are also other mechanisms for transcriptional repressors to oppose transcriptional activators. These mechanisms include competitive DNA binding with the activator, masking the activation surface of the activator, and direct interaction with the general transcription factors. Some transcription factors can be both activator and repressor, depending on the protein complex that they are present in. Sometimes the DNA sequence to which the transcription factor binds, can affect the role of the transcription factor. An example for this condition is the steroid hormone receptor (Alberts et al., 2002).

Several models (looping, tracking, facilitated tracking and linking) have been postulated by which regulatory proteins can control transcription at different distances from the promoter. In the looping model the regulatory complex forms a loop on the DNA to get into close proximity to the promoter. In the tracking model the regulatory complex migrates linearly to the promoter. The facilitated tracking model is a mixture of the looping and the tracking models. Finally, the

linking model suggests that the regulatory proteins bind to each-other, with the help of non-DNA binding proteins, along the DNA from their binding site to the promoter (Li et al., 2002a).

VII. Transcription factors involved

Several studies indicate that, Olig1 and Olig2, two basic helix-loop-helix transcription factors, and Sox10 act upstream of the *PDGFRa* gene in OPCs and have a major role in the process of oligodendrocyte lineage development. They start to be expressed one day before *PDGFRa*, in the zone where OPCs arise. Then, their pattern of expression overlaps that of *PDGFRa*. Ectopic expression of Olig1 *in vitro* in OPCs can increase their number. Olig2 knock-out in mice results in the lack PDGFRa^+ OPCs and oligodendrocytes (Lu et al., 2002; Zhou and Anderson, 2002).

Sox10 also has binding sites in the promoters of some of the myelin genes such as MBP and P0. In the absence of Sox10 the development of Schwann cells and oligodendrocytes is blocked (Britsch et al., 2001; Stolt et al., 2002).

Nkx transcription factors have also been shown to play a role in OPC development. The *Nkx2.1*-expressing region of the ventral forebrain gave rise to migratory OPCs that populated the embryonic forebrain including the cortex, but most of these were eliminated after birth (Kessaris et al., 2006). *Nkx2.2* has been shown to be expressed in some Olig$^+$/PDGFRa^+ cells during development

(Vallstedt et al., 2005; Wu et al., 2006). Nkx6 mutation experiments in mice show lack of expression of *Olig1*, *Olig2*, *Sox10* and *PDGFRa*. It has also been shown that *Olig2* expression by *Nkx6* genes is dose dependent and is mainly dependent on *Nkx6.1*. (Cai et al., 2005; Vallstedt et al., 2005)

VIII. HPRT transgenesis

The HPRT destination vector is a targeting vector for the hypoxanthine phosphoribosyltransferase (*Hprt*) locus on the X chromosome. This strategy has the benefit of integrating only one copy of the construct into a predetermined site in the genome, overcoming the variation associated with random transgenesis including the transcriptional environment and enhancer activities associated with different integration sites and variable copy number. The *Hprt* docking site has been shown to be suitably neutral and permissive to reveal the regulatory activity of numerous promoters and enhancers (Bronson et al., 1996; Ciavatta et al., 2006; Denarier et al., 2005; Farhadi et al., 2003; Kakoki et al., 2004; Touw et al., 2007; Yurchenko et al., 2007).

Embryonic Stem cells deficient for part of the *Hprt* gene are transfected by the targeting vectors. Only ES cells with *Hprt* restored by the homologous recombination event are able to grow in HAT (hypoxanthine, aminopterin, thymidine) selection media (Bronson et al., 1996). Selected ES clones are used to generate transgenic mice.

IX. BAC Recombineering

BACs are large cloning vectors that can contain genomic DNA sequences extending up to 300kb which makes them suitable for the functional analysis of large segments of genomic DNA. Recombineering is a powerful tool that enables us to effectively modify BACs to use them for transgenic studies. It is based on phage-based *Escherichia coli* homologous recombination systems and uses very small homology arms usually ranging between 30bp to 50bp. This system is very effective and faster than conventional methods, in which the cloning vectors cannot tolerate large inserts. Also, use of restriction enzymes in the classical methods makes it almost impossible to manipulate large pieces of DNA since even rare restriction sites are present frequently throughout large DNA molecules.

First, a cassette is PCR amplified with 30bp to 50bp of homology arms to the target DNA. Then, the BAC is introduced into the DH10B *Escherichia coli* strain that carries recombination functions, by having a lambda prophage integrated into its DNA. In the next step, the PCR amplified cassette is transformed in to the bacteria that contain the BAC and the recombineering function. As a result, the recombinant will be produced. The recombineered BAC can then be purified easily and used for downstream applications (Copeland et al., 2001; Montigny et al., 2003; Yang et al., 1997; Yu et al., 2000).

X. Statement of purpose

To design new therapeutic strategies for demyelinating diseases, it is crucial to understand the process of oligodendrocyte development from OPCs. As mentioned above, in the CNS, PDGFR*a* is expressed by OPCs and the activity of this receptor, stimulated by its soluble ligand, affects proliferation, migration and final maturation of OPCs. Hence, I believe that identifying the mechanism regulating *PDGFRa* transcription in OPCs, will contribute to a better understanding of oligodendroglial lineage specification, OPC proliferation, migration and final maturation.

Complementary studies on the regulation of the *PLP/DM20* gene in OPCs will strengthen the information obtained on *PDGFRa*. The concurrent analysis of two enhancers, both driving expression in OPCs can lead to a significantly greater understanding of the element requirements for OPC expression. Thus, it is excellent that the *PLP/DM20* wmN2 enhancer found by Claire Tuason (Tuason et al., 2008) captured elements responsible for OPC expression of that gene.

The overall goal of this study is to locate and characterize more of the regulatory sequence that controls expression of the *PDGFRa* gene. The specific objectives of this investigation are to locate and characterize the regulatory element(s) driving expression in OPCs of *PDGFRa* and *PLP/DM20*. As a result, this study opens a window onto the regulatory system modulating the expression of these two genes in the oligodendrocyte lineage.

To refine the search in a predefined 380kb region of *PDGFRa* (Sun et al., 2000), a transgenic mouse line containing a bacterial artificial chromosome (BAC), containing a *lacZ* reporter gene, was generated, using a recombineering strategy. This line, which contains an 87kb BAC, demonstrates robust lacZ expression in brain regions where OPCs originate. Based on interspecies sequence comparison and the presence of relevant transcription factor binding sites, four candidate non-protein coding modules were identified within the 87kb defined by the BAC. These were cloned and are being evaluated for *in vivo* function in the context of a *lacZ* reporter gene docked at the hypoxanthine phosphoribosyl transferase (*HPRT*) locus. One of these constructs, which contains 600bp of intron 21 of the *PDGFRa* gene, shows a similar pattern of expression to the BAC, suggesting that the sequence containing the OPC enhancer may have been captured out of a 380kb region. Deletions have been made in this 600bp, to discover the elements responsible for the activity of this enhancer.

The 1200bp wmN2 enhancer drives expression of *PLP/DM20* gene in four cell types of the nervous system, including OPCs during embryonic development. A 313bp fragment (wmN2(313)) contains most of the conservation of the larger 1200bp region. Transgenic mice containing this module driving a reporter with the minimal hsp promoter show a similar expression pattern to the larger sequence. Thus, the enhancer function was localized to this smaller sequence. Sequence comparison among different species has revealed four highly conserved regions in this enhancer. These conserved sequences are now under analysis *in vivo*, individually and in combinations with each other, by generating

constructs docked at the mouse *HPRT* locus, to reveal their role in *PLP/DM20* expression. These experiments have already gone a long way towards, completing the objective of finding unique enhancers that drive expression in the OPCs during embryonic and postnatal life.

Materials and methods

Interspecies sequence comparison

The University of California, Santa Cruz (UCSC) genome browser (available at http://genome.ucsc.edu/) was used for interspecies sequence comparison. The Human March 2006 (hg18), and Mouse February 2006 (mm8) assemblies were used for the purpose of this study (Figure 1).

Screening for conserved transcription factor binding sites

Dr. Denarier, a visiting scientist in our lab, developed a program that enables us to screen the genome for the presence of any combination of desired transcription factor binding sites in the mammalian aligned sequence. He used this program to locate potential Schwann cell enhancers in the genome. He first defined crucial motifs, in an enhancer from the Myelin Basic Protein (MBP) locus that controls expression in Schwann cells myelinating axons in the PNS. Then, he showed that these specific motifs are shared in all Schwann cell enhancers known to be functional in transgenic mice (Denarier et al., 2005). I used this program to

screen for the presence of the potential binding sites for Sox10 and Olig transcription factors in conserved modules in the *PDGFRa* locus.

Along with the above mentioned program, I also used the Predicted Regulatory Modules, PReMOD, program to search for modules that contain multiple conserved transcription factor binding sites in them (Blanchette et al., 2006). This program is available at http://genomequebec.mcgill.ca/PReMod.

Isolation of human *PDGFRa* sequence

Human BAC clone CTD-2243G22, which contains the human *PDGFRa* gene, was obtained from Invitrogen and used to amplify the regions of interest. Selected sequences of *PDGFRa* DNA were amplified by PCR from the CTD-2243G22 BAC, using primers listed in table 1. Sequences were amplified using the Taq polymerase from Invitrogen in the following program: Initial dentaturation for 4 minutes at 94ºC, 45 cycles of amplification with denaturation at 94ºC for 1 minute, annealing temperature at 55ºC for 30 seconds, extension at 72ºC for periods of 60 seconds per 1000bp of sequence, and final extension at 72º for 10 minutes. The PCR products were purified using the PureLink™ PCR Purification Kit from Invitrogen.

Isolation of mouse *PLP* sequences

Selected sequences of *PLP* DNA were amplified by PCR from the wmN2 construct (Tuason et al., 2008), using primers listed in table 2. Sequences were

amplified using the Taq polymerase from Invitrogen in the following program: Initial dentaturation for 4 minutes at 94ºC, 45 cycles of amplification with denaturation at 94ºC for 1 minute, annealing temperature at 55ºC for 30 seconds, extension at 72ºC for 30 seconds, and final extension at 72º for 10 minutes. Because of the small size of wmN2_2 and wmN2_4 constructs, the positive and negative strands were ordered and annealed to each other. The PCR products were purified using the PureLink™ PCR Purification Kit from Invitrogen.

BAC Recombineering

Human BAC clone CTD-2243G22, which contains the human *PDGFRa* gene, was obtained from Invitrogen. The bacteria containing the BAC were grown on LB-broth media containing 12.5ug/ml chloramphenicol. The DNA was extracted using the Phaseprep BAC DNA kit from SIGMA.

The recombineering agents were kindly provided by Dr. N.G. Copeland's laboratory. Their exact protocol was followed for BAC recombineering (Recombineering). A postdoctoral trainee, Omar Akhouayri, from Dr. Rene St-Arnaud's laboratory, in Shriner's hospital, helped me at the beginning to become more familiar with the recombineering technology. With this technology a cassette containing a *LacZ* reporter gene and a neomycin selection marker (named LacZNeofrt cassette) was inserted into the CTD-2243G22 BAC in a way that the translation start site of the *LacZ* reporter gene replaces the translation start site of the *PDGFRa* gene (Figures 2 & 3).

The SW105 bacterial clones (Recombineering) that now contained the recombineered CTD-2243G22 BAC, were PCR screened at multiple locations by the primers listed in table 3. Clone number 34 which contained the complete recombineered BAC was selected and the DNA was extracted using the PhasePrep BAC DNA Kit from Sigma-Aldrich. The integrity of the extracted DNA was again confirmed by PCR screening with the primers listed in table 3, and enzymatic digestion with XhoI. Prior to transfection into ES cells, the BAC was linearized with NruI.

To generate the LacZNeofrt cassette first, the PL451 vector (Recombineering) was digested with MlyI and NotI, to obtain the neomycin selection marker flanked by two frt sites. The Neofrt cassette was ready to be ligated into a vector containing the *LacZ* reporter gene, pENhlKN, which was digested with SmaI and NotI. The purification of both of the digested products was performed by using the Purelink PCR Purification kit from Invitrogen. The Neofrt cassette was ligated to the pENhlKN vector using the Rapid ligation kit from Roche. As a result, the Neofrt cassette was placed after the *LacZ* reporter gene, and the new vector was named pENhlKNNeofrt. The ligation was transformed into DH5 bacteria which were grown on kanamycin plates. DNA was extracted from some of the clones using the Miniprep Kit from Invitrogen. The extracted DNA from the selected clones was screened by enzymatic digestion by HindIII and NotI. One good clone which contained the complete new vector was selected and the DNA was extracted using the Purelink Hipurelink Plasmid DNA

Purification Kit from Invitrogen. The LacZNeofrt cassette was PCR amplified using the Expand long template PCR system from Roche. The primers are listed in table 4.

Generation of LacZ reporter plasmid constructs

For the *PDGFRa* constructs, first some desired restriction sites were cloned into a *LacZ*Gateway cloning vector from Invitrogen, pENTRlA. It was already modified by a former student in the lab (Samar Dib) to contain *LacZ* and some additional cloning sites. The new vector was named pENTlNRS. The -3.5kb of *PDGFRa* which was PCR amplified was digested at its ends with NotI and KpnI as was the pENlNRS vector. Both of the digestion products were purified with the PureLink™ PCR Purification kit from Invitrogen. The -3.5kb cassette was ligated into the pENTlNRS vector using T4 DNA ligase. This new construct was named -3.5pENTlNRS. This construct served as the control construct and the other proposed regulatory elements were cloned into this vector upstream of the -3.5kb promoter.

These constructs were moved into the hprt-targeting, Gateway Destination vector using the LR clonase reaction kit from Invitrogen. The Gateway Destination vector contains *HPRT* homology arms for recombination in ES cells at the *HPRT* locus. The final destination vectors were amplified and sequenced across the insert. In order to be transfected into ES cells, these constructs were linearized by AgeI or SalI. The ES cells used, bear a deletion of the promoter and

first exon of *HPRT*. Recombination with the homology arms in the Destination vector restores the gene and allows clones to grow in HAT selection media (Bronson et al., 1996; Forghani et al., 2001).

For the PLP constructs, the proposed regulatory elements were cloned into pENhel, an Entry vector that contains the hsp promoter and an *eGFPLacZ* fusion reporter gene (Tuason et al., 2008). The next steps were similar to those described above.

Sequencing

Sequencing of the constructs was performed at the Genome Quebec Innovation Center, Montreal, Quebec, using the primers listed in tables 5 to 10. Later, sequences were analyzed by MacVector 7.2.

Cell culture and electroporation

The ES cell lines in our laboratory, bearing the HPRT docking site, were newly derived from mice grown from BK deletion ES cells (Bronson *et al*., 1996). The ES cells were grown on gamma-irradiated MEF in 15% heat-inactivated FBS (Invitrogen cat. No. 10439-024), 1% GlutaMAX-I supplement (Invitrogen cat. No. 35050-061), 1% MEM amino acids (Wisent), 1% sodium pyruvate, 1% Gentamycin, 0.1 mM β-mercaptoethanol (0.1mM, 4µl), and 28.75µL of LIF (1000 units/ml, Life Technologies). Approximately, 5-7 x 10^6 ES cells were electroporated with 40 µg of linearized DNA (250 V and 500 µF, Gene-Pulser II;

Bio-Rad Laboratories). ES cells which had a complete restoration of the *HPRT* gene through the inserted constructs were selected on HAT-supplemented medium, containing 0.1 mM hypoxanthine, 0.004 mM aminopterin, and 0.016 mM thymidine (Sigma). HAT-resistant colonies were picked 14-21 days later for generating transgenic mice.

For the BAC construct, the ES cells which were used were complete in their genome, meaning there was no deletion in the *HPRT* locus. The electroporated ES cells were grown on G-418 200μl/ml for neomycin selection. The DNA was extracted from the grown ES clones and the integrity of the BAC was checked by long range PCR, using the Expand Long Template PCR kit from Roche. At the start, for better designing the primers and also optimizing the PCR conditions, Vince Forgetta and Andrei Verner, from Dr. Ken Dewar's laboratory in Quebec Genome Center, had a significant contribution. Figure 4 shows a schematic picture of the long range PCR screening; the primers are listed in table 11.

Generation of transgenic mice

For plasmid constructs, ES cells containing the complete constructs were injected into C57Bl/6-derived blastocysts that were then transplanted into the uteri of recipient females. Some of the resulting chimeric mice were analyzed for expression at different embryonic and postnatal ages. The rest of the male chimeras were bred with C57Bl/6 females, and the F1 agouti female offspring

were backcrossed with C57Bl/6 males to establish a line. Genotyping was performed by PCR analysis of genomic DNA, extracted from tail, using primers present in the *LacZ* coding sequence and the *HPRT* locus.

For the BAC construct, the same procedure was followed. The only difference was that since it was random insertion, both male and female chimeras were used to establish a heterozygous line.

All of the above experiments were performed in accordance with McGill University animal care guidelines.

Histochemical detection of β-galactosidase activity

Selected Mice were first anaesthetized with avertin intraperitoneally. They were then perfused intracardially with a 4^{o}C fix solution made of 0.5% paraformaldehyde, 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). Tissues to be analyzed were taken out and postfixed for an additional hour at 4^{o}C. Embryos were put directly into fix. Samples were then rinsed in 0.1 M phosphate buffer and incubated at 37ºC overnight in stain consisting of 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 0.4 mg/mL Bluo-Gal (Gibco/BRL, Burlington, Ontario), in 0.1M phosphate buffer.

Results

An 87kb BAC expresses in brain regions where OPCs are found

As a tool to narrow down the region of interest, I used bacterial artificial chromosomes (BACs), containing different regions of the *PDGFRa* locus, in *in vivo* transgenic assays. I have modified two human BACs containing the *PDGFRa* gene and different lengths of upstream and downstream sequence. These were recombineered to insert the E. coli *lacZ* reporter gene, encoding the β–galactosidase enzyme, under the control of the *PDGFRa* regulatory sequences. One of the BACs (CTD-2243G22) contains the sequence from -16kb upstream of the transcription start site to +2kb downstream of the 3' UTR. The other BAC (RP11-626H4) starts from -3.2kb upstream of the transcription start site and ends at +110kb downstream of the 3'UTR. The PCR screening showed that the RP11-626H4 BAC was rearranged in the ES cells. Therefore, only ES cells that contained the complete CTD-2243G22 BAC were injected into C57BL/6 blastocysts to generate transgenic mice.

Chimeras containing the recombineered CTD-2243G22 BAC were analyzed at postnatal days 5 and 10. *lacZ* expression was detected in brain, in the subventricular zones and in some of the white matter tracts (Figure 5). At this age OPCs should be present everywhere in the brain, but *lacZ* expression was only detected in the subventricular zone. Also, mice at different stages of embryonic

development were analyzed but no expression was seen in the spinal cord or the brain.

This could suggest that there is a combinatorial network for expression of *PDGFRa* in OPCs and I have been able to capture part of it, in a much smaller region which is 300kb less than the previous candidate sequence, and contains only less than 10 conserved non-protein coding sequences.

Intron 21 may contain an OPC regulatory element

First, the -3.5kb *PDGFRa* promoter was PCR amplified and cloned into a plasmid containing the *lacZ* reporter gene. This construct was used as control. Then, four candidate OPC regulatory sequences, based on interspecies conservation and the presence of transcription factor binding sites, were PCR amplified, and cloned into the control plasmid in front of the –3.5kb *PDGFRa* promoter and the *lacZ* reporter. The constructs were transferred into an HPRT destination vector by homologous recombination mediated by phage recombinase. These four candidate sequences were as follows: Int1_1: a 2.7kb sequence from intron 1, Int1_2: a 5.2kb sequence from intron 1, Int21: a 600bp sequence from intron 21, Ex23: a 3.6kb sequence which is almost the whole sequence from exon 23 (Figure 6).

Embryonic stem cells deficient for part of the *Hprt* gene were transfected by the targeting vectors. As mentioned before, only ES cells with *Hprt* restored

are able to grow in HAT (hypoxanthine, aminopterin, thymidine) selection media (Bronson et al., 1996). Selected ES cells were injected into C57BL/6 blastocysts. The blastocysts were transplanted into pseudo-pregnant female mice and the resultant male chimeras were mated with C57BL/6 female mice. The F1 germline females were backcrossed with C57BL/6 males to establish transgenic lines. The first two lines that were successfully established were the Int21-3.5 and the Ex23-3.5 lines.

Whole mounts and tissue sections from transgene bearing mice at different stages of embryonic and postnatal development were fixed and stained for β–galactosidase (Farhadi et al., 2003; Forghani et al., 2001).

The control construct showed *lacZ* expression almost everywhere that *PDGFRa* is expressed other than CNS. The Ex23-3.5kb did not show any difference from the control construct. Interestingly, the Int21-3.5kb showed a similar pattern of expression to the BAC (Appendix A.). Images were from initial samples designed to determine only if the transgene was expressing and not to characterize the cell types that express. Nonetheless, the expressing cells seem to be a layer a single cell thick and in a position consistent with the distribution and location of OPCs in the subependymal layer (Ligon et al., 2006; Liu and Rao, 2004; Pringle and Richardson, 1993; Pringle et al., 1996). Thus, it was extremely fortunate that my first construct, containing only the promoter and a 600bp conserved module from intron 21, possibly contained an OPC regulatory element.

Sequence comparison within the Int21 construct showed that 275bp out of the 600bpwas more highly conserved. This 275bp (Int21V) also contains eleven consensus binding sites for Olig, Sox and Nkx2.2 transcription factors (Figure 7). Four additional constructs have been made out of this 275bp region to further refine our search for OPC regulatory elements (Figure 8).

A 313bp enhancer drives *PLP/DM20* expression in OPCs

A 1200bp sequence (wmN2) from intron 1 of the *PLP/DM20* gene, fused to a minimal hsp promoter, drives expression of a lacZ reporter at E9.5 in several regions of the developing neural tube including the prosencephalic neural crest, trigeminal placode, epibranchial placodes, otic placode, and in neural crest cells migrating to form dorsal root ganglia. Expression in OPCs was first detected at E12.5 at the ventral part of the cervical neural tube. The expression was more robust at E14.5 and E16.5 including the more dorsal parts. Within this region, a 313bp module was located, based on sequence homology and TFBS abundance. A new transgene construct, (wmN2(313)) was generated using this 313bp fused to the minimal hsp promoter and the *lacZ* reporter gene. In mice, this enhancer construct drives expression of the reporter gene in the same pattern as the full wmN2 (Tuason et al., 2008) (Figure 9).

Sequence comparison among different species has revealed four highly conserved regions in this enhancer numbered 1 to 4. In order to reveal their role in *PLP/DM20* expression in different cell types especially the OPCs which are my

primary interest, nine constructs were made out of these conserved sequences individually and in combinations (Figure 10). All of these constructs have been transfected into ES cells to generate *HPRT*-docked transgenes and clones are ready to be used to generate transgenic mice. There are eleven conserved motifs, of at least 5bp, present in the enhancer. Some of these motifs exactly match the binding sites for transcription factors such as Sox-10 and Krox-20 which play important roles in gene expression in the central and peripheral nervous systems (Figure 11) (Kuhlbrodt et al., 1998; Stolt et al., 2002; Topilko et al., 1994).

Discussion

Oligodendrocytes are responsible for producing myelin in the Central Nervous System (CNS) (Martini and Schachner, 1997; Quarles, 2002). Oligodendrocytes differentiate from oligodendrocyte progenitor cells (OPCs), which proliferate and migrate in response to platelet derived growth factor-A (PDGF-A), the ligand for PDGFR*a*. In the oligodendroglial lineage, *PDGFRa* is exclusively expressed in OPCs. Upon differentiation into mature oligodendrocytes, PDGFR*a* downregulates (Reynolds and Hardy, 1997; Woodruff et al., 2001). Some OPCs do not differentiate into mature oligodendrocytes, and stay as a reserved pool in the CNS. After a demyelinating insult, these OPCs proliferate once again and differentiate into new oligodendrocytes capable of restoring normal myelin. However, in progressive demyelinating diseases like multiple sclerosis (MS), OPC proliferation and maturation eventually fails leading to permanently demyelinated lesions and axonal loss (Chang et al., 2000;

Franklin, 2002; Nait-Oumesmar et al., 2007; Wilson et al., 2006; Wolswijk, 1998; Woodruff et al., 2004). I believe that identifying the regulatory elements and transcription factors controlling *PDGFRa* and *PLP/DM20* expression in OPCs will contribute to a better understanding of oligodendroglial lineage specification and OPC proliferation, migration and final maturation. My hope is that such understanding will lead eventually to new tools that can modulate oligodendrocyte development and their capacity to repair.

Due to the critical functions that PDGF and its receptors play in the normal development of many tissues and in many cancers, considerable effort already has been made to locate and characterize its regulatory sequences. This has exposed the general location of enhancers driving expression in cartilage, lung, heart and some other tissues which mainly lie within the -6kb upstream the transcription start site (Afink et al., 1995; Wang and Stiles, 1994). Despite these insights, the enhancer(s) driving expression in oligodendrocytes were previously only known to be located somewhere within a 380kb yeast artificial chromosome (YAC) (Sun et al., 2000). This left us with a very large region to look for the OPC regulatory sequences of *PDGFRa.*

As the first step to narrow down my search, an analysis of conservation within non-protein coding sequence was used, which has been shown to be a powerful technique in locating functional regulatory sequences (Hardison, 2000). Unfortunately, an initial search of the 380kb sequence defined by the expressing YAC revealed 80 such conserved modules making it impractical to test each one

individually in transgenic mice. Therefore, to refine my search, I decided to begin by using large pieces of that 380kb sequence. For this purpose I generated a transgenic mouse line containing an 87kb human bacterial artificial chromosome (BAC). This BAC (CTD-2243G22) starts –16kb upstream of the transcription start site, and ends +2kb downstream of the 3'UTR. To modify this BAC to contain the *lacZ* reporter gene, a novel technology called recombineering was used. Recombineering utilizes phage-based Escherichia coli homologous recombination systems and requires very small homology arms usually ranging between 30bp to 50bp. It is very effective and faster than conventional methods (Copeland et al., 2001; Montigny et al., 2003; Yang et al., 1997). Using the recombineering strategy, a 5.2kb cassette containing a *LacZ* reporter gene and a neomycin selection marker (named LacZNeofrt cassette) was inserted into the CTD-2243G22 BAC in a way that the transcription start site of the *LacZ* reporter gene replaced the transcription start site of the *PDGFRa* gene (Figures 2 & 3). There is a PGK promoter in this cassette upstream the neomycin selection marker. PGK and neomycin are flanked by frt restriction sites. The PGK promoter drives neomycin expression in eukaryotic cells and was used as a selection marker in embryonic stem (ES) cells. Chimeras containing the recombineered CTD-2243G22 BAC were analyzed at postnatal days 5 and 10. *lacZ* expression was detected in brain, in the subventricular zones and in some of the white matter tracts (Figure 5). At this age OPCs should be present everywhere in the brain, but *lacZ* expression was only detected in the subventricular zone. Also, mice at different stages of embryonic development were analyzed but no expression was seen in the spinal cord or the brain. This could suggest that there is a

combinatorial network for expression of *PDGFRa* in OPCs and I have been able to capture part of it, in a much smaller region which is 300kb less than the previous candidate sequence. One point to keep in mind is that this recombineered BAC is bearing a PGK promoter (driving the neo selectable marker). The PGK promoter has been shown to have adverse effects on the normal pattern of expression (Pham et al., 1996). It would be of great value to remove the PGK-Neo sequence and study the pattern of expression again. The PGK-Neo sequence in this cassette is flanked by frt restriction sites which are the target for the FLP recombination enzyme. By mating these mice with FLP expressing mice the PGK-Neo sequence can be removed (Kanki et al., 2006).

To find the responsible regulatory sequence in this 87kb BAC, a bioinformatics approach was chosen. Dr. Denarier, a visiting scientist in our lab, developed a program that enables us to screen the genome for the presence of any combination of desired transcription factor binding sites in the mammalian aligned sequence (Denarier et al., 2005). I used this program to screen for the presence of the potential binding sites for relevant transcription factor binding sites such as Sox10 and Olig, in conserved modules in the *PDGFRa* locus (Kuhlbrodt et al., 1998; Lu et al., 2002; Zhou et al., 2000). Along with the above mentioned program, I also used the Predicted Regulatory Modules, PReMOD, program to search for modules that contain more of the conserved transcription factor binding sites in them (Blanchette et al., 2006). This program is available at http://genomequebec.mcgill.ca/PReMod. Using these programs, I have identified 4 candidate non-protein coding modules within the 87kb defined by the BAC.

These were evaluated for *in vivo* function in the context of a *lacZ* reporter gene docked at the *HPRT* locus. This strategy enables us to insert only one copy of the construct in a ubiquitously permissive site for expression and also overcome the variability of expression in random insertions. (Bronson et al., 1996; Ciavatta et al., 2006; Denarier et al., 2005; Farhadi et al., 2003; Kakoki et al., 2004; Touw et al., 2007; Yurchenko et al., 2007). One of these constructs, which contains 600bp of intron 21 of the *PDGFRa* gene and -3.5kb *PDGFRa* promoter, shows a similar pattern of expression to the BAC. It is expressed in the subependymal layer (Appendix A.) suggesting that a sequence containing the OPC enhancer may have been captured out of a 380kb region. This also suggests that the PGK-Neo sequence did not affect the expression in the context of the recombineered BAC, since there is no PGK-Neo in this intron 21-3.5 construct. This is also the first time that lacZ expression, under the control of *PDGFRa* regulatory elements has been detected possibly in OPCs and this unique marker is expected to facilitate numerous investigations on oligodendrocyte lineage progression. Sequence comparison within the Int21 construct showed that 275bp out of the 600bp was more highly conserved. This 275bp (Int21V) contains eleven consensus binding sites for Olig, Sox and Nkx2.2 transcription factors (Figure 7). Four constructs have been made out of this 275bp region to further refine our search for OPC regulatory elements (Figure 8).

Since these results drew our attention more towards the Int21-3.5 construct, ES cell lines from Int1_1-3.5 and Int1_2-3.5 constructs have not been

established yet. Therefore, it would be valuable to further analyze these constructs and evaluate their role in *PDGFRa* expression.

Again, it is worth mentioning, despite this apparent success, the expression phenotype realized by this enhancer is more restricted to OPCs present in the postnatal brain. This suggests that I may have captured some, but not all of the regulatory sequence required to confer the normal *PDGFRa* expression program in OPCs. The rest of the regulatory sequences should lie somewhere in the next downstream 300kb that was not investigated in this study. A similar approach could be used as the first step; a few overlapping BACs, covering this region can be recombineered to drive *lacZ* expression. Since there is no *PDGFRa* promoter in these downstream sequences, the *lacZ* cassette should also contain the *PDGFRa* promoter. Once a smaller region is defined by the expression pattern of the BACs, bioinformatics approaches, as mentioned above, would be of great value to localize the target sequence.

I have also extended the studies of a former student in our lab on the regulation of *PLP/DM20* gene expression. In the CNS, the DM20 isoform is expressed by OPCs (Dickinson et al., 1996; Timsit et al., 1992). As OPCs differentiate into mature oligodendrocytes, the expression of the DM20 isoform decreases while the PLP isoform expression increases (LeVine et al., 1990; Verity and Campagnoni, 1988). I have located a 313bp enhancer, wmN2(313), out of a predefined 1200bp region (wmN2). This enhancer drives expression of *PLP/DM20* gene in several cell types of the nervous system including OPCs

during embryonic development, similar to the wmN2 construct (Tuason et al., 2008) (Figure 9). Sequence comparison among different species has revealed four highly conserved regions in this enhancer. Constructs have been made to analyze the function of these conserved regions, *in vivo* individually and in combinations with each other. These constructs will also target the mouse *HPRT* locus. There are also eleven conserved motifs, of at least 5bp, present in the wmN2(313) enhancer. Some of these motifs exactly match the binding sites for transcription factors such as Sox-10 and Krox-20 which play important roles in gene expression in the central and peripheral nervous systems (Kuhlbrodt et al., 1998; Stolt et al., 2002; Topilko et al., 1994) (Figure 11). This will help to define the essential regulatory elements for expression in OPCs.

One other complimentary approach to define the essential regulatory elements for expression in OPCs, for the enhancers discovered in this study would be to make 5-6bp motif deletion constructs, docked at the *HPRT* locus. This will help in revealing the role of the transcription factors involved in expression of *PDGFRa* and *PLD/DM20* in OPCs.

Cuprizone model can be used, to study the role of these potential regulatory regions in remyelination. In this model, addition of cuprizone to the diet induces demyelination in CNS, especially corpus callosum. With three weeks of cuprizone treatment the amount of myelin in CNS reaches to its minimum. After this, remyelination starts after six weeks. Population of OPCs has been shown to increase in corpus callosum between one to three weeks of cuprizone

treatment. The number of OPCs decreases after six weeks, when the number of oligodendrocytes increases and remyelination starts (Hiremath et al., 1998; Mason et al., 2000; Morell et al., 1998).

Conclusion

Expression of *PDGFRa* in OPCs seems to be controlled by more than one regulatory sequence. Generating recombineered BAC transgenic mice was a very useful approach to narrow down the 380kb candidate sequence to an 87kb sequence. Interspecies sequence comparison and searching for the presence of relevant transcription factor binding sites in this 87kb region led me to only four candidate sequences. Transgenic mice generated from these candidate sequences, docked at the *HPRT* locus, showed that a 600bp sequence (Int21 construct) expresses in some but not all of regions where OPCs are present, similar to the pattern of expression of the 87kb BAC. This suggests that the rest of the regulatory sequences responsible for expression of *PDGFRa* in OPCs, lie somewhere downstream of the 87kb BAC. Experiments are underway to further define the role of elements present in this 600bp sequence.

A 313bp sequence (wmN2(313)) was defined out of a 1200bp expressing region, which shows the same pattern of expression. It expresses in several cell types in the CNS including OPCs. The four conserved regions in this 313bp sequence could function individually in these four cell types or in combination

with each other. Constructs have been made out of these sequences to address this question.

This study and other similar studies may open windows to our knowledge about oligodendroglial lineage development and could possibly enable us to generate therapeutic interventions, in situations where oligodendrocyte development is not progressing normally, such as in multiple sclerosis.

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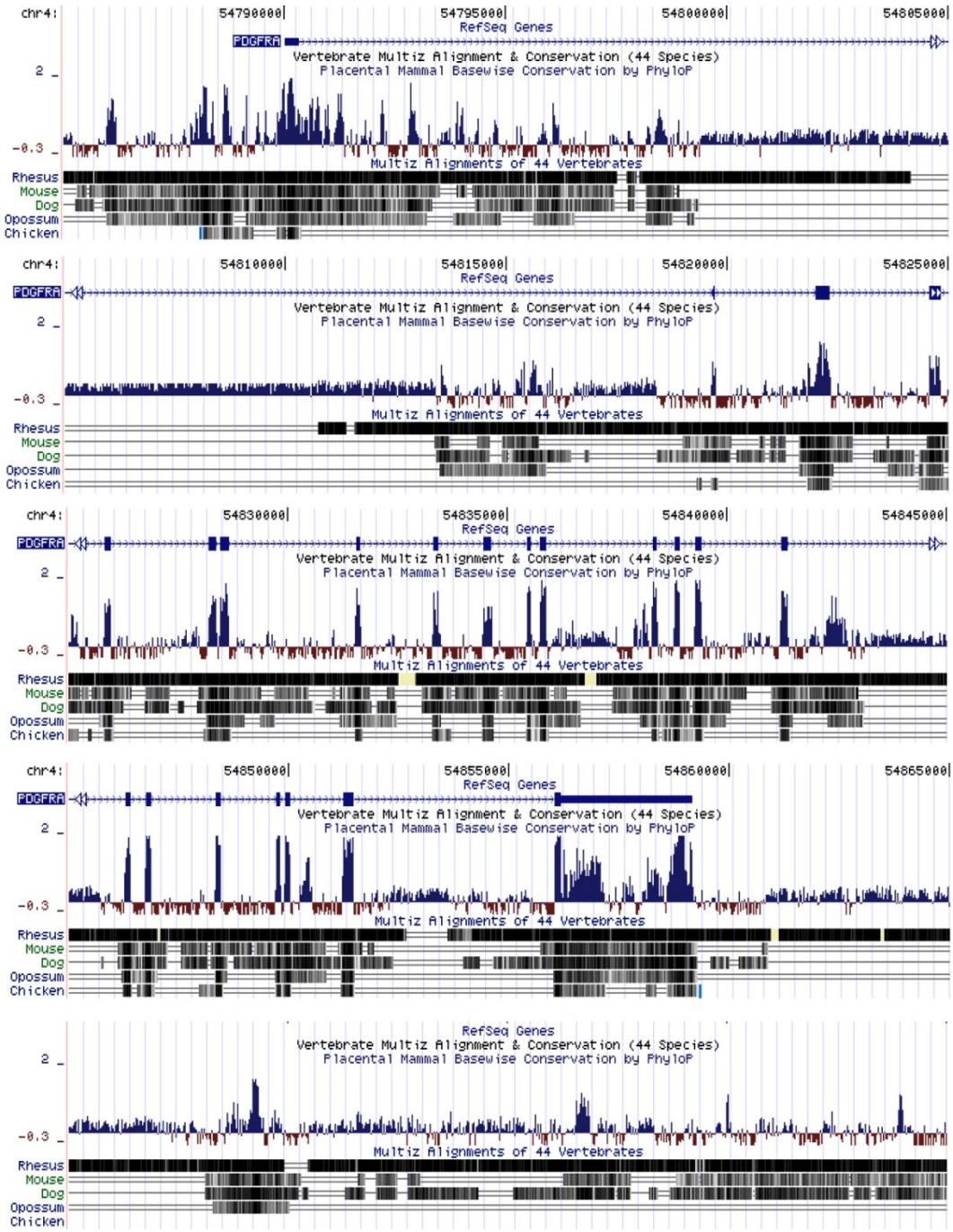
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Multiz Alignments of 44 Vertebrates
Rhesus
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Dog
Opossum
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RefSeq Genes
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Chicken

Figure 1. Interspecies sequence comparison.

Schematic representation of the *PDGFRa* locus in the human genome, from the UCSC genome browser website. The figure covers –5kb upstream from the transcription start site of the *PDGFRa* gene to +26kb downstream of the end of the last exon. The Human March 2006 assembly (hg18), which was used for the purpose of this study, is shown. The top numbers in each section show the genomic locus of that region. The *PDGFRa* gene is shown in blue. The blue vertical bars show the degree of interspecies conservation. A taller line indicates higher degree of interspecies conservation. *PDGFRa* genes of Rhesus, Mouse, Dog, Opossum and Chicken are represented at the bottom of each section. The black vertical lines indicate the presence of that part of the sequence in the species, and the density of the black line represents the degree of conservation.

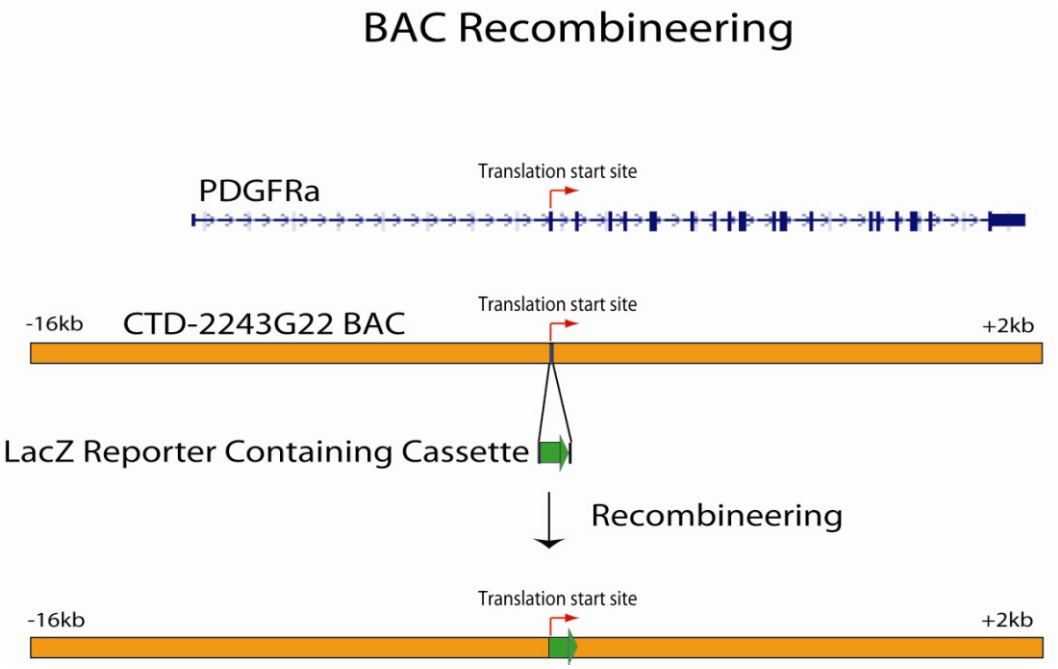

BAC Recombineering
PDGFRa
Translation start site
-16kb
CTD-2243G22 BAC
Translation start site
+2kb
LacZ Reporter Containing Cassette
Recombineering
Translation start site
-16kb
+2kb

Figure 2. BAC Recombineering.

Schematic representation of CTD-2243G22 BAC Recombineering. The human *PDGFRa* gene is shown at the top, in blue. The red arrow represents the translation start site which is located in exon 2. The orange bar represents the CTD-2243G22 BAC which covers the *PDGFRa* locus, from -16kb upstream of the transcription start site to +2kb downstream of the end of last exon, exon 23. The green arrow represents the *LacZ* reporter-containing cassette, with a homology arm at both ends, which was inserted in the CTD-2243G22 BAC, using recombineering technology. As the result, the translation start site of *LacZ* replaced the *PDGFRa* translation start site.

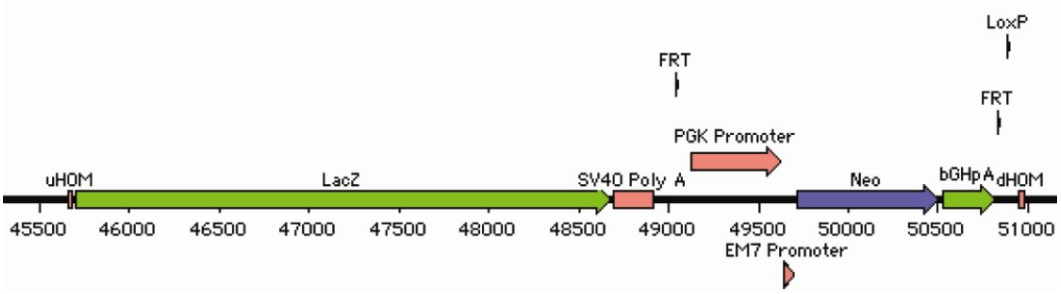

LoxP
FRT
FRT
PGK Promoter
uHOM
LacZ
SV40 Poly A
Neo
bGHp A
dHOM
45500
46000
46500
47000
47500
48000
48500
49000
49500
50000
50500
51000
EM7 Promoter

Figure 3. *LacZ* reporter-containing cassette.

Schematic representation of the *LacZ* reporter-containing cassette which was used to recombineer the CTD-2243G22 BAC. This 5.2kb insert has replaced a 50bp region (exon 2) in the original sequence of the BAC. The 50bp upstream homology arm (uHOM) is located exactly upstream of the ATG in exon 2 and the 50bp downstream homology arm (dHOM) is located exactly downstream of exon 2. FRT: FRT site-specific recombination site, LoxP: LoxP site-specific recombination site. The cassette also contains a neomycin selection marker.

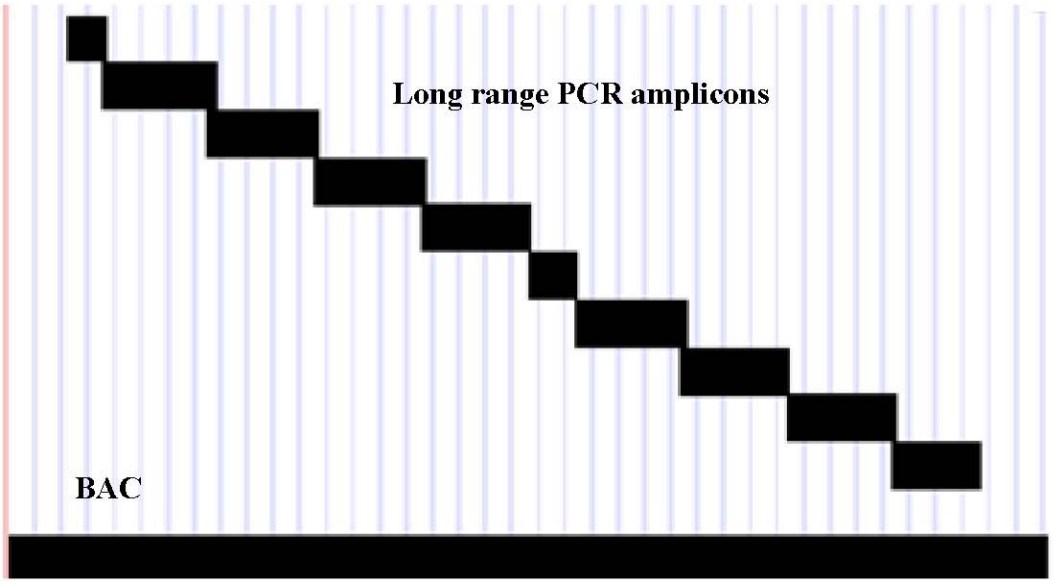
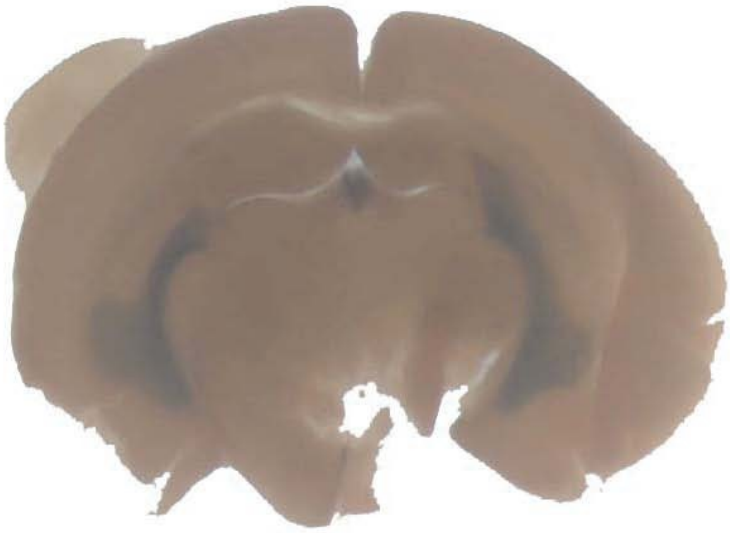

Long range PCR amplicons
BAC

Figure 4. PCR screening of recombineered CTD-2243G22 BAC in ES cells.

Schematic representation of the Long Range PCR, used to screen the integrity of the recombineered CTD-2243G22 BAC after transfection into ES cells. Since the BACs are so big, they tend to break after transfection into ES cells. Therefore, the ES cells were screened by long range PCRs for the integrity of the BAC. Transgenic mice were made with a good clone from the CTD-2243G22 BAC.



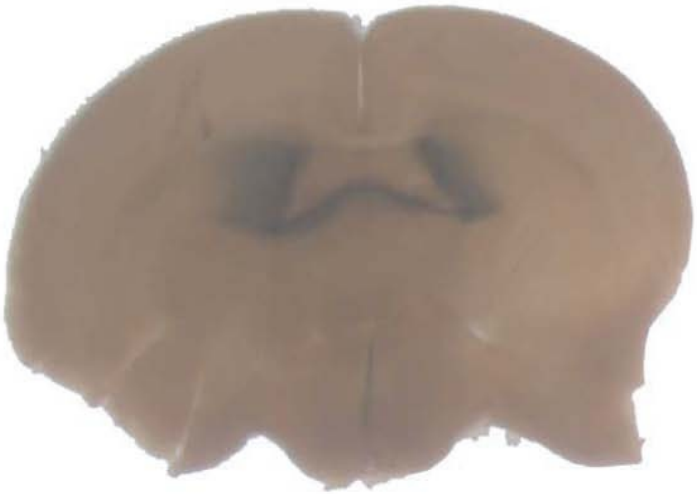
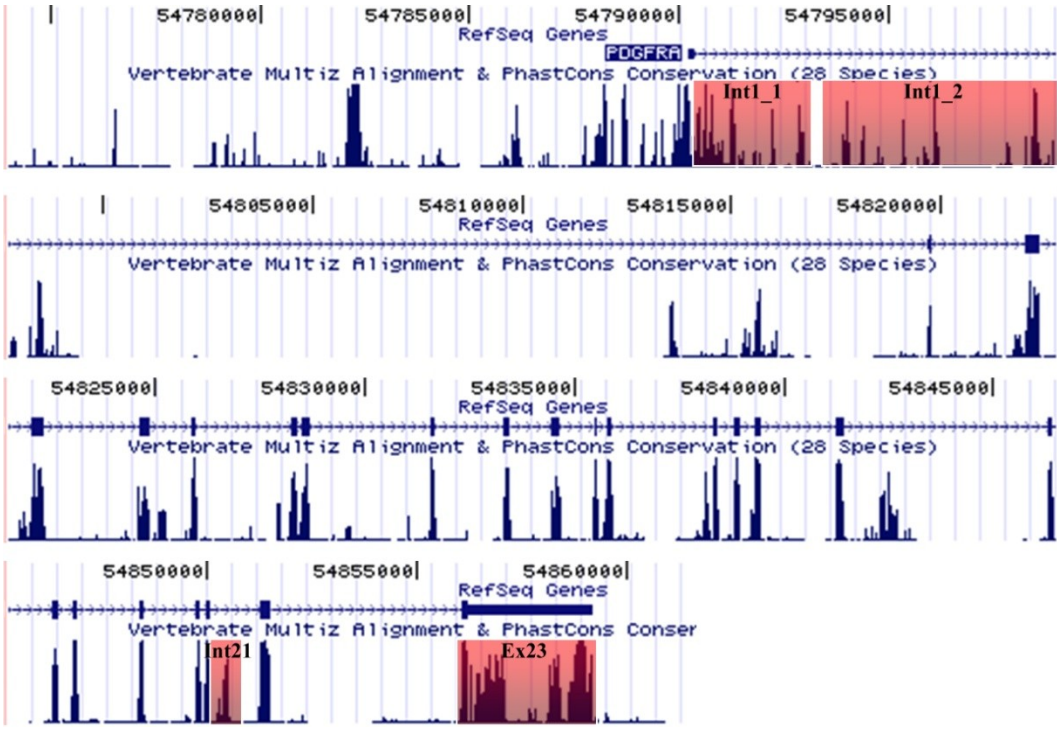


Figure 5. The CTD-2243G22 BAC expresses in brain regions where OPCs are found.

Results from brain of P5 & P10 chimeras of the CTD-224322 BAC, shows expression of *lacZ* in the subventricular zones (dark blue). This area is the area that the OPCs mainly originate from. It should be mentioned that at this age OPCs should be present everywhere in the brain, but *lacZ* expression in only detected in the subventricular zone. This picture shows two brain sections of a P10 chimera.


54780000
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RefSeq Genes
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Vertebrate Multiz Alignment & PhastCons Conservation (28 Species)
Int1_1
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RefSeq Genes
Vertebrate Multiz Alignment & PhastCons Conservation (28 Species)
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54845000
RefSeq Genes
Vertebrate Multiz Alignment & PhastCons Conservation (28 Species)
54850000
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Vertebrate Multiz Alignment & PhastCons Conser
Int21
Ex23

Figure 6. Four candidate sequences in the CTD-2243G22 BAC.

The figure shows a schematic representation of the *PDGFRa* gene in the UCSC genome browser web site. The *PDGFRa* gene is shown as a blue horizontal line at the top. The blue vertical lines represent the interspecies conservation. Based on the interspecies conservation and presence of transcription factor binding sites, four candidate regions were selected. The candidate regions are highlighted in red boxes.

Formatted Alignments

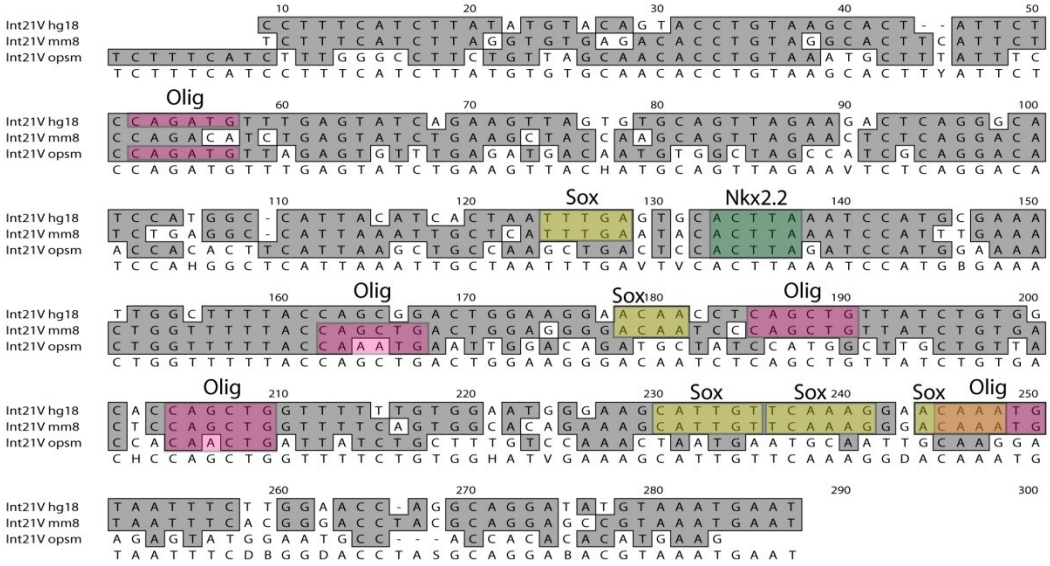
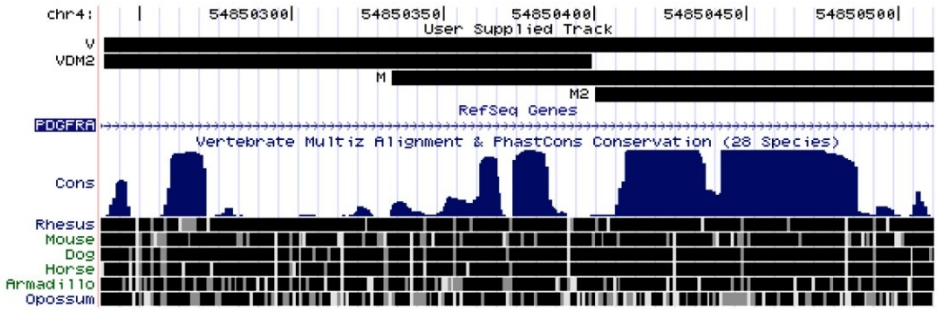


Figure 7. 275bp of the Int21 construct shows more conservation and contains relevant transcription factor binding site.

The figure shows the sequence of a more conserved 275bp (Int21V) region in the Int21 construct. Sequences from human, mouse and opossum are shown. The comparison has been made by Mac Vector program. The red boxes show consensus binding sites for Olig transcription factors. The yellow boxes show consensus binding sites for Sox transcription factors. The green box shows a consensus binding site for Nkx2.2 transcription factor.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Vertebrate Multiz Alignment & PhastCons Conservation (28 Species)
Cons
Rhesus
Mouse
Dog
Horse
Armadillo
Opossum

Figure 8. Consrtucts made out of the 275bp, Int21V sequence.

The figure shows a schematic representation of the Int21V sequence, part of the Int21 of human *PDGFRa* gene, from the UCSC genome browser website. Four different constructs were made out of this region; Int21V, Int21VDM2, Int21M and Int21M2. Each construct is shown with a black bar.

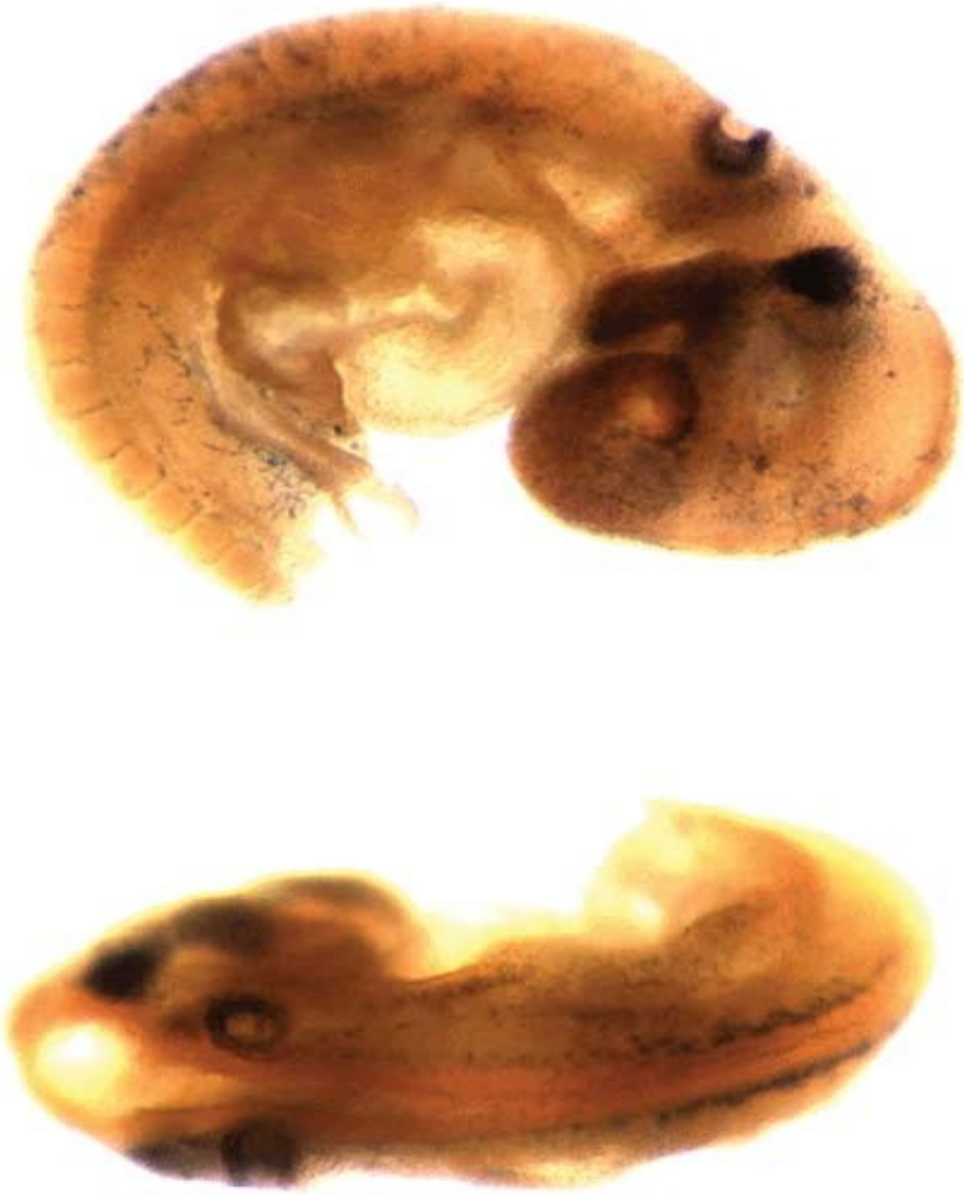
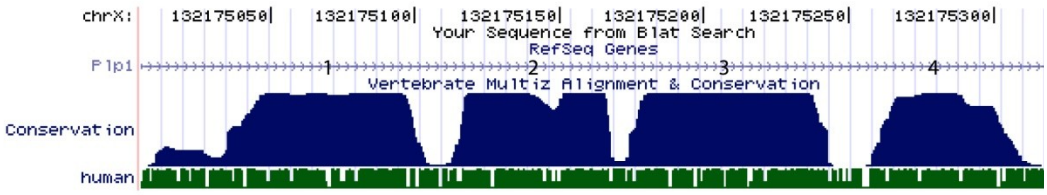


Figure 9. Expression of wmN2(313) construct.

Expression of the wmN2(313bp) construct in an E9.5 chimera. Intense expression was detected at E9.5 in several regions of the developing neural tube including the prosencephalic neural crest, trigeminal placode, epibranchial placodes, otic placode, and in neural crest cells migrating to form dorsal root ganglia.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Conservation
human

Figure 10. Interspecies comparison of wmN2(313) sequence.

Interspecies conservation across the 313bp wmN2 sequence. In a multiple species comparison, 4 well defined blocks of conservation are detected (large blue blocks). In order to reveal their role in *PLP/DM20* expression in OPCs, nine constructs were made out of these conserved sequences individually and in combinations. For example, wmN2_23 construct contains only blocks 2 and 3. Comparison between only human and mouse in contrast, reveals few substitutions (small green blocks at the bottom).

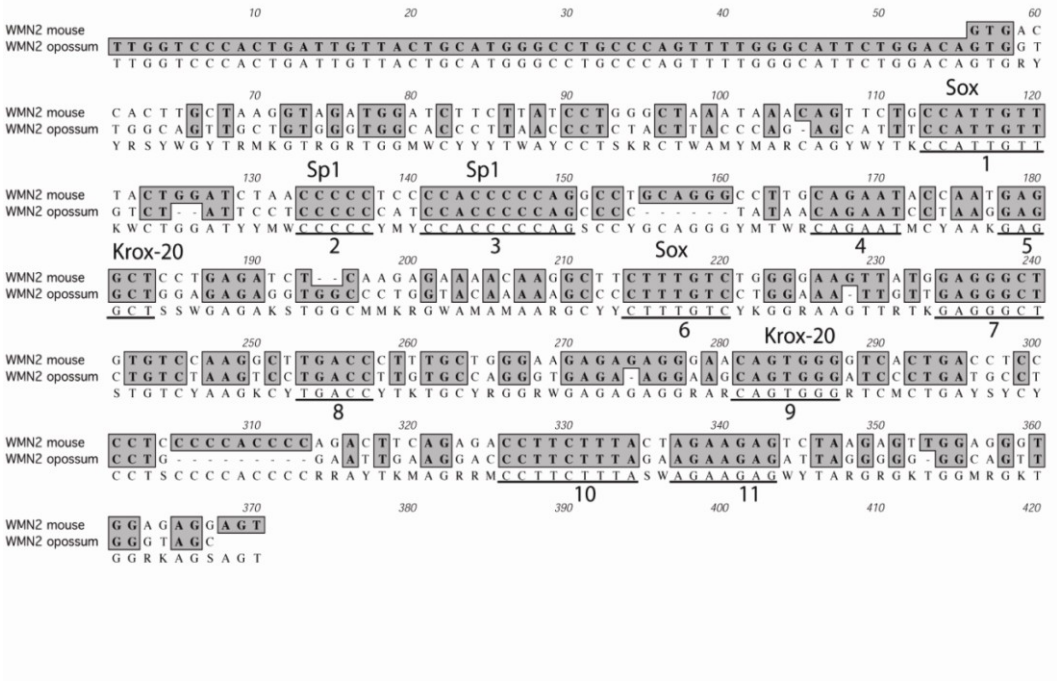

WMN2 mouse
WMN2 opossum
Sox
Sp1
Sp1
Krox-20
Sox
Krox-20
1
2
3
4
5
6
7
8
9
10
11

Figure 11. Conserved motifs within the wmN2(313) sequence.

Alignment of wmN2 sequence from mouse and opossum, by Mac Vector program. The alignment reveals 11 invariant motifs extending at least 5bp (underlined).

Table 1. Primers used for amplification of *PDGFRa* sequences from BAC DNA

Construct		
-3.5	**F**	ATCGATCGGCGGCCGCCCTTTTCCAGGACTAGTGCCGTTCTGAA
	R	ATCGGGTACCGAGTAAAGAGCGTGCCCACGGCCT
Int1_1	**F**	ATGGCGCGCCAATAACATCGGAGGAGAAGGTAAGGGAAAAG
	R	ATGCGGCCGCGAATTGTCACTTTTCCACCAGCAACGTATAAC
Int1_2	**F**	ATGGCGCGCCGCACTTTTGTGTGTGTGTGTGACAAGAGTC
	R	ATGCGGCCGCAGGAGAAACACAATTACAGTGCTGTGAACC
Int21	**F**	ATGGCGCGCCTGGAAAGGTCTGGATAAAGCTGGAAGTTATAC
	R	ATGCGGCCGCCACACAATCTCCTAACACTATTCCTCCTTGAG
Int21V	**F**	ATGGCGCGCCCCTTTCATCTTATATGTACAGTACCTG
	R	ATGCGGCCGCATTCATTTACATATCCTGCCTGG
Int21M	**F**	ATGGCGCGCCGGCCATTACATCACTAATTTGAG
	R	ATGCGGCCGCATTCATTTACATATCCTGCCTGG
Int21M2	**F**	ATGGCGCGCCAGGAACAACCTCAGCTGTTATCT
	R	ATGCGGCCGCATTCATTTACATATCCTGCCTGG
Int21VDM2	**F**	ATGGCGCGCCCCTTTCATCTTATATGTACAGTACCTG
	R	ATGCGGCCGCTCCAGTCCGCTGGTAAAAG
Ex23	**F**	ATGGCGCGCCTGTGCAGGAGTTGTAATATTTGCTCTTCTCTC
	R	ATGCGGCCGCAAGTCAAATACTGTGTCGTGGAACAATCTAGC

F: Forward primer, R: Reverse primer. Underlined letters represent sequence added to include restriction sites.

Table 2. Primers used for amplification of sequences within the original wmN2 construct from the *PLP* gene.

Construct		
wmN2(300)	**F**	ACGGCGCGCCGTGACCACTTGCTAAGGTAGATG
	R	ACCTCGAGACTCCTCTCCACCCTCCAAC
wmN2_1	**F**	ACGGCGCGCCGTGACCACTTGCTAAGGTAGATG
	R	ACTGACTGCCCGGGGCAGGCCTGGGGGT
wmN2_12	**F**	ACGGCGCGCCGTGACCACTTGCTAAGGTAGATG
	R	ACTGACTGCCCGGGCAGACAAAGAAGCCTTGTTTTC
wmN2_23	**F**	ACGGCGCGCCAGGGCCTTGCAGAATACCA
	R	ACCTCGAGAGGGGAGGTCAGTGACCC
wmN2_34	**F**	ACTGACTGCCCGGGAAGTTATGGAGGGCTG
	R	ACCTCGAGACTCCTCTCCACCCTCCAAC
wmN2_123	**F**	ACGGCGCGCCGTGACCACTTGCTAAGGTAGATG
	R	ACCTCGAGAGGGGAGGTCAGTGACCC
wmN2_234	**F**	ACGGCGCGCCAGGGCCTTGCAGAATACCA
	R	ACCTCGAGACTCCTCTCCACCCTCCAAC
wmN2_3	**F**	ACTGACTGCCCGGGAAGTTATGGAGGGCTG
	R	ACCTCGAGAGGGGAGGTCAGTGACCC
wmN2_2	+	CGCGCCAGGGCCTTGCAGAATACCAATGAGGCTCCTG AGATCTCAAGAGAAAACAAGGCTTCTTTGTCTGCCC
	-	GGGCAGACAAAGAAGCCTTGTTTTCTCTTGAGATCTC AGGAGCCTCATTGGTATTCTGCAAGGCCCTGG
wmN2_4	+	GGGCCCCCACCCCAGACTTCAGAGACCTTCTTTACTAG AAGAGTCTAAGAGTTGGAGGGTGGAGAGGAGTC
	-	TCGAGACTCCTCTCCACCCTCCAACTCTTAGACTCTTC TAGTAAAGAAGGTCTCTGAAGTCTGGGGTGGGGGCCC

F: Forward primer, R: Reverse primer. +: Positive strand, -: Negative strand. Underlined letters represent sequence added to include restriction sites.

Table 3. Primers used for screening the CTD-2243G22 BAC after recombineering.

Primers		
2.4_F	**F**	CGATCTCGAAGGATCTTAGAACGACACTG
1.2_R	**R**	ACGCACTCTGAAAATGGCCAAAGTG
Int1_end_F	**F**	CTAGGCTCCAGGGTTGTTTCT
3' LacZ	**R**	AATGTGAGCGAGTAACAACCCG
SHGC-	**F**	ACAGACATTGTATCAGCTGGGGT
SHGC-	**R**	AACTGATCCCGAGACTCCTGTAA
SHGC-	**F**	AAATGGGTGCTAAATTGATTGG
SHGC-	**R**	GCACATCTTTAGCAGGAGCC
RH68733_L	**F**	AGCAAATCATCTCAGCTACC
RH68733_R	**R**	AAATAAAGCAATTCACAGAAACTAC
GDB:626070_L	**F**	TGGCACCCCTTACCCCGGCA
GDB:626070_R	**R**	ACTTCACTGGTAGCGTGGT
SHGC-8069_L	**F**	AAACATGAACAGGGGCATTC
SHGC-8069_R	**R**	TAATCCCCACAGGCACATTAA

F: Forward primer, R: Reverse primer.

Primers starting from SHGC-85185 are STS marker primers.

Table 4. Primers used for PCR amplification of LacZNeofrt cassette.

Primers		
LacZ_F_uHOMhPDGFR*a*	**F**	TTGCTAATGCTGTTTCTGTTGACTTTTGACTTTTCTAGTTTCCCAGAGCTATGGGGGATCCCGTCGTTTTACAACG
PL451_R_dHOMhPDGFR*a*	**R**	AAAAAACAAGACACCCAAACAAGGAACTCAGAGAGGACTGGGCTCCGTACGGCCGCTCTAGAACTAGTGGATCCACCTA

F: Forward primer, R: Reverse primer. The underlined sequences represent the upstream and downstream homology arms to the human *PDGFRa* sequence, making the cassette suitable for recombineering. The upstream homology arm is located exactly upstream of the ATG in exon 2 and the downstream homology arm is located exactly downstream of exon 2.

Table 5. Primers used for sequencing the -3.5pENTlNRS construct.

Primers		
3.5_F	**F**	CCTTTTCCAGGACTAGTGCCGTTCTGAA
2.4_R	**R**	CAGTGTCGTTCTAAGATCCTTCGAGATCG
2.4_F	**F**	CGATCTCGAAGGATCTTAGAACGACACTG
1.2_R	**R**	ACGCACTCTGAAAATGGCCAAAGTG
1.2_F	**F**	CACTTTGGCCATTTTCAGAGTGCGT
3.5_R	**R**	GAGTAAAGAGCGTGCCCACGGCCT

F: Forward primer, R: Reverse primer.

Table 6. Primers used for sequencing the Int1_1-3.5pENTlNRS construct.

Primers		
Int1_1-1_F	**F**	ATCGGAGGAGAAGGTAAGG
Int1_1-2_F	**F**	AGCGGTTTGTTGGGTCAGTG
Int1_1-3_R	**R**	TCCTCAAATCCCAGTTGC
Int1_1-4_F	**F**	TCTCCTTCCTCTGCTGTCTAC
Int1_1-5_R	**R**	AAGTCCTTCCCGACCAAGAC
In1_1-6_F	**F**	AATGAACCCGTCGTTAGCC
Int1_1-7_R	**R**	CTCCTTCAAGTCCTTCTCG
pENT3'	**R**	CATCAGAGATTTTGAGACACGG

F: Forward primer, R: Reverse primer.

Table 7. Primers used for sequencing the Int1_2-3.5pENTlNRS construct.

Primers		
In1_2-1_R	**R**	TATGGTGAAACCCCGTCTC
Int1_2-2_F	**F**	GGAACGGTTCTTAAATGC
Int1_2-3_F	**F**	TGGATTGAGAATGGGACG
Int1_2-4_R	**R**	TGGAAACGACTGTTACCG
Int1_2-5_F	**F**	ACTGAGGTCACCACGAAAG
Int1_2-6_R	**R**	ACCTTCCTAATGGTCTCCG
Int1_2-7_F	**F**	AAAACAGGCTCTGGTAGGG
Int1_2-8_R	**R**	GATTCAGATTTCCGAGGC
Int1_2-9_F	**F**	GGCATCCCTCACTTTCTTAG
Int1_2-10_R	**R**	TTTCCACCATCCTCTTGGC
Int1_2-11_F	**F**	AAAGGGGGTTGTTCTCACGG
Int1_2-12_R	**R**	GCTCAGCGTGTTTGTGAAG
Int1_2-13_F	**F**	CCAGGGAACCACTGTTATC
Int1_2-14_R	**R**	AGGAGAATGGCTTCAACC
Int1_2-15_F	**F**	AAAAGCAGTGAGCCCTTC
pENT3'	**R**	CATCAGAGATTTTGAGACACGG

F: Forward primer, R: Reverse primer.

Table 8. Primers used for sequencing the Int21-3.5pENTlNRS constructs.

Primers		
Int21-1_F	**F**	TGGAAAGGTCTGGATAAAGC
Int21-2_F	**F**	ATTGGCTTTTACCAGCGG
pENT3'	**R**	CATCAGAGATTTTGAGACACGG

F: Forward primer, R: Reverse primer.

Table 9. Primers used for sequencing the Ex23-3.5pENlNRS construct.

Primers		
Ex23-1_F	**F**	CTTCATCAAGAGAGAGGACG
Ex23-2_R	**R**	TGACTTCCTGGACAACACTAC
Ex23-3_F	**F**	TAGGTTCCCCAATCCATCG
Ex23-4_R	**R**	GCATCCTTCTGACCCTTTTC
Ex23-5_F	**F**	AGATGCTACTTCCCACTGTATG
Ex23-6_R	**R**	GAATGCCAATGAACCCAC
Ex23-7_F	**F**	CCTAACAAATGCTCCCACC
Ex23-8_R	**R**	CCCTCTTCACAAAAATAGGACC
Ex23-9_F	**F**	TGTATTACGAATGCCCCTG
Ex23-10_R	**R**	TCATCTAAGGTCAGGAGTTCG
pENT3'	**R**	CATCAGAGATTTTGAGACACGG

F: Forward primer, R: Reverse primer.

Table 10. Primer used for sequencing the wmN2pENThel constructs.

Primers		
pENT3'	**R**	CATCAGAGATTTTGAGACACGG

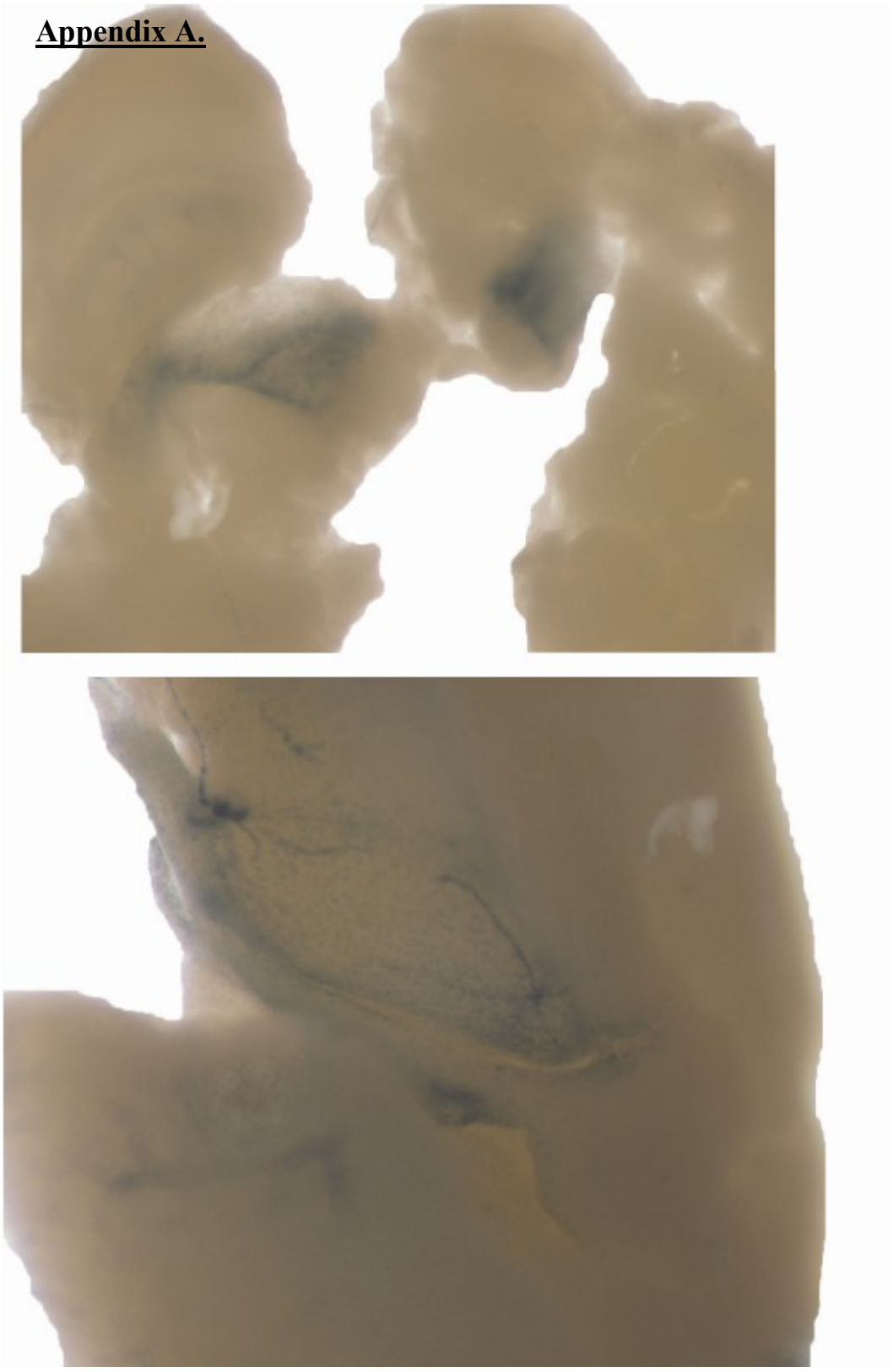
F: Forward primer, R: Reverse primer.

Table 11. Primers used for Long Range PCR screening of the recombineered CTD-2243G22 BAC, in ES cells.

Primers		
SCL01_F	**F**	TTTAGAAATCACTGCAGGCGGGTTAAAGAC
SCL01_R	**R**	CAAATTAAACTTCCAGGTTGACCTCTTGGG
SCL02_F	**F**	ACGTGGAGAAATCCCGTCTCTACTAAAAATAC
SCL02a_F	**F**	AGAGGAGGAGAAGACACAAAAAGCCAACTC
SCL02b_F	**F**	AGTGAGAAGCACAAGAAGGGGAGAAAAGAC
SCL02_R	**R**	ACTCAGGGCCAAATGTGTTTGTCATTATTC
SCL02c_F	**F**	GAAGGCCAAAAGTGTGCAGACTGTAAGAATAG
SCL02e_R	**R**	GAATTCTCTAAAAAGATCGAGATGGGTCTGTG
SCL02d_F	**F**	GTCCATAAAATCTAGGAGGAAAGGGCTGTATC
SCL02f_R	**R**	ATATGGTTTACCTCCCACCTTTCTGTCTTACC
SCL03_F	**F**	GAAGGCGACAGGTTTTAAATGAGTGTTTTC
SCL03_R	**R**	GAGGTCACTTTCATCACCATCTTCGTTTTG
SCL04_F	**F**	TCAAGATGAGCCTGGACAACATAGTGAGAC
SCL04_R	**R**	GTTTGTAGTTCTTGAAGAGGTCCTTCACGTC
SCL05_F	**F**	TTGTATATCTAGAAAACCCCATTGTCTCAGCC
SCL05_R	**R**	ATCATAGTTCACTGTATCCTTGAACTCCTGGG
SCL06_F	**F**	GTGGTTTCACAAGATACGGGTGATAAAGACC
SCL06_R	**R**	AACACTCAAATAGACAGAGGATGTCTGATTCC
SCL07_F	**F**	ACCTCACAAAGTGCTGGGATTATAGACATGAG
SCL07_R	**R**	CATGGTGAAACCCCCATCTCTACTAAAAATAC
SCL08_F	**F**	TTATGCACATACATACACACACACACACACAC
SCL08a_F	**F**	ATGTCTCAAACATCATCACGGAGATCCACT
SCL08_R	**R**	TAACTCACTCTCATTCAAACCTATCAGCAAGG
SCL08b_F	**F**	CCTGTTATGAGCCAGTGAATACAAAGATGAAC
SCL08c_R	**R**	GTATTTTCTCCAAAGTTGCTCCAACAGTAACC
SCL09_F	**F**	ATCACAAATGGAAAGACCCATGTCCTGATAG
SCL09_R	**R**	CAGGTTATCTTAACACTGTCTACATCCTGGGG
SCL10_F	**F**	ACACTGAGTGATGTCTGGTCTTATGGCATTC
SCL10_R	**R**	TAATGCACATCCAGCCTCCACTAAAGAATATC
SCL10a_R	**R**	CTCCTTACGTACTAGAGAAGTGGCTGAAAGGG
SCL11_F	**F**	GAGGCATAAACCTGTGCTGAACATAACTTCTC
SCL11_R	**R**	GGGTTCTGTATTCTGTTCCATTGGTCTATGTG
SCL12_F	**F**	AGAGCCTGCATAGCCAAAGCAAGACTAAAC
SCL12_R	**R**	AGACTTTTGAGGAGCCTACTGCAGTCATACAG

F: Forward primer, R: Reverse primer. The SCL 11 & 12 primers were used as negative controls.

Appendix A.



Appendix A. The Int21-3.5 construct expresses in the surface of brain ventricles.

Results from brain of P5 chimeras of the Int21-3.5 construct shows expression of *lacZ* in the subventricular zones (dark blue). This area is a single cell layer thick and is in a position consistent with the subependymal layer. The expressing cells putatively represent OPCs. This picture shows two different brain views from two separate P5 chimeras.

Appendix B.

ACTION	✓	DATE
COs		
DB	✓	April 1 '07
APPROVED		

www.mcgill.ca/research/compliance/animal/forms/ COPY

1

McGill University Animal Care Committee

RENEWAL of Animal Use Protocol

For: Research ☒ Teaching ☐ project

For Office Use Only:
Protocol #: 5038
Approval end date: MARCH 31, 2008
Facility Committee: RVH
Renewal#: 1st (2nd)

Principal Investigator: Alan C. Peterson
Protocol # 5038
Protocol Title: Regulation of the oligodendrocyte genome
Phone: 934-1934 ext. 35846
Unit, Dept. & Address: Molecular Oncology Group RVH H-5
Fax: 843-1478
Email: Alan@devbiol2.molonc.mcgill.ca **Level:** C
Funding source: 2004-2006 4466
Start of Funding: 2007 (approved-not funded during 2006) **End of Funding:** 2010
Emergency contact #1 + work AND home phone #s: Alan C.Peterson RVH ext. 35846 Home 937-3102
Emergency contact #2 + work AND home phone #s: Irene Tretjakoff RVH ext. 35850 Home 642-9035

1. Personnel and Qualifications

List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/ graduate student, fellow). If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to *www.animalcare.mcgill.ca* for details. Each person listed in this section must sign. ***(Space will expand as needed)***

Name	Classification	Animal Related Training Information	Occupational Health Program *	Signature *"Has read the original full protocol"*
Alan Peterson	Principal Investigator	Theory course, April 2005, McGill		Alan C. Peterson
Samar Dib	Ph.D. student	Mouse workshop McGill University		Samar Dib
France Bourdeau	Technician (AHT)	Mouse workshop McGill University		France Bourdeau
Julie Quinn-Pacquette	Technician (AHT)	Mouse workshop McGill University		
Irene Tretjakoff	Technician (AHT)	Mouse workshop McGill University		Irene Tretjakoff

AT cted Fuji

*** Indicate for each person, if participating in the local Occupational Health Program, see http://www.mcgill.ca/research/compliance/animal/occupational/ for details.**

Approved by: FACC approved GPB Dr Nazano for Dr. Peterson

2. Approval Signatures

Principal Investigator/ Course Director	Alan C. Peterson	**Date:** Jan 29, 2007
Chair, Facility Animal Care Committee		**Date:** 29 March, 2007
UACC Veterinarian		**Date:** 11 April 2007
Chairperson, Ethics Subcommittee *(D level or Teaching Protocols Only)*	—	**Date:** —
Approved Animal Use Period	**Start:** April 1, 2007	**End:** MARCH 31, 2008

3. Summary (in language that will be understood by members of the general public)

AIMS AND BENEFITS: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge ***(was section 5a in main protocol).***

ML RiC 11/04/07

25 MAR 2007

4. Has an unanticipated problem occurred?	YES ☐ NO ☒ if yes, supply details:

5. If creating genetically modified animals or new combinations of genetic modifications, complete and attach a *Phenotype Disclosure form* (http://www.mcgill.ca/research/compliance/animal/forms/)

6. Procedures

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES☒ NO ☐

IF NO, complete the following:
Detail new procedures that are different from section 10a of the original protocol *(include a copy of the entire revised procedure section 10a of the original protocol with the changes and/or new procedures in CAPS):*

b) For D level of invasiveness,

Include here ALL procedures except transgenic procedures, including the ones described in the original protocol as well as new and changed procedures in CAPS *(was section 10a in main protocol); Please only attach SOPs related to new and changed procedures to this renewal form.*

7. Endpoints

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES☒ NO ☐

IF NO, supply new endpoints that are different from the original protocol:

b) For D level of invasiveness,

Include here ALL endpoints, including the ones described in the original protocol as well as new and changed endpoints in CAPS:

8. Hazards (check here if none are used: ☐)

a) Are the hazards different from original protocol? (infectious, radioactive, toxic, carcinogen, tumours)

YES ☐ NO ☒ if yes, supply details (material, risks, precautions):

b) Have the cell lines been tested for human and animal pathogens? YES:☐ NO:☐ None used:☒

9. Description of Animals to be used in the coming year (only):

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals. *If more than 6 columns are needed, please attach another page*

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	mouse dmod3 mouse dmod4	mouse d Inter M a and b	mouse Prm-cre	mouse Plp-CreERT2	mouse P0-Cre	C57Bl/6 C3H 129 Balb/C DBA/2 hybrids + BC
Supplier/Source	in house	in house	Jackson Lab	Dr. Isom, U. of Michigan	Dr. Isom, U. of Michigan	in house
Strain						
Sex						
Age/Wt						
# To be purchased			6	6	6	
# Produced by in-house breeding	768 (includes breeders)	768 (includes breeders)	30	30	80	75
# Other (e.g.field studies)						
TOTAL# /YEAR	**768**	**768**	**36**	**36**	**86**	**75**

10. Justification of Animal Numbers:

BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear.

Based on prior HPRT based transgenic evaluations, we expect that deletion of modules 3 and 4will reduce mbp expression in the central and peripheral nervous systems while deletion of intermodular (IM) sequences a and b should be of little or no consequence. Should altered MBP expression levels occur in any of these mice, myelin sheath formation or maintenance abnormalities may occur. However, mice bearing the fully null mbp allele "shiverer" are viable and breed.

Mice bearing floxed regulatory sequences are now established. 140 mice will be required to maintain these multiple lines and generate mice with the appropriate combinations of Cre expressing transgenes and floxed regulatory sequences.

For both module and intermodular knockout lines, 3 homozygous and 3 heterozygous mice, at four time points during development (P10,P18,P90,P270), will be analyzed for myelin ultra-structure, myelin proteins and mRNA levels and compared to wild type control mice. Thus, mice of 9 genotypes will be compared. 9 (genotypes) x 4 (time points) x 3 (assay types) x 3 (replicate samples) = 324.

Homozygous knockout mice will be analyzed for remyelination capacity in the peripheral nervous system in regenerating sciatic nerves. After sciatic nerve crush injury, four time points will be analyzed (7, 14, 21 and 28 days post surgery) spanning the full recovery period. 3 mice will be sacrificed for each assay (mbp mRNA, protein levels and myelin structure). 5 (genotypes: control, M3, M4 IMa, IMb) x 4 (recovery stages) x 3

(replicate samples) = 60

To generate the above number of mice (324+60), 5 breeding cages (2 females and 1 male per cage) will be kept per line. 4 lines (M3, M4, IMa, IMb) x 15 mice x 2 (replacement of breeding trios every 6 mo) = 120

Homozygous knockout mice will be analyzed for remyelination potential in the central nervous system using the well extablished cuprizone demyleination/remyelination model and compared to wild-type (listed as strain 6) and heterozygous mice. Remyelination success will be evaluated at 10, 20 and 30days following the start of remyelination spanning the full recovery period. At the low doses of cuprizone used in our experiments, no other toxic effects except for limited weight gain are encountered. However, significant inter-animal variation in demyelination is observed. Therefore, we will analyze 5 mice per time point x 9 (different genotypes as above) x 3 (recovery stages) x 5 (replicate samples) = 675
To generate the above number of mice (675), 24 breeders will be kept per line. 4 lines (M3, M4, IMa, IMb) x 24 mice x 2 (replacement of breeders every 6 mo) = 192

To generate the described KO lines, Cre expressing transgenic mice (129TgPrm-cre, P0-CRE and PLP-CreERT2) will be mated with lines bearing "floxed"mbp regulatory sequences causing an in situ deletion of the targeted regulatory sequence. In one case this will occure in the zygote and in the others, it will be conditional and either oligodendrocyte OR SCHWANN CELL SPECIFIC, but require an injection of tamoxifen. Following these crosses, lines of the respective KO regulatory domains will be established to support the experiments described here. Minimally sized breeding colonies of these Cre expressing lines will be maintained to support the KO experiments. 6 mice of each genotype will be imported and breeding colonies will be established. 18 imported + 30 in each of 3 breeding colonies = 108

KO-CrERT and P0-CRE homozygous mice will be treated with tamoxifen to stimulate CRE expression to cause knock out of the floxed regulatory sequence. Mice will be analyzed 5, 10 and 60 DAYS after the last tamoxifen administration for myelin ultrastructre, myelin proteins and mRNA levels and compared to heterozygous and wild type mice. Five (genotypes) x 3 (time points) x 3 (assay types) x 3 (replicate samples) = 135 mice. To generate the above number of mice (135), 1 breeding trio (2 females and 1 male) will be kept per line. Five genotypes x 3 mice=15.

Summary of "Strain 1" and "Strain 2":
Strain 1: Seven hundred and sixty eight mice bearing deletions of modular sequences. Floxed lines @ 70 mice. Developmental study @ 162 mice. Sciatic nerve remyelination studies @ 30 mice. Breeding colonies @ 161 mice. Cuprizone treatments @ 300 mice. Conditional cre expression @ 45 mice. Strain 2: requirements are identical to strain 1 but the mice involved here have deletions of intermodular sequences.

Total requirements of investigation: 140 + 324 + 60 +120 + 675 + 192+ 108 + 135 +15 = 1769.

NOTE: P0-CRE mice provide a refinement to the experiment and were approved under an ammendment.

.

Submit to your local Facility Animal Care Committee. Please note that after two renewals, a full protocol needs to be submitted.

This approval does not imply that space will be made available. If a major increase of space needs is anticipated, please contact the appropriate animal facility manager.

McGill University Animal Care Committee
AMENDMENT to Animal Use Protocol

www.mcgill.ca/research/compliance/animal/

ACTION	✓	DATE
CCs		
DB	✓	May 8 '07
APPROVED		

Principal Investigator: Alan C. Peterson Protocol # 5038 May '08

Protocol Title: Regulation of the oligodendrocyte genome Phone: (514) 934-1934 ext. 35846

Unit, Dept. & Address: Molecular Oncology Group Fax: (514) 843-1479

Email: alan@devbiol2.molonc.mcgill.ca Level: C Funding: MSSC 2007-2009

1. ADDITIONAL ANIMALS REQUESTED: *(justify additional animal numbers in box 5 below)*

Species	Strain	Supplier / Source	# Animals Purchased	# By Breeding	Age	Sex	Weight	# Needed at One Time	#/Cage	Total Per Year

2. ADDITIONAL PERSONNEL: *If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed. Refer to www.animalcare.mcgill.ca for details. Each person listed in this section must sign to indicate that s/he has read the main protocol.*

Name	Classification	Animal Related Training	Occupational Health & Safety Program	Signature
Farnaz Forghani	Ph.D. student	Mouse workshop, McGill	no	
Ali Rastikerdar	Ph.D. student	Mouse workshop, McGill	no	

3. CHANGE IN FUNDING SOURCE AND/OR TITLE *(title of grant must appear on the cover page of the animal protocol)*

4. OTHER: *(including housing, procedure, anaesthetic/analgesic, problems anticipated) If additional procedures, specify how many and which animals are to be used.*

5. WHY ARE THESE AMENDMENTS NECESSARY? *If requesting additional animals, justify the numbers*

Farnaz was unavailable to sign renewal form at last submission.
And Ali were

Approval Signatures:

Principal Investigator/ Course Director	Alan C Peterson	Date	24 April 2007
Chair, Facility Animal Care Committee		Date	24 April 2007
UACC Veterinarian		Date	–
Chairperson, Ethics Subcommittee (D level, cloning or Teaching Protocols Only)	–	Date	–

Form revised December 2005

Note: the above modifications are valid until the expiration date of the main protocol.

SIMPLE LETTER OF AGREEMENT FOR THE TRANSFER OF MATERIALS

In response to the RECIPIENT's request for the MATERIAL, **strains:** SW102, SW105, SW106 and **plasmids:** pTamp, p1GCN21, pEL04, pL451, pL452, pL253, pgalK the PROVIDER asks that the RECIPIENT and the RECIPIENT SCIENTIST agree to the following before the RECIPIENT receives the MATERIAL:

1. The above MATERIAL is the property of the PROVIDER and is made available as a service to the research community.

2. THIS MATERIAL IS NOT FOR USE IN HUMAN SUBJECTS.

3. The MATERIAL will be used for teaching or not-for-profit research purposes only.

4. The MATERIAL will not be further distributed to others without the PROVIDER's written consent. The RECIPIENT shall refer any request for the MATERIAL to the PROVIDER. To the extent supplies are available, the PROVIDER or the PROVIDER SCIENTIST agree to make the MATERIAL available, under a separate Simple Letter Agreement to other scientists for teaching or not-for-profit research purposes only.

5. The RECIPIENT agrees to acknowledge the source of the MATERIAL in any publications reporting use of it.

6. Any MATERIAL delivered pursuant to this Agreement is understood to be experimental in nature and may have hazardous properties. THE PROVIDER MAKES NO REPRESENTATIONS AND EXTENDS NO WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED. THERE ARE NO EXPRESS OR IMPLIED WARRANTIES OF THE MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, OR THAT THE USE OF THE MATERIAL WILL NOT INFRINGE ANY PATENT, COPYRIGHT, TRADEMARK, OR OTHER PROPRIETARY RIGHTS. Unless prohibited by law, Recipient assumes all liability for claims for damages against it by third parties which may arise from the use, storage or disposal of the Material except that, to the extent permitted by law, the Provider shall be liable to the Recipient when the damage is caused by the gross negligence or willful misconduct of the Provider.

7. The RECIPIENT agrees to use the MATERIAL in compliance with all applicable statutes and regulations.

8. The MATERIAL is provided at no cost, or with an optional transmittal fee solely to reimburse the PROVIDER for its preparation and distribution costs. If a fee is requested, the amount will be indicated here: _____

The PROVIDER, RECIPIENT AND RECIPIENT SCIENTIST must sign this letter and return to the PROVIDER. The PROVIDER will then send the MATERIAL.

PROVIDER INFORMATION and AUTHORIZED SIGNATURE
Provider Scientist: Drs. Neal Copeland and Nancy Jenkins
Provider Organization: National Cancer Institute
Address: NCI-Frederick, P.O. Box B, Bldg. 539
Frederick, Maryland 21702, U.S.A.
Name of Authorized Official: Dr. Neal Copeland
Title of Authorized Official: Senior Investigator

Certification of Authorized Official: This Simple Letter Agreement __ has/ √ has not been modified. If modified, the modifications are attached.

_____
Signature of Authorized Official/Date

RECIPIENT INFORMATION and AUTHORIZED SIGNATURE

Recipient Scientist: Dr. Alan Peterson
Recipient Organization: McGill University
Address: 687 Pine Ave. West
Royal Victoria Hospital
Molecular Oncology Group H5-35
Montreal, PQ, H3A 1A1

Name of Authorized Official: Dr. Alan Peterson

Title of Authorized Official: Principal Investigator

Signature of Authorized Official: _____

Date: 9 JA06

Certification of Recipient Scientist: I have read and understood the conditions outlined in this Agreement and I agree to abide by them in the receipt and use of the MATERIAL.

Alan C. Peterson Jan 3, 2006
Recipient Scientist Date