CONDITIONAL REGULATION OF *Hoxa2* GENE EXPRESSION IN CG4 CELLS

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By

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ABSTRACT

Oligodendrocytes (OLs) are the glial cells responsible for the synthesis and maintenance of myelin in the central nervous system. Recently, *Hoxa2* was found by our laboratory to be expressed by OLs and down-regulated at the terminal differentiation stage during oligodendrogenesis in mice (Nicolay *et al*., 2004b). To further investigate the role of *Hoxa2* in oligodendroglial development, a tetracycline regulated controllable expression system was utilized to establish two stable cell lines where the expression level of *Hoxa2* gene could be up-regulated (CG4-SHoxa2 [sense *Hoxa2*]) or down-regulated (CG4-ASHoxa2 [Antisense *Hoxa2*]) in CG4 glial cells. Morphologically, no obvious differences were observed between CG4-SHoxa2 and CG4 wild-type cells, whereas CG4-ASHoxa2 cells exhibited much shorter processes compared with those of CG4 wild-type cells. Data from BrdU uptake assays indicated that an up-regulation of *Hoxa2* gene promoted the proliferation of CG4-SHoxa2 cells. PDGFαR (Platelet-derived growth factor [PDGF] receptor alpha), a receptor for the mitogen PDGF that enhances the survival and proliferation of OLs, was assessed at the mRNA level in both CG4 and CG4-SHoxa2 cells, but no significant differences were observed between *Hoxa2* up-regulated cells and wild-type CG4 cells with respect to the mRNA level of PDGFαR. In addition, specific investigations of the differentiation of CG4-SHoxa2 cells were carried out by characterizing the composition of stage specific oligodendroglial subpopulations in culture. Our immunocytochemical study did not indicate the differentiation course of the genetically engineered cells was significantly altered compared to CG4 wild-type cells, although results from semi-quantitative RT-PCR of oligodendrocyte-specific ceramide galactosyltransferase (CGT) and myelin basic protein (MBP) indicate that the differentiation of CG4-SHoxa2 cells was delayed when *Hoxa2* gene was up-regulated.

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2DM	cell culture grew in differentiation medium for 2 days
3DM	cell culture grew in differentiation medium for 3 days
ANOVA	analysis of Variance
A-P	anterior-posterior
ASHoxa2	antisense *Hoxa2*
B104 CM	B104 cells conditioned medium
bHLH	basic helix-loop-helix
BM	bone marrow
BMP(s)	bone morphogenetic protein(s)
bp	base pair
BrdU	bromodeoxyuridine
cAMP	cyclic adenosine monophosphate
Cdks	cyclin-dependent protein kinases
CGT	oligodendrocyte-specific ceramide galactosyltransferase
CMV	human cytomegalovirus
CNPase	2, 3-cyclic nucleotide 3-phosphodiesterase
CNS	central nervous system
CREB	cyclic AMP response element binding protein
DM	differentiation medium
DMEM	Dulbecco's modified eagle medium
Dox	doxycycline
Dpp	decapentaplegic
D-V	dorsal-ventral
E.coli	*Escherichia coli*
ECM	extracellular matrix
EDTA	ethylenediaminetetra acetic acid
EGF	epidermal growth factor
EM	electroporation medium
FBM	facial branchimotor neurons
FGF	fibroblast growth factor
FGF-2	fibroblast growth factor-2
FITC	fluorescein isothiocyanate
g	gravity
GalC	galactocerebroside C
GFAP	glial fibrillary acidic protein;
GM	growth medium
GRP(s)	glial restricted precursor(s)
h	hour
HD	homeodomain
Hyg	Hygromycin
ID2/4	inhibitor of DNA binding 2/4

IGF-1	insulin-like growth factor 1
IN(s)	interneuron(s)
LSD	least significant difference
µL	microliter
MBP	myelin basic protein
mg	milligram
min	minute
mL	milliliter
mm	millimeter
µm	micrometer
MN(s)	motor neuron(s)
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger ribonucleic acid
Myc	human c-myc protein
NC	neural crest
NCC(s)	neural crest cell(s)
Nkx2.2	NK2 transcription factor related, locus 2
Nkx6.1	NK6 transcription factor related, locus 1
NSC	neural stem cell
O-2A	oligodendrocyte-type 2 astrocyte
OD	optimal density
OL	oligodendrocyte
Olig1/2	oligodendrocyte lineage gene 1/2
OPC(s)	oligodendrocyte precursor cell(s)
p27/Kip1	kinase inhibitory protein 1
Pax	paired box gene
PBS	phosphate buffer saline
PBS-T	PBS with 0.08% Tween-20
PDGF	platelet derived growth factor
PDGFαR	platelet derived growth factor receptor α
PDGFβR	platelet derived growth factor receptor β
P-D-L	Poly-D-Lysine
PVDF	polyvinylidene difluoride
PKA	protein kinases A
PKC	protein kinases C
PLB	passive lysis buffer
PLP	myelin proteolipid protein
PNS	peripheral nervous system
r	rhombomere
RA	retinoic acid
RIPA	radioimmunoprecipitation assay
rlu	relative light unit
RM	recovery medium

R-mAb	Ranscht monoclonal antibody
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
s	second
Shh	sonic hedgehog
SHoxa2	sense *Hoxa2*
SM-PBS	skim milk in PBS
Sox	SRY-box containing gene
T3	triiodothyronine
Taq	*Thermus aquaticus*
Tet	tetracycline
*t*TA	tetracycline transcriptional activator
TF(s)	transcription factor(s)
TH	thyroid hormone
TPPS	transferrin-putrescine-progesterone-sodium selenite
TRs	thyroid hormone receptors
Ubx	*Ultrabithorax*
V	voltage
+ve	positive
VZ	ventricular zone

I INTRODUCTION

The vertebrate central nervous system (CNS), a highly organized complex structure is comprised of two fundamental cell types: neurons and glial cells. Neurons interconnect to establish the neuronal circuitry, facilitating electrical activity to process information, while glial cells are generally considered to provide physical or nutritional support to neurons. The two most abundant classes of CNS glia are astrocytes, which are important for nurturing neighboring neurons, and oligodendrocytes (OLs), which are responsible for myelin formation. Research interest on OLs has increased significantly in recent years, and has revealed that OLs play an essential role in neuronal functions, and are involved in processes ranging from neuron development (reviewed by Du and Dreyfus, 2002), to synaptic regulation (reviewed by Fields, 2005), to CNS degenerative diseases (reviewed in Ludwin, 1997; Compston, 2004b).

Significant efforts have been made by researchers to provide detailed information on when and where OL-lineage cells originate, migrate and differentiate. In vertebrate brain, oligodendrocyte precursor cells (OPCs) originate from restricted ventricular regions (Warf *et al*., 1991; Noll and Miller, 1993; Levison *et al*., 2003) during embryonic development and then follow guidance cues from surrounding tissues to reach their final destination by long distance migration. Regulated by both intrinsic timing modulators and extrinsic signals from nearby environment, OPCs withdraw from cell cycling and start cell differentiation and maturation to give rise to myelin-producing OLs. In cultures, the generation of mature OLs has been well characterized, with several known cell surface markers (A2B5, PDGFαR [platelet-derived growth factor receptor-α], O4, GalC, and MBP [myelin basic protein]) and corresponding cell morphologies that depict specific developmental stages (Fig. 1).

Of particular importance is to understand the transcriptional regulation during oligodendrogenesis, which governs the orderly gene expression that orchestrates the differentiation of this cell lineage. A number of transcription factors (TFs) essential for OL specification and maturation have been identified (Wegner, 2000; Marquardt and Pfaff, 2001; Gokhan *et al*., 2005; Wegner and Stolt, 2005; Ligon *et al*., 2006). For example, two basic helix-loop-helix (bHLH) TFs *Olig1* and *Olig2* are key factors that determine the origins of OPC, as well as their relationship to motor neurons (MNs)

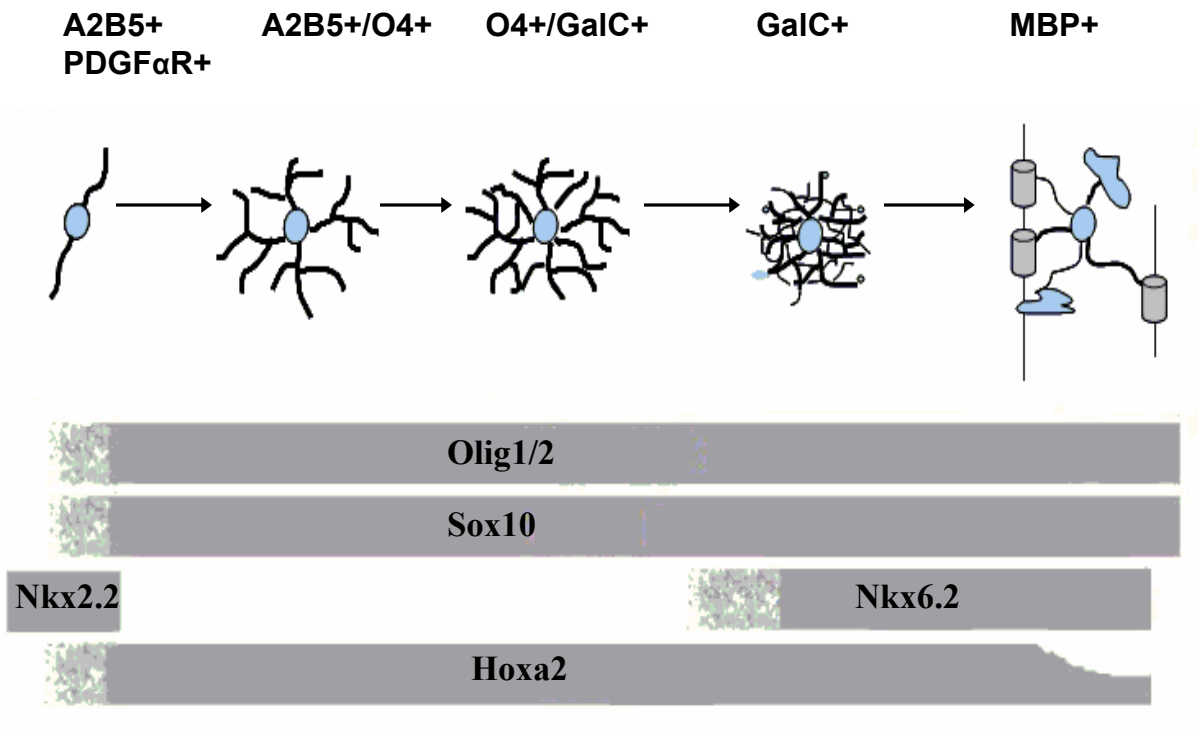

A2B5+
PDGFαR+
A2B5+/O4+
O4+/GalC+
GalC+
MBP+
Olig1/2
Sox10
Nkx2.2
Nkx6.2
Hoxa2

Figure 1. Schematic illustration of the developmental course of OL-lineage cells and the expression profiles of several transcription factors along the differentiation pathway. The differentiation of OLs from their progenitors follows a stepwise morphological transformation from bipolar appearance to multiple processes, then to a membrane-sheath-bearing appearance. In addition to the phenotype change there is a sequential expression of lineage specific markers: A2B5 antigen, platelet-derived growth factor receptor-α (PDGFαR) in early progenitor cells, O4 antigen in late progenitor cells, GalC in pre-mature OLs, and myelin basic protein (MBP) in myelinating OLs. Olig1, Olig2 and Sox 10 are expressed throughout the development of OL cells, whereas Nkx2.2 is only expressed during the early stages and Nkx6 homeodomain proteins are temporally expressed during maturation stages. *Hoxa2* has been shown to be down-regulated at late developmental stages (Nicolay *et al*, 2004). The grey shaded bars indicate the temporal expression of the particular TF during oligodendrogenesis (Adapted from Zhang, 2001 with permission).

(Takebayashi *et al*., 2000; Lu *et al*., 2000, 2002; Zhou and Anderson, 2002). TFs of the homeobox gene family, in particular NKx2.2, play an important role in normal differentiation of OLs (Qi *et al*., 2001; Fu *et al*., 2002).

More recent studies have identified the presence of *Hox* genes in OL-lineage cells (Hao *et al*., 1999; Nicolay *et al*., 2004a, b). In vertebrates, *Hox* genes encode a family of TFs that play an critical role in determining cell identity (Trainor and Krumlauf, 2001; Gaufo *et al*., 2004), establishing body segmentation (Rijli *et al*., 1998; Dubrulle and Pourquie, 2002), modulating facial patterning (Rijli *et al*., 1998; Creuzet *et al*., 2005), and regulating cell proliferation and differentiation (Ford, 1998; Del Bene and Wittbrodt, 2005). In the vertebrate hindbrain, *Hox* genes exhibit a highly segment-restricted expression profile and are believed to be crucial for specifying the identities of the reiterated units (rhombomeres) and the tissues originating from them (Rijli *et al*., 1998; Dubrulle and Pourquie, 2002; Creuzet *et al*., 2005). Particularly, in a role that resembles their function in regional specification, *Hox* genes participate in determining neuronal cell fates in the CNS, as well as in peripheral tissues derived from the neural crest (NC) (Trainor and Krumlauf, 2001; Gaufo *et al*., 2000,2004; Briscoe and Wilkinson, 2004). Furthermore, mounting evidence suggests *Hox* genes are also involved in directing and maintaining the precise projection of axons in the CNS (Gavalas *et al*., 1997; Arenkiel *et al*., 2003, 2004; Gaufo *et al*., 2004), possibly by playing key roles in both neurons and glial cells (Arenkiel *et al*., 2003, 2004). Although early development of both neurons and Schwann glial progenitor cells appear normal with respect to specification and cell population number, loss of *Hoxb1* in peripheral glial cells results in incorrect projection of the facial nerve, likely by interfering at the later developmental stage of these glial cells. An additional interesting finding from Arenkiel *et al.*'s work (2003, 2004) is that the majority (over 95%) of the myelin sheaths that wrap around the axons of the facial nerve are derived from rhombomere 4 (r4) neural crest cells (NCCs) that also express *Hoxb1*, implying *Hox* genes might serve as a marker for correct contact and ensheathment between the neurons and Schwann cells.

An important question that remains unresolved is whether *Hox* genes also participate in programming the fate of OLs. Nicolay *et al.* (2004b) conducted the first attempt to address this question by investigating the role of *Hoxa2* gene, but no

substantial abnormalities of early OL specification were observed in the *Hoxa2* knockout mice (Nicolay *et al.,* 2004b). The seemingly unaltered expression profiles of Olig2, Nkx2.2 and Pax6, and the detection of O4+ve (positive) or MBP+ve OL-lineage cells are likely due to gene compensation for *Hoxa2*, since 39 *Hox* genes are expressed in vertebrate CNS, and redundancy is well known in this family (reviewed in Akin and Nazarali, 2005). Alternatively, the *Hoxa2* gene could function at a later stage in the myelination process which was not considered in Nicolay *et al.*'s (2004b) study.

In the brain, space restriction calls for tight organization among different types of cells. Hence, the regulation of cell division, cell number, as well as cell differentiation of OPCs is critical for the ensheathment and survival of axon bundles (Burne *et al.,* 1996; Ishii *et al.,* 2001; McTigue *et al.,* 2001; Kotter *et al.,* 2006). Although a coordinated orchestration of intracellular modulators and extracellular signals has been suggested to regulate the proliferation and differentiation of OL-lineage cells (Durand and Raff, 2000), our knowledge of the underlying mechanism(s) remains limited. *Hoxa2* appears to be down regulated in more mature OLs that express myelin basic protein (MBP) (Nicolay *et al.,* 2004b). In addition, primary OPCs isolated from *Hoxa2*-null mice display a somewhat more mature phenotype in culture than their counterpart derived from wild-type mice (unpublished data from our laboratory). Taken together, *Hoxa2* might participate in modulating cell-cycle and/or differentiation timing of oligodendroglial progenitor cells.

To examine the potential role for *Hoxa2* gene, I have used the CG4 cell line (Louis *et al.,* 1992), a pure cell population with well characterized properties resembling oligodendrocyte-type 2-astrocyte (O-2A) in culture. An inducible expression approach (Tet-off) was employed to either increase or decrease the expression level of Hoxa2 protein in CG4 cells. Once a stable expressing cell line was developed, the impact of over-expression of *Hoxa2* gene on the proliferation and differentiation of genetically modified CG4 cells was examined.

1.1 Hypothesis

The hypothesis to be tested is that up or down regulation of the expression level of *Hoxa2* gene will influence the proliferation and differentiation of the genetically modified CG4 cells.

1.2 Objectives

1.2.1 To develop a tetracycline controlled *Hoxa2* sense and antisense expression system, respectively, in the CG4 cell line

One limitation of using *Hoxa2* null mice as study model is the effect of gene compensation, where homologous gene products may compensate for the lost function of the disrupted gene and thus, obscure the function of the target gene. Additionally, *Hoxa2* gene knockout causes severe developmental defects and animals die soon after birth, which prevents the investigation to be carried further. Therefore, the ability to establish loss-of-function and gain-of-function in a controllable and inducible manner is particularly important and relevant so as to overcome these limitations.

1.2.2 To examine the impact of an up-regulation of *Hoxa2* gene on the proliferation and differentiation of CG4 cells

Experiments carried out by Nicolay *et al*., (2004) employed *Hoxa2* null mice as study subject, examining the role of *Hoxa2* gene in OL-lineage cells using a loss-of-function approach. Also, given limited time, I decided to focus on investigating the impact of over-expressing *Hoxa2* gene on the proliferation and differentiation of OL-like cells.

II LITERATURE REVIEW

2.1 Oligodendrogenesis

Astrocytes, microglia and OLs are commonly known as glial cells, and constitute the majority of the cell population in the central nervous system (CNS) of the vertebrates. OLs form the fatty sheath surrounding nerve fibres known as myelin. Accumulating evidence indicates that myelin plays a key role in the structure, function and maintenance of axons, providing essential growth factors (Byravan *et al*., 1994; Dai *et al*., 1997; Raabe *et al*., 1997) and guidance cues (Kuhn *et al*., 1999; Sandvig *et al*., 2004; Goldberg *et al*., 2004). Damage of myelin via X-irradiation (Colello *et al*., 1994), pathological attack (Martin *et al*., 1992) or genetic instability (Jacobs, 2005) can lead to severe neural degeneration, functional deficits, or animal lethality. As a result, significant efforts are being taken to explore the many biological aspects of oligodendrogenesis, and to identify the underlying mechanisms that modulate the developmental course of the OL-lineage cells, aiming to develop potential therapeutic approaches for myelin related neural disorders.

Mature OLs are derived from highly mitotic and migratory precursor cells (OPCs), which undergo a sequential process of cell development. This embryonic advancement entails a complex combination of events established through a program of gene expression and controlled by master transcriptional regulators. I provide below a review of the transcriptional mechanisms controlling the cell cycle progression, cell growth, and differentiation of OL-lineage cells, with specific focus on understanding the potential role of *Hox* genes in these processes.

2.1.1 Stepwise development of OL-lineage cells

Progress in morphological techniques and the discovery of cellular markers by immunocytochemical techniques have greatly expanded our knowledge of OL-lineage cells. OL cells are morphologically distinguishable from other glial cells, possessing ample amount of microtubules in their extending processes, but lacking in intermediate filaments and glycogen (Peters *et al*., 1991; Baumann and Pham-Dinh, 2001). In addition, extending from the cell body of a myelinating OL are a variable number of processes, which contact and repeatedly envelop 1-2 mm segments of as many as 40 different axons during early postnatal development in rodents (Bray *et al*., 1981; Fanarraga *et al*., 1998).

Prior to maturation and myelin formation, OL-lineage cells undergo a series of phenotypic stages of development, characterized by the expression of distinct profiles of antigenic markers (Fig.1), as well as a change in their mitotic and migratory status (reviewed in Zhang, 2001; Miller, 2002). In culture, the OL lineage has been subcategorized into 4 stages: progenitors (A2B5+), pre-oligodendrocytes (late progenitors, O4+), pre-myelinating (immature, GalC+) oligodendrocytes and myelinating (mature, MBP+) oligodendrocytes (Woodruff *et al*., 2001; Zhang, 2001; Miller, 2002). OPCs are also known as oligodendrocyte-type 2-astrocyte (O-2A) cells, because they can differentiate into both OLs and type 2 astrocytes depending on the culture medium (Raff *et al*., 1984). OPCs are highly mitotic and migratory, featuring cell surface markers that include A2B5 and platelet derived growth factor receptor alpha (PDGFαR) (Raff, 1989). Late-progenitor cells retain their mitotic ability, and gradually lose anti-A2B5 antibody reactivity, while they begin to express the antigen recognized by the O4 monoclonal antibody (Pfeiffer *et al*., 1993). Upon reaching the target destination, single OL cells become postmitotic and start to express galactocerebroside (GalC or O1 antigen) on their surface. At this stage, non-myelinating OLs have an extremely dense network of processes that contain primary (emerging from the cell body), secondary (branched on the primary process), and tertiary (interconnecting primary and secondary filaments) outgrowths. Eventually, myelinating OLs are defined by the expression of myelin-specific proteins, including myelin basic protein (MBP), myelin proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) (Baumann and Pham-Dinh, 2001; Le Bras *et al*., 2005). In comparison to the non-myelinating stage, the morphology of a myelinating OL is dramatically altered. The myelinating OLs have less primary and secondary processes, no tertiary processes, but bear whorls of myelin-like structures at their tips (Fig. 1).

In summary, throughout the maturational stages of an OL, a number of cell lineage specific markers are sequentially expressed as others are lost. The well defined profile of stage-specific cell surface markers has allowed the progressive differentiation and maturation of OL-lineage cells to be followed, providing insights into particular functions carried out by these cells.

Given that the orderly changes of gene expression are under the coordination of signaling molecules and cell intrinsic transcription factors (TFs), elucidating the transcriptional control cascade that governs the development of oligodendroglial cells has been of considerable interest. In this context, several families of TFs that are essential for the generation, specification and maturation of OLs have been identified, in particular the homeodomain (HD) protein family (Nkx2.2, Nkx6.1, Nkx6.2, Hoxa2, Hoxb1), the zinc finger protein family (Olig1, Olig2, Id2, Id4) and the HMG-domain protein family (Sox10) (reviewed in Wegner, 2000; Zhou *et al*., 2000; Qi *et al*., 2001; Zhou and Anderson, 2002; Wei *et al*., 2004, 2005). Notably, research suggests some of these known TFs, including Olig1, Olig2 and Sox10, are restricted to OL-committed cells in the CNS (Kuhlbrodt *et al*., 1998; Zhou *et al*., 2000), and thus these TFs are frequently utilized in research as OL cell lineage markers.

2.1.2 Generation and specification of OL-lineage committed precursors

Glial cells and neurons are both derived from multipotent neural stem cells (NSC) (Wu *et al*., 2006), but the intermediate steps that NSCs follow to generate the OL-lineage cells are still undetermined. Based on *in vivo* and *in vitro* studies, models of different lineage perspectives have been proposed for the genesis of oligodendrocytes (reviewed in Anderson, 2001; Stiles, 2003). All of these models agree that some known TFs, such as Olig1, Olig2, and Nkx2.2, mediate the switch from intermediate progenitors to OPCs.

2.1.2.1 Dorsal-ventral debate of oligodendrocyte precursor cell origin

Mature OLs that disperse widely in the white matter of the CNS are derived from highly proliferative and mobile progenitors, the origins of which have been extensively discussed and examined (Warf *et al*., 1991; Pringle and Richardson, 1993; Fok-Seang and Miller, 1994; Hall *et al*., 1996; Hardy and Friedrich, 1996; Birling and Price, 1998; Tekki-Kessaris *et al*., 2001). With the recent discovery of cell-lineage specific markers and increasing understanding of the transcriptional patterning in the developing neural tube, researchers have been able to locate the birth place of OPCs (Fig. 2).

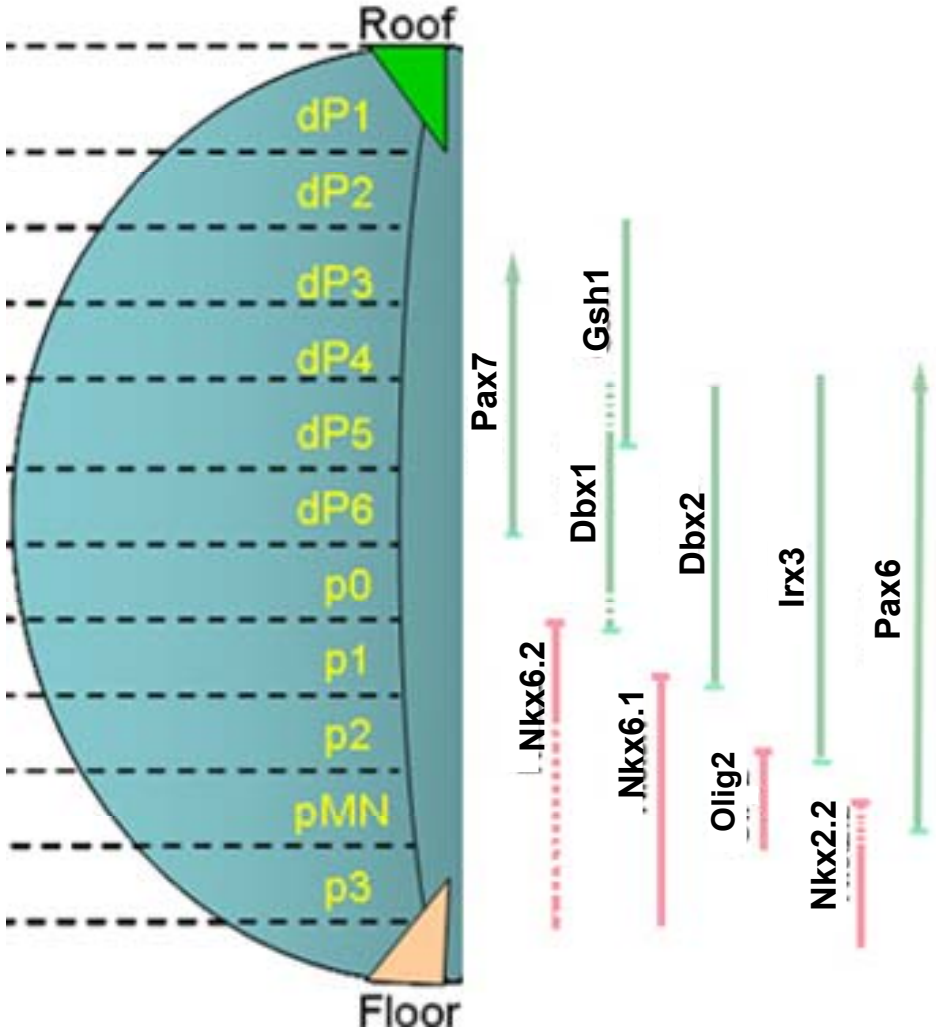

Roof
dP1
dP2
dP3
dP4
dP5
dP6
p0
p1
p2
pMN
p3
Floor
Pax7
Gsh1
Dbx1
Dbx2
Irx3
Pax6
Nkx6.2
Nkx6.1
Olig2
Nkx2.2

Figure 2. The embryonic spinal cord is divided into various domains that give rise to specific neural progenitor cell types. Several homeodomain (HD) families of proteins were identified in the spinal cord, the expression profiles of which are featured with a regional-restricted characteristic associated with sonic hedgehog (Shh) signalling. The cell types generated from each domain are indicated on the left. Transcription factors belonging to the Class I factors are repressed by Shh, and are indicated in green on the right. The expression patterns of Class II factors are depicted in red on the right. P1–dP6, dorsal progenitor domains; pMN and p0-p3, ventral progenitor domains; Olig2, oligodendrocyte lineage gene 2; Pax6,7, paired box gene 6,7; Dbx1,2, developing brain homeobox 1,2; Irx3, iroquois homeobox protein 3 (Modified from Richardson *et al*., 2006).

One of the best studied systems of neural patterning is the embryonic spinal cord, where regional restricted expression profiles of particular TFs have been depicted (Fig. 2) (Reviewed by Woodruff *et al*., 2001; Kessaris *et al*., 2001; Marquardt and Pfaff, 2001; Richardson *et al*., 2006). During early neurogenesis, a gradient of the morphogen sonic hedgehog (Shh), emanating from notochord and floor plate, set up the expression of two classes of patterning cues. Class I molecules, including Pax6, Dbx1 and Irx3, are repressed by Shh, whereas the expression of Class II members, Nkx2.2, Nkx6.1 and Olig1/2 requires Shh (Briscoe *et al*., 2000; Briscoe and Ericson, 2001). In addition, these TFs exhibit a nested pattern of expression in the spinal cord, partitioning the ventricular zone of the spinal cord into 5 progenitor domains, namely p0, p1, p2, pMN and p3, respectively (Fig. 2) (Briscoe *et al*., 2000; Jessell, 2000). Intriguingly, all of the patterning molecules, with the possible exception of Olig2, are homeodomain TFs, and they likely function as transcriptional repressors (Jessell, 2000). Since the appearance of Olig2 and PDGFαR expressing cells are first seen in the pMN domain (Pringle and Richardson, 1993; Hall *et al*., 1996), and dorsally derived E14 neuroepithelium cells are unable to generate GalC+ve cells after 9 days *in vitro* (Warf *et al*., 1991), it has been suggested that OPCs originate only from a restricted ventral region (pMN) and subsequently migrate to colonize the whole spinal cord.

Instead of employing the usual *in vitro* approach, embryologists have used chick-quail chimeras to assess whether the dorsal regions of the spinal cord are able to generate OPCs (Cameron-Curry and Le Douarin, 1995; Pringle *et al*., 1998). However, inconsistent results were obtained from these chimeric studies, which might be due to the variation in dissecting techniques between different research laboratories. Interestingly, recently published articles have provided convincing evidence supporting multiple origins, including a dorsal birthplace, for OL-lineage cells in the spinal cord (Hardy *et al*., 1996; Soula *et al*., 2001; Fu *et al.,* 2002; Gregori *et al*., 2002; Cai *et al*., 2005; Vallstedt *et al*., 2005; Richardson *et al*., 2006). In particular, Cai *et al*. (2005) show dorsal oligodendrogenesis exists in *Nkx6.1/Nkx6.2* and *Shh* mutant mice, where the early generation of OPCs at the pMN domain is diminished and the possibility of ventral-dorsal migration of OPCs is eliminated. Most importantly, Cai *et al.* (2005) demonstrate the expression of *Pax7* and *Mash1* in these dorsally derived Olig2+ve cells *in vitro*, thus

overcoming the argument that the dorsal cells might have undergone a ventral fate switch in *Nkx6* mutant mice. Consistent with these findings, Vallstedt *et al.* (2005) found dorsally derived OPCs do not express Nkx2.2 but Pax7, and vice versa for the ventral OPC counterpart (Fig. 3).

In the ventral zone of the spinal cord, an independent population of OPCs derived from the p3 domain has been reported in addition to the group of OPCs from the pMN domain (Soula *et al*., 2001; Zhou *et al*., 2001; Fu *et al*., 2002). The p3 domain features the expression of Nkx2.2, which sets the ventral limit of Olig2, or the pMN domain, during neurogenesis (Fig. 2). Upon completion of neuron generation, the boundary between p3 and pMN no longer exists, and Olig2 and Nkx2.2 are co-expressed with the onset of oligodendrogenesis. However, double immunolabelling techniques have revealed two distinct populations of OPCs that originate from the pMN and p3 domains, respectively, where the pMN region gives rise to Olig2+ve/Nkx2.2-ve OPCs and the p3 gives rise to Nkx2.2+ve/Olig2-ve progenitors (Fig. 3) (Zhou *et al*., 2001; Fu *et al*., 2002).

Taken together, the above findings indicate multiple sources of OPCs in the spinal cord, although the onset of a specific progenitor population begins at a defined embryonic stage (Fig. 3). It has therefore been proposed that the emergence of different pools of OPCs might be under the control of regional signals and this is probably associated with their later functions (Miller, 2005). This interpretation of the findings also sheds new light on how these regionally dominated HD proteins participate in oligodendrogenesis. The cross-repressive interactions between Class I and Class II proteins likely establish the dorsal-ventral (D-V) identity of the early domestic OPCs and may play a role in initiating the timing of OPC generation, as this D-V organization of cell subtype specification in the spinal cord has clearly been demonstrated in the spatial patterning of neurons (Lumsden and Krumlauf, 1996; Pattyn *et al*., 2003).

2.1.2.2 OPCs are generated along the rostrocaudal axis of the embryonic brain

The battle of ideas on the dorsal origin of OPCs, however, overshadows another dimension of the developing neural tube - the anterior-posterior (A-P) axis. Are tissues along the A-P axis all able to produce OPCs? Although studies of this A-P distribution of OPCs within CNS remain rare, several lines of evidence demonstrate that progenitor cells can be generated along the rostrocaudal axis in both mouse and chick (Hardy and

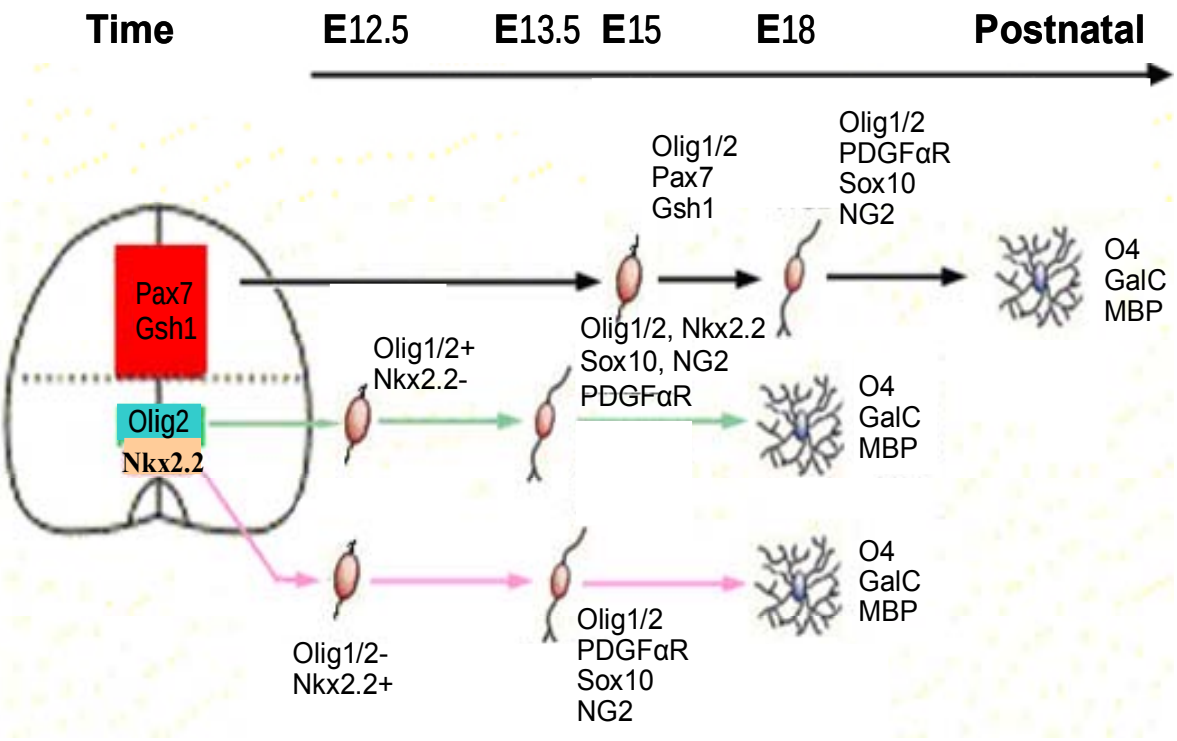

Time
E12.5
E13.5
E15
E18
Postnatal
Pax7
Gsh1
Olig2
Nkx2.2
Olig1/2+
Nkx2.2-
Olig1/2-
Nkx2.2+
Olig1/2
Pax7
Gsh1
Olig1/2, Nkx2.2
Sox10, NG2
PDGFαR
Olig1/2
PDGFαR
Sox10
NG2
O4
GalC
MBP
O4
GalC
MBP
Olig1/2
PDGFαR
Sox10
NG2
O4
GalC
MBP

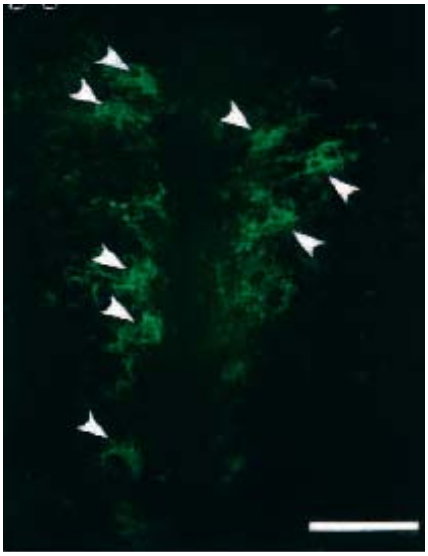
Figure 3. Ventral and dorsal sources of Olig2+ve cells. At least three independent sources have been suggested in the spinal cord. At E15 in mice, a few Olig2+ve cells start to originate from the Pax7 and Gsh1 patterned dorsal region (red rectangular) (Cai *et al*., 2005; Vallstedt *et al*., 2005). The dorsally derived Olig1/2+ve cells express other OL-lineage markers, including PDGFαR, Sox10 and NG2, but not Nkx2.2. The dorsally obtained OPCs progress to MBP expressing oligodendrocytes well after birth (Vallstedt *et al*., 2005). Further, in the ventricular region of the spinal cord, OPCs can be generated from both the pMN domain and p3 domain (Fu *et al*., 2002; Zhou *et al*., 2001). At the early stages of oligodendrogenesis, before the dorsal expansion of the Nkx2.2 expressing domain, the Olig2+ve cells arise and initially do not express Nkx2.2; vice versa for cells that originate from the p3 region (Based on Figure 4Y from Vallstedt *et al*., 2005).

Friedrich, 1996; Olivier *et al*., 2001; Vallstedt *et al*., 2005; Le Bras *et al*., 2005) in the hindbrain and spinal cord. Tissues of all regions along the A-P axis in the CNS, including coronal slices of hindbrain, cervical, and lumbar spinal cord, as well as the dorsal telencephalon of embryonic mice (E10.5-E12.5), were transplanted into the left cerebral hemisphere of postnatal mice, at the level of the subcortical white matter (Hardy and Friedrich, 1996). Unexpectedly, all of these regions taken from E10.5 mice, prior to any expression of putative OPC markers, gave rise to MBP+ve cells following transplantation. In addition, Le Bras (2005) and Hardy (1996), respectively, show that at least along the A-P axis of the hindbrain, there are discrete expression foci of the markers PLP in chick and O1 in mouse.

By using immature OL markers, O1 and R-mAb (Ranscht monoclonal antibody, Ranscht *et al*., 1982), the stage specific maps of differentiated oligodendrocytes were depicted (Hardy and Friedrich, 1996). At E14.5, immunostained positive cells were first observed along the midline of the posterior hindbrain and at the restricted ventral region of anterior hindbrain. In contrast, the first appearance of O1+ve cells is detected from the posterior forebrain at E18.5, whereas in the anterior forebrain they are observed after birth. From these observations, the first conclusion of Hardy and Friedrich (1996) was that two discontinuous groups of OLs exist in the brain: one caudal group in the spinal cord, hindbrain and midbrain; and a rostral group in the anterior diencephalon and the telencephalon. Furthermore, they suggested that the maturation of OPCs starts caudorostrally within the CNS, indicating OLs might also have spatial programmed identity along the A-P axis of the neural tube, a theory which is supported by several other research groups (Fanarraga *et al*., 1998; Spassky *et al*., 1998; Olivier *et al*., 2001).

Olivier *et al*. (2001) used the quail-chick chimeras to address the question of whether OPCs developed metamerically in the hindbrain, as observed in many other cell types (reviewed by Rijli *et al*., 1998; Lumsden, 2004). In fact, discrete populations of OLs are compartmentalized within the caudal hindbrain by inter-rhombomeric boundaries (Figure 4A) (Hardy and Friedrich, 1996). Additionally, the migration and myelination of the OPCs derived from each rhombomere (r) remains confined within the compartment (Figure 4B) (Olivier *et al*., 2001). Yet, it is not clear if this spatial position identity is correlated with their myelination of axons in a specific region, since optic

A



B

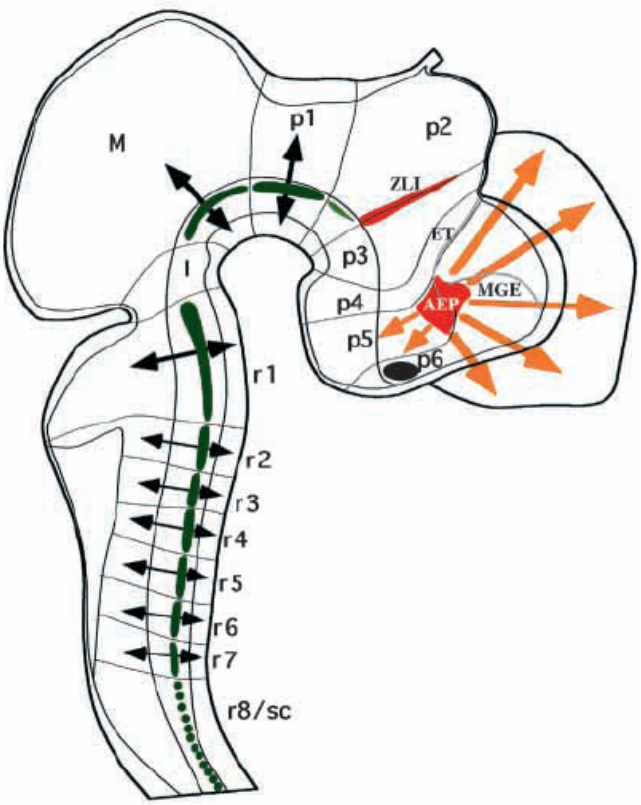


Figure 4. Compartments of oligodendrocytes within the hindbrain rhombomeric segments. A. Pre-mature OLs were recognized using O1 antibody, and displayed regionally restricted patterns, corresponding to each rhombomeric unit (Hardy and Friedrich, 1996). B. Specific migration routes of independent oligodendrocyte populations along the anterior-posterior axis in the CNS (Olivier *et al*., 2001). r1-r8, rhombomere1-8; p1-p6, prosomere1-6; sc, spinal cord; ZL1, zona limitans intrathalamica; ET, eminentia thalami; I, Isthmus; AEP, anterior entopeduncular area; M, mesencephalon; MGE, medial ganglionic eminence. Images are taken from Hardy and Friedrich (1996) and Olivier *et al*. (2001) with permission.

nerve derived OLs are able to myelinate axons in the spinal cord (Fanarraga *et al*., 1998). It is very likely that under normal conditions OPCs are generated at multiple foci and follow specific radial or tangential migration paths within a small coronal slice of tissue to ensure efficient myelination of the sophisticated neural network. On the other hand, regional OPCs or late-progenitors could have the capacity to migrate and myelinate axons in other regions, or in cultures, so that damage (e.g. demyelination) can be repaired quickly. However, it will be interesting to explore the molecular mechanisms that constrain their radial migrations within the confined segments along the A-P axis of the neural tube.

2.1.2.3 Commitment of OL-lineage cells

In the developing rodent spinal cord, expressions of early OL marker genes, such as Olig2, PDGFαR and Sox 10, are first detected in the pMN domain, where this same region gives rise to the MNs at an earlier stage (Sun *et al*., 2001; Lu *et al*., 2002; Zhou *et al*., 2001; Fu *et al*., 2002). Thus, MNs and OLs are believed to be sequentially generated from the same precursor cells at different time windows (Richardson *et al*., 1997, 2000). However, the controversy about the relationship between neurons and OLs was compounded by the finding of glial restricted precursors (GRPs) in primary cell cultures derived from different parts of the spinal cord (Rao *et al*., 1998; reviewed in Liu and Rao, 2003), which gave rise to the hypothesis that OL-committed progenitor cells come from lineage restricted precursors. As a result, multiple types of intermediate glial precursors were proposed and various models of neural cell lineage relationships were described (Richardson *et al*., 2000; Rowitch *et al*., 2002; Liu and Rao, 2003).

Regardless of the model debate, two bHLH transcriptional factors - Olig1 and Olig2 - have been conclusively found to play a key role in the neuronal switch and OL specification (Lu *et al*., 2000; Nery *et al*., 2001; Takebayashi *et al*., 2002; Zhou and Anderson, 2002; Lu *et al*., 2002). In Olig1/2 double-mutant mice, MNs and OL are largely eliminated, whereas V2 interneurons and astrocytes are produced from the pMN region (Zhou and Anderson, 2002). Additional evidence indicates Olig1 and Olig2, respectively, exhibit different expression profiles within the CNS (Takebayashi *et al*., 2000), indicating they do not simply compensate each other but may participate in different embryonic events in various cell types. Consistent findings from a loss of

function study suggest that Olig1 is dispensable for OPC formation, but is required for terminal maturation and proper myelination of OLs (Lu *et al*., 2000, 2002; Xin *et al*., 2005). In contrast, Olig2-null animals fail to produce OPCs in the spinal cord, although small patches of OPCs in the forebrain and relatively normal numbers of PDGFαR+ve cells in the hindbrain were reported (Lu *et al*., 2002). Taken together the proposed multiple origins for oligodendrogenesis in the CNS, OPCs originating from different regions might be under the control of local mechanisms rather than under one global regulatory mechanism.

Sonic Hedgehog is a potent inducer and is obligatory for OL generation in both rostral and caudal neural tube (Orentas *et al*., 1999; Nery *et al*., 2001; Tekki-Kessaris *et al*., 2001). Knocking down Shh signalling results in the absence of OPCs at normal embryonic stages; conversely, ectopic OPCs can be induced in dorsal loci by Shh. At least part of the pro-oligodendrocyte function of Shh can be attributed to its temporal-specific regulation of Olig (Olig1 and Olig2) genes (Lu *et al*., 2000; Nery *et al*., 2001; Cai *et al*., 2005). Unlike Shh, members of the bone morphogenetic proteins (BMPs) have been proposed as inhibitors of OL fate even in the presence of Shh, possibly by repressing the transcription of *olig* genes (Gross *et al*., 1996; Mekki-Dauriac *et al*., 2002). From a neural patterning point of view, the antagonistic roles played by Shh and BMP can be attributed to their activity gradient patterns along the D-V axis of the neural tube (Jessell, 2000).

A cell intrinsic timing control mechanism has also been proposed, where Neural Stem Cells (NSCs) orderly generate neurons and various types of glial cells by counting time and/or cell division (Ibarrola *et al*., 1996). Suggestions for the identity of the internal timer include telomere length, the cyclin-dependent kinase inhibitor p27, and receptors for epidermal growth factor (EGF) and fibroblast growth factor (FGF) (Morrison, 2000). Lastly, an intermediate hypothesis proposes the interplay between extrinsic local signals, including Shh, BMP, and Notch, and cell intrinsic cues determines the emergence of spatially distributed OPCs (Fig.2) (Billon *et al*., 2002b), particularly in the cell fate decisions of retinal cells (reviewed by Cepko, 1999).

2.1.2.4 Homogeneity vs. heterogeneity in OPCs

Morphologically, four types of mature OLs have been reported (Bjartmar *et al*., 1994). We are accustomed to thinking of OPCs as a functionally homogenous population, which, in fact, might not be the case, as increasing evidence suggests. For example, OPCs isolated from different brain regions exhibit distinct properties in terms of maturation timing and responsiveness to thyroid hormone (TH) (Power *et al*., 2002). However, this discrepancy might be due to OPCs undergoing various cycling programs while migrating to reach their destination in the CNS, rather than due to inherent differences.

However, two recent studies provide strong evidence of a dorsal origin of OPCs, demonstrating the TF profiles of the dorsal pool of Olig2+ve cells are distinguishable from that of their ventral counterpart (Cai *et al*., 2005; Vallstedt *et al*., 2005). Kitada and Rowitch (2006), with the aid of new fluorescent technique and confocal microscopy, dissected the expression patterns of several TFs (Olig1, Olig2, Nkx2.2 and Sox10) in both adult OPCs and mature OLs, revealing that various combinations of these four molecules exist within subpopulations of OL-lineage cells. In particular, a co-localization study of Olig1 and Olig2 indicated a distinct cytoplasmic distribution of Olig1 in stage specific OLs.

More intriguingly, several lines of evidence from the study of Olig2 (Lu *et al*., 2002) and Nkx6.1 (Liu *et al*., 2003; Vallstedt *et al*., 2005) imply heterogeneity in oligodendrogenesis also occurs along the A-P axis. Along with the fact that OL-lineage cells are generated along the A-P body axis, it is therefore important to see if mature OLs also bear spatial identity corresponding to the regions they are derived from, as observed in neurons whose identity is regulated by regional signals, or '*Hox* genes', in the hindbrain. Although TF heterogeneity of OL-lineage cells is described, how these expression patterns regulate cellular function has not been established. Further investigations are necessary to determine whether TF expression profiles correlate with the morphology and function specificity of OLs in distinct regions of the CNS.

2.1.3 Differentiation and maturation of OPCs

Once committed to the OL lineage, precursors undergo a massive but specific pattern of migration to colonize the entire CNS (Olivier *et al*., 2001). These migrating progenitors actively proliferate and respond to growth factors such as PDGF and FGF

(Raff *et al*., 1989; Richardson *et al*., 1988). After dividing a limited number of times, OPCs transform into pre-myelinating OLs, and stop at their defined destination. As differentiation progresses, newly formed immature OLs start to produce GalC but lose the expression of O4. Finally, the synthesis of 2', 3'-Cyclic nucleotide 3'-phosphodiesterase (CNPase) demarks the onset of terminal maturation, whereas MBP is expressed 2-3 days later along with PLP (Barradas *et al*., 1995; Reynolds and Wilkin, 1988), immediately before myelin formation. Significant efforts have been directed at discovering the cellular and molecular cues that control this sophisticated schedule of development.

Terminal differentiation of OLs is a carefully orchestrated event *in vivo* to ensure timely myelination of the axons. Both cell intrinsic timing mechanisms (reviewed in Raff *et al*., 1998; Durand and Raff, 2000) and extracellular mitogenic factors, including PDGF (Raff *et al*., 1998; Richardson *et al*., 1988) and thyroid hormone (Rodriguez-Pena, 1999; Billon *et al*., 2002a), have been proposed to play a role in the regulation of the differentiation and maturation of OLs.

2.1.3.1 Mitogenic factors influence the development of oligodendrocytes

A number of soluble factors can regulate the proliferation and differentiation of OLs *in vivo* and *in vitro*, and one major player appears to be PDGF (Richardson *et al*., 1988; Raff *et al*., 1998; Calver *et al*., 1998). In culture, PDGF is able to inhibit premature differentiation and restore the development of isolated O-2A cells as in their *in vivo* counterparts (Raff *et al*., 1989). In mice where the PDGF-A gene has been inactivated, severe reduction of OPCs and mature OLs, along with a dysmyelinating phenotype, were reported (Fruttiger *et al*., 1999). Recently, Baron and co-workers (2002) have further demonstrated that PDGF promotes the proliferation of OPCs by activation of αvβ3 integrin in the extracellular matrix (ECM), which in turn induces OPC proliferation via a phosphatidylinositol 3-kinase-dependent signalling pathway. In addition to PDGF, fibroblast growth factor-2 (FGF-2) and insulin-like growth factor 1 (IGF-1) also have been shown to enhance the proliferation of OPCs in cultures.

The story does not end here, since in PDGF containing cultures O-2A precursor cells keep on differentiating, corresponding to their normal development timeline. Addition of excess PDGF does not alter the timing of OL development, and newly

formed OLs continue to express large amounts of PDGFαR, thus excluding the possibility of PDGF signalling blockage (Temple and Raff, 1986). This finding raised the possibility that the progenitor cells have a cell-intrinsic mechanism which governs their proliferative capacity and controls when they should withdraw from the cell cycle and start terminal differentiation. In support of this concept, Gao *et al*. (1997) demonstrated that purified embryonic OPCs underwent the same developmental course as their *in vivo* counterparts, even when the cells were maintained in a relatively constant environment and in the virtual absence of other cell types.

2.1.3.2 The role of thyroid hormone

Another important factor that regulates OL development is thyroid hormone (TH). Hypothyroidism in rat increases proliferation of NG2+ve cells in the subventricular zone (SVZ) and delays the differentiation of OPCs (Fernandez *et al*., 2004). In contrast, enhanced TH levels exert opposite effects on OL-lineage cells. In cultures containing mitogens (PDGF, FGF) and TH, OPCs develop on time (Gao *et al*., 1998), whereas very few precursors display a more advanced morphology if TH (T3 and T4) is omitted from this defined medium (Barres *et al*., 1994). Furthermore, several independent studies confirm that thyroid hormone receptors are expressed on both OPCs and OLs (Barres *et al*., 1994; Gao *et al*., 1998), implying TH directly regulates OL-lineage cells. Retinoic acid (RA) has been shown to have the same impact on OL-lineage cells as TH (Barres *et al*., 1994).

Surprisingly, OPCs purified at different developmental stages react differently to TH. P7 precursor cells stop dividing and differentiate rapidly in the presence of T3, whereas E18 progenitor cells remain in cell cycle until 3-4 days later in cultures with T3 (Gao *et al*., 1998). However, when cells are cultured in medium without any mitogens (PDGF, FGF etc.), no obvious difference is observed in the morphology or rate of advancement in cell groups treated with or without TH within a 2-day examination period (Barres *et al*., 1994). Based on above findings, it was concluded that TH does not trigger differentiation, but rather it activates the effector component of the built-in timer of OL-lineage cells to overcome the influence of mitogens, which have to be persistently produced *in vivo* to promote OL survival.

2.1.3.3 Transcriptional regulations for an intrinsic timing clock

Abundant evidence from the study of mitogens and hydrophobic signals indicates an internal circadian system exists and masters the development of OLs (Temple and Raff, 1985, 1986; Barres *et al*., 1994; Gao *et al*., 1997, 1998). Specifically, two components have been described for this timing mechanism: (1) a timing mechanism that might depend on PDGF or other growth factors to measure elapsed time, and (2) an effector element, which is regulated by TH or other signals, to initiate cell differentiation when the time is up.

It is conventionally believed that such a timer should interact with cell cycling in counting the elapsed time. Therefore, proteins including cyclins or Cdks (cyclin-dependent protein kinases) that normally drive the cycling machine and molecules that suppress cell-cycle operation, like p27/Kip1, were carefully examined. Although accumulated data suggest p27/Kip1 participates in the timing event (Durand *et al*., 1997), the puzzle is far from solved. Therefore, researchers have focused on the transcriptional control of OL-lineage cells which govern the sequential expression of key genes during cell commitment and differentiation, in attempts to determine the timing regulation.

Available evidence strongly suggests that the Notch pathway plays a critical role in the differentiation of OL-lineage cells (reviewed by Wang and Barres, 2000). In the rat optic nerve system, Notch1 is expressed in OPCs while Jagged, a Notch ligand is present on the optic nerves (Wang *et al*., 1998). Activation of Notch1 by Jagged can powerfully inhibit the differentiation of OPCs in culture (Wang *et al*., 1998). Moreover, the expression of Jagged on optic nerves is controlled developmentally *in vivo* such that Jagged levels decrease while embryonic development progresses, thus allowing myelination to occur in a controlled manner (Wang *et al*., 1998). Additionally, Notch activation generally leads to the expression of *Hes* family inhibitory basic helix-loop-helix TFs (Kageyama and Nakanishi, 1997). The expression of one *Hes* family member, *Hes5*, was found in optic nerve OPCs. Ectopic expression of *Hes5* can block the differentiation of OPCs in culture (Kondo and Raff, 2000), indicating Notch1 might act through *Hes5* to inhibit the terminal differentiation of OL-lineage cells in the optic nerve. *Id2*, another inhibitory bHLH factor, was also found to negatively regulate OPC differentiation (Wang *et al*., 2001). As both Hes and Id family bHLH factors are known

to antagonize the functions of tissue specific bHLH transcription factors (Kageyama and Nakanishi, 1997), it was hypothesized that unknown E-box bHLH factors might positively promote the terminal differentiation of OPCs (Kondo and Raff, 2000; Wang *et al*., 2001). Whether the candidates might be Olig1 and/or Olig2 remains to be determined, although Gokhan (2005) shows the combination of Sox10, E47 and Olig2 can greatly enhance MBP promoter activity. Moreover, the above findings are mostly based from observations in culture or using optic nerve as the *in vivo* model system. In the spinal cord and brain, Notch receptors and ligands, *Hes* and *Id* family members are expressed in complicated patterns (Kageyama and Nakanishi, 1997; Artavanis-Tsakonas *et al*., 1999; Andres-Barquin *et al*., 2000), and are not specific to the OL-lineage cells. Whether some or all of them participate in controlling the timing of myelination in distinct brain regions is yet to be elucidated.

In addition to the *Id* proteins, Sox10 (Wei *et al*., 2004; Gokhan *et al*., 2005) and Nkx2.2 (Wei *et al*., 2005; Gokhan *et al*., 2005), two crucial TFs during OPC early specification and commitment, have also been suggested as important for the normal timing of differentiation. In particular, Gokhan *et al*. (2005) revealed a transient down-regulation of Nkx2.2 in early OL progenitor cells (NG2+ve/O4+ve), followed by a transient up-regulation of this TF in more advanced progenitor cells (NG2-ve/O4+ve, GalC+ve/O1+ve) before terminal differentiation. The second wave of expression of Nkx2.2 indicates two distinct roles for this gene at different developmental stages, but with one ultimate goal - to regulate the expression of myelin proteins and the onset of maturation program at the right time (Watanabe *et al*., 2004). Consistent with this suggestion, Qi *et al*. (2001) found that the appearance of MBP and PLP/DM20 are severely reduced and delayed in Nkx2.2 null animals. This retarded differentiation does not appear to be due to the delay of early OPC specification, since Olig1/Olig2 double positive cells and PDGFαR+ve cells are observed at normal developmental stages. Thus, homeodomain-containing protein Nkx2.2 may directly regulate the expression of myelin protein encoding genes, supported by the findings reported by Wei *et al*., (2005) that two regulatory elements in the promoter of *mbp* interact with Nkx2.2 *in vitro.*

Another transcription factor, CREB (cyclic AMP response element binding protein) has been demonstrated to play a role during the final differentiation of OLs

(Sato-Bigbee *et al*., 1999; Afshari *et al*., 2001). CREB regulation and its downstream molecular signalling pathways are under extensive investigation (Sato-Bigbee *et al*., 1999). Several laboratories have shown that OL differentiation is stimulated by cyclic adenosine monophosphate (cAMP) (Raible and McMorris, 1989), while Montminy *et al*. (1990) demonstrated that the expression of cAMP is under the transcriptional control of CREB. Interestingly, elevated expression of CREB immediately precedes the peak in myelin synthesis (Sato-Bigbee *et al*., 1994). Recently, Garcia *et al*. (2004) provided more convincing evidence that this cAMP/CREB mediated pathway can induce exit from cell cycling by increasing p27/Kip1 levels and diminishing cyclin D1 expression at a specific stage during oligodendrogenesis. In fact, CREB phosphorylation is required for CREB-mediated transcription. Shiga *et al*. (2005a, b) report this phosphorylation is principally regulated by protein kinases A (PKA) in the progression from OPCs to late-progenitor, but by protein kinases C (PKC) in subsequent differentiation to mature OLs. Therefore, studying this phosphorylation serves to identify events upstream of CREB, and help to advance our understanding of how mitogen stimulations are connected to cytoplasmic event cascades.

The impact of THs on the maturation of OLs are known to be mediated by specific thyroid hormone receptors (TRs), which are ligand-activated transcription factors that modulate the expression of certain down stream targets. Three of these receptors - α_1, β_1, and β_2 - are expressed in OL-lineage cells, with α_1 found only in progenitor cells and β_1 mostly present in mature OLs, suggesting specific functions for both types of TRs during the development of OL-lineage cells. Furthermore, Billon *et al*. (2002a) propose TR α_1 might be associated with the timing of cell differentiation, since OPCs derived from TR α_1 -/- mouse fail to differentiate in response to TH. The transcriptional regulation of thyroid hormone-receptor is likely to directly interact with the MBP promoter region, as has been demonstrated by Farsetti *et al*. (1991).

Importantly, this circadian clock is based on the interconnections of various TFs, which via positive and/or negative feedback loops may mediate the pace and function of the clock. To fully illustrate this internal timing mechanism in OL-lineage cells, in addition to identifying factors that participate in this event, further investigations of the

relationships within this transcriptional arch, as well as how the TF cascades associate with cellular behavior and physiology will be needed.

2.1.3.4 Proliferation and differentiation of OPCs contribute to cell number control

The number of cell divisions and the timing of cell differentiation onset are controlled tightly, not only for myelinating axons in a timely manner but also for the regulation of the population size of mature OLs (Barres and Raff, 1994; Casaccia-Bonnefil *et al*., 1997; Raff *et al*., 1998). The population size of OL-lineage cells in a specific region must be generated and maintained properly so that they match the number and length of axons, allowing the correct establishment and function of the CNS. For example, lack of a sufficient number of OLs often results in loss of nearby axons, as revealed by many demyelinating and degenerative neural diseases (reviewed in Compston, 2004a). In general, the quantity of cells depends on the orchestration of the duration of cell division, timing of cell differentiation and cell death. As summarized above, cell proliferation and the onset of differentiation as well as the regulation of cell numbers are regulated by cell autonomous intracellular timing program, which interplays with dynamic extracellular signals. Extracellular signals, such as PDGF, TH, RA and Notch1 pathway have been demonstrated as effectors in regulating the proliferation and the maturation of OL-lineage cells. The internal mechanisms that regulate cell cycle machinery involve a tissue specific transcriptional system, which may directly modulate components within this timing system and/or interact with mitogen pathways that drive the G1 progression. In neuron progenitors, knock out of Pax6, a homeodomain TF, has been shown to interrupt cell-cycle kinetics in mice cortex, resulting in a large increase in the length of S phase and extension of the overall cell cycle (Warren *et al*., 1999; Estivill-Torrus *et al*., 2002). During oligodendrogenesis, the TF Sox10 has been shown to activate the promoter of MBP, at least partially via regulating the expression of a secondary transcription factor Sp1 (Wei *et al*., 2004). In parallel the up-regulation of Sp1 results in an elevation of p27/Kip1, an inhibitor of the cell cycling system in OLs.

2.1.3.5 Presence of the Hox transcription factors during oligodendrogenesis

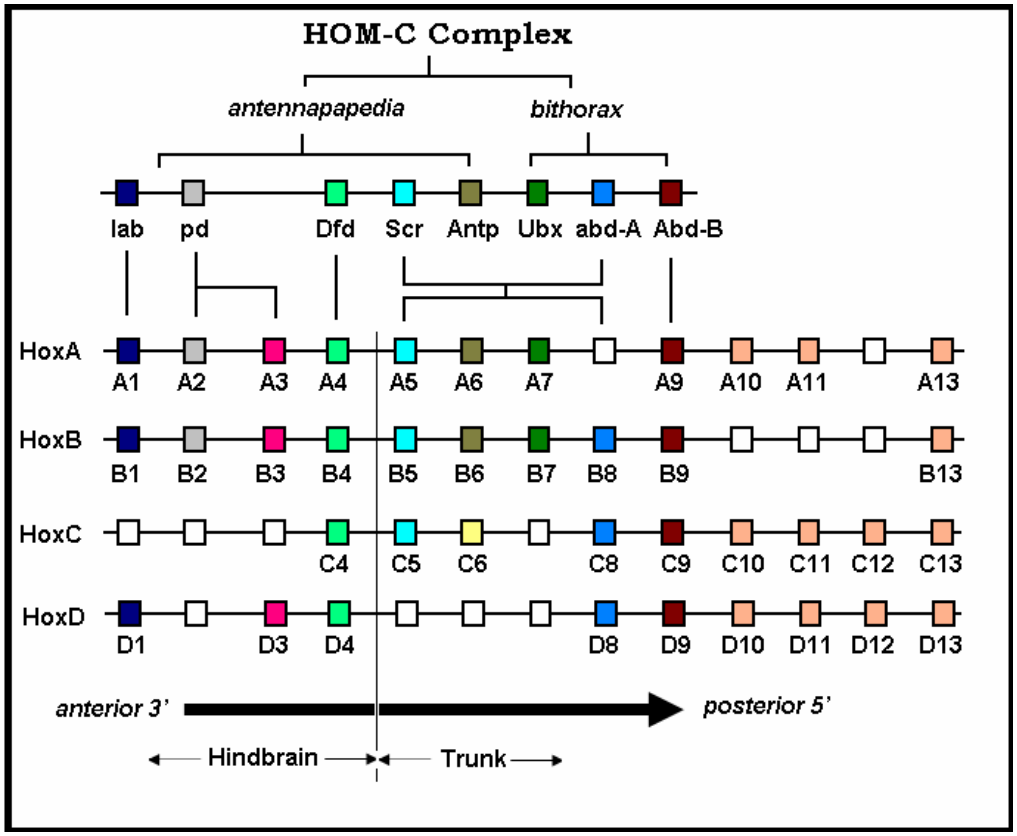
Hox genes are TFs that are expressed in almost all embryonic tissues, including the nervous system (reviewed in Lumsden and Krumlauf, 1996; Briscoe and Wilkinson, 2004), determining cell fate and providing positional information for individual cells

(Lumsden and Krumlauf, 1996; Trainor and Krumlauf, 2000). Several laboratories including ours have recently identified the presence of *Hox* genes in OLs in the CNS and Schwann cells in the PNS (Hao *et al*., 1999; Arenkiel *et al*., 2003, 2004; Nicolay *et al*., 2004a, b). In the periphery, although early development of Schwann progenitor cells appeared normal in the absence of *Hoxb1* with respect to specification and population number, abnormal projection of the VII nerve occurs at a later stage (Arenkiel *et al*., 2003, 2004). In addition, these researchers demonstrated that the majority (over 95%) of the myelin sheath that wraps around the VII nerve is derived from rhombomere 4 (r4) *Hoxb1* expressing neural crest cells (NCCs), implying *Hox* genes might serve as a marker for correct contact and ensheathment between neurons and Schwann cells.

Our laboratory has demonstrated the presence of *Hoxa2* (Hao *et al*., 1999; Nicolay *et al*., 2004b), *Hoxb4* (Nicolay *et al*., 2004a) and *Hoxd1* (Booth *et al*., 2007) during the development of OLs. Although the early development and specification of OPCs appear normal in the absence of *Hoxa2*, primary OPCs isolated from *Hoxa2 -/-* mice displayed a much more advanced phenotype than the wild-type controls (unpublished data), which implies a role of *Hoxa2* in regulating the proliferation and differentiation of OL-lineage cells.

2.2 *Hox* genes in neural development and cell proliferation

Hox genes form a subgroup of the homeobox gene family, which all contain a highly conserved homeobox of 183 nucleotides encoding 61 amino acids. The amino-acid-specified homeodomain is a DNA binding domain that regulates specific DNA-protein interactions, thereby influencing DNA transcription (reviewed by Akin and Nazarali, 2005). In vertebrates, these genes are arranged in four distinct chromosomal clusters, whereas their homologs in *Drosophila* form only one cluster, the *HOM*-C complex (Fig. 5). Interestingly, the chromosomal organization of the genes in the *Hox/HOM-C* cluster usually reflects its A-P expression in the body plan, known as spatial colinearity (reviewed by Holland and Garcia-Fernàndez, 1996; Akin and Nazarali, 2005). In general, vertebrate *Hox* genes also display temporal colinearity (Duboule, 1998; Gaunt and Strachan, 1996). Thus, the 3´ genes within a chromosomal cluster are activated much earlier and expressed more anteriorly than genes located close to the 5´ end, with respect to the rostrocaudal axis of the embryo. This colinearity and ordered arrangement of


HOM-C Complex
antennapapedia
bithorax
lab
pd
Dfd
Scr
Antp
Ubx
abd-A
Abd-B
HoxA
A1
A2
A3
A4
A5
A6
A7
A9
A10
A11
A13
HoxB
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C8
C9
C10
C11
C12
C13
HoxD
D1
D3
D4
D8
D9
D10
D11
D12
D13
anterior 3'
posterior 5'
Hindbrain
Trunk

Figure 5. Organization and evolutionary relationships between the *Hox* clusters (below) in vertebrates and *HOM-C* homeotic complexes (top). Each square represents one Hox or Homeotic gene, with its name noted underneath. Only one *HOM-C* complex is found in *Drosophila*, consisting of two regions: Antennapedia and Bithorax. Unlike *Drosophila*, four paralogous groups exist in vertebrates, presumably derived by duplication of an ancestral cluster. In most cases, vertically arranged squares share the same color, which indicates they are structurally and/or functionally homologous to each other. At the bottom, the anterior-posterior (A-P) order of each *Hox* member on the chromosome is denoted, together with the colinear expression pattern of *Hox* genes in the CNS of vertebrates (Image is taken from Cobourne, 2000 with the permission of author).

expression have been observed for *Hox* genes in a variety of developing tissues, particularly in the CNS (Keynes and Krumlauf, 1994; Gaufo *et al*., 2003; Briscoe and Wilkinson, 2004), neural crest and branchial arches (Creuzet *et al*., 2005), as well as the limb buds (Zakany and Duboule, 1999). Homeobox genes are often referred to as "master regulatory genes", setting in motion a complex series of processes necessary for the formation of, for example, heads or limbs (Akin and Nazarali, 2005).

2.2.1 *Hox* genes are master genes in patterning the neural system

The vertebrate neural system is a complex and highly compact organization; however, it also undergoes regional subdivision during early embryonic development, providing a convenient basis for the generation and assembly of diversity. A series of transitory segmental elevations, or neuromeres, are formed in the early neural tube. Particularly in the hindbrain, morphological repeated units (7 in mouse and 8 in chick), also known as rhombomeres (r), have been described (Gaufo *et al*., 2004). Each r generates defined types of neurons according to its A-P position (Fig. 6). Moreover, the hindbrain partitioning may underlie spatial establishment of the neural circuitry (Fig. 6) and the segmental specification of NCCs, which contribute to the patterning of cranial sensory ganglia and the branchial arches (Lumsden *et al*., 1991; Kontges and Lumsden, 1996).

Hox genes are important regulators that control this segmentation and specification. In the developing hindbrain (Fig. 6) and the spinal cord (reviewed by Lumsden and Krumlauf, 1996; Briscoe and Wilkinson, 2004), *Hox* genes are expressed in overlapping domains along the A-P axis, in a fashion colinear with their positions within the cluster on the chromosomes (Fig. 5). The 3' anterior *Hox* paralogs (1-4) have varying rostral expression limits in the hindbrain (Fig. 6), whereas 5' *Hox* members (5-13) generally are detected in the spinal cord, with *Hoxa5*, *Hoxb5* and *Hoxc5* having the most the most anterior boundary (Carpenter, 2002).

Accumulating evidence demonstrates that the specific identity of each r and its derived cells is regulated by the differential expression of *Hox* genes (Trainor and Krumlauf, 2000). Marshall *et al*. (1992) report that an alternation in the expression profiles of '*Hox* code' [by retinoic acid (RA)] in mice hindbrain will induce transformation of r2/3 into an r4/5 identity. Similarly, disruption of *Hoxa1* results in the

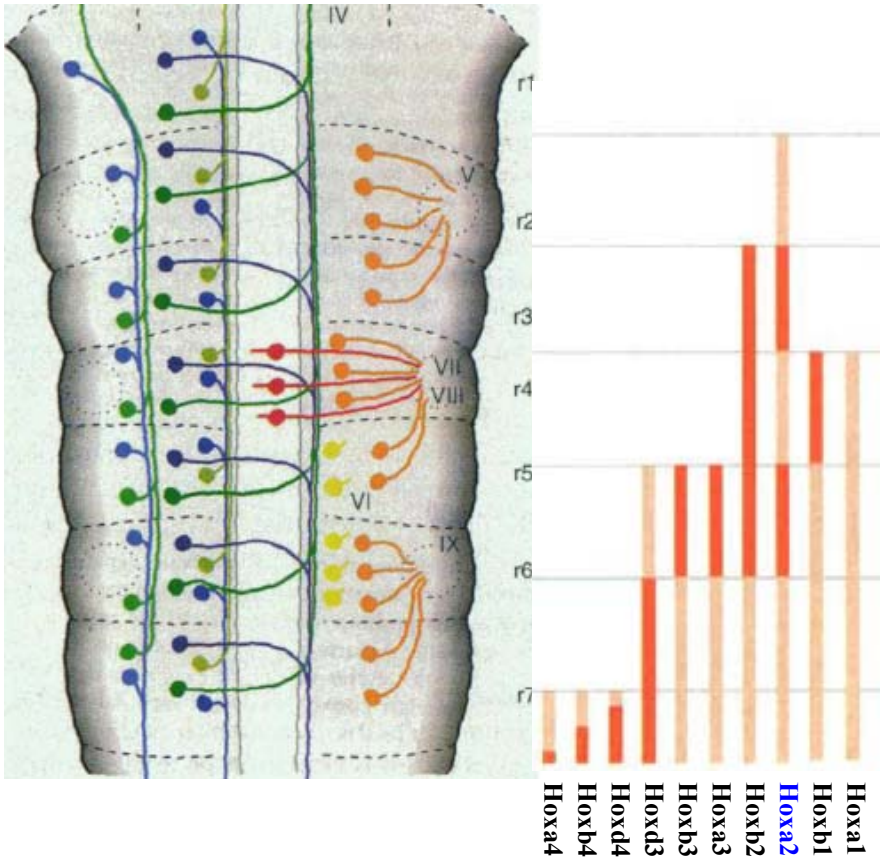

IV
V
VII
VIII
VI
IX
r1
r2
r3
r4
r5
r6
r7
Hoxa4
Hoxb4
Hoxd4
Hoxd3
Hoxb3
Hoxa3
Hoxb2
Hoxa2
Hoxb1
Hoxa1

Figure 6. Patterns of cell organization and expression profiles of *Hox* genes in chick hindbrain. This schematic diagram represents the hindbrain of a chick embryo, which during development is subdivided into clearly demarcated units called rhombomeres (r). Each r contains reticulospinal neurons (blue or green), the axon trajectories of which are related to the rhombomeric divisions. The motor nuclei (in the right side basal plate) are IV-trochlear, V-trigeminal, VI-abducens, VII-facial, IX-glossopharyngeal. The bars mark the anterior rhombomere boundaries of *Hox* gene expression, with the name of each gene identified at the bottom. The red regions denote higher levels of expression; orange regions denote lower levels of expression. *Hoxa2* displays the most anterior expression domain, up to the boundary of r1/r2. [Based on Figures 2 and 3 in Lumsden and Krumlauf (1996) with the permission of the author]

reduction of r4 with an absence of most of r5, a decrease of caudally migrating facial MNs, as well as misprojection of some axons (Carpenter *et al*., 1993; Gavalas *et al*., 2003). In comparison, *Hoxb1* has a restricted high level of expression at r4 and loss of *Hoxb1* leads to a seemingly partial transformation of the r4 to an r2 identity (Studer *et al*., 1996; Gavalas *et al*., 2003). Furthermore, the posterior migratory behavior of the r4-derived neurons is abnormal in *Hoxb1* mutants, adopting a lateral migration similar to that of the trigeminal neurons in r2 (Studer *et al*., 1996). Recently, Arenkiel and co-workers (2003, 2004) demonstrated that *Hoxb1* is also expressed in r4-derived NCCs that are fated to generate glia that myelinate the VIIth cranial nerve. Loss of *Hoxb1* in the peripheral nervous system (PNS) does not alter the early development and specification of Schwann progenitor cells, although fewer overall numbers of glial cells are observed with the loss or absence of facial branchimotor neurons (FBM) at later stages.

Hoxa2 is located at the second foci of the 3' end of the paralog group-A chromosomal cluster, and exhibits the most anterior boundary of expression in the hindbrain of mice and chick, up to the interface of r1/r2 (reviewed in Krumlauf, 1994). More recently, data collected from our laboratory uncovered its presence in the diencephalon and the forebrain during late embryonic stages and postnatal life in mice (Tan *et al*., 1992; Wolf *et al*., 2001). In mice that harbor a null mutation in *Hoxa2*, the r1/2 boundary is missing, the sizes of r2 and r3 are reduced and trigeminal MN axons display abnormal projections (Krumlauf, 1994; Gavalas *et al*., 1997; Barrow *et al*., 2000).

Within the hindbrain, diverse groups of interneurons (INs) and MNs are located in stereotypic positions in a rhombomere-specific manner. Trigeminal (V) MNs are derived from r2/r3 and belong to a class of branchiomotor neurons; facial (VII) MNs are generated from r4/r5, and the abducens (VI) MNs are of r5 origin. *Hox* genes have also become prime molecular candidates for providing positional information to neural cells. These studies have focused primarily on the specification of MNs (Pattyn *et al*., 2003; reviewed in Briscoe and Wilkinson, 2004; Dasen *et al*., 2003, 2005). For example, *Hoxb1* is required for the specification of r4-branchial MNs (Studer *et al*., 1996). Abnormal patterns of neural circuitries depicted in other *Hox* gene mutants indicate that either the initial specification of regional MNs is altered, or their differentiation and/or migration are changed. Gaufo *et al*. (2003) demonstrated that the precursors for somatic sensory

INs are under the control of *Hox* genes. According to these researchers, a distinct combination of *Hox* genes in r4 and r5 of the hindbrain are required for the generation of precursors for visceral sensory INs.

Notably, the expression profiles of some *Hox* genes within the developing spinal cord also exhibit a dorsoventral (D-V) pattern along the transverse plane. At E12.5, *Hoxa4*, *a5* and *a6* genes exhibit a ventral domain of expression in the spinal cord, as do *Hoxc5* and *Hoxd5* (reviewed in Gaunt, 1991). Expression analysis of genes in the B cluster reveals dynamic dorsoventral patterns of expression in various coronal sections along the A-P axis (Graham *et al*., 1991). Particularly, *Hoxb3* and *Hoxb4* have dorsally restricted expression, whereas some members that belong to the *HoxA* and *HoxC* cluster genes exhibit a specific ventral appearance, suggesting a Hox-mediated D-V specification of cell populations. This concept is further supported by Hao *et al*. (1999) whose data show dynamic D-V patterns of *Hoxa2* in the developing neural tube, which is believed to instruct the spatial arrangement of certain groups of neurons.

2.2.2 *Hox* genes regulate cell proliferation and differentiation

Accumulating evidence suggests certain homeobox genes may provide an important link between the processes of cell proliferation and tissue development (reviewed in Ford, 1998; Levine and Green, 2004; Del Bene and Wittbrodt, 2005; Crickmore and Mann, 2006). Much of this evidence comes from cancer studies, where many cancers exhibit abnormal levels of *Hox* genes (reviewed in Ford, 1998; Del Bene and Wittbrodt, 2005). For example, ectopic *Hox* expression obtained by transfection of *Hox* genes into murine and rat cells induces tumorigenicity (Hawley *et al*., 1997; Thorsteinsdottir *et al*., 1997). Similarly, enforced expression of *HOXB4* is associated with elevated keratinocyte proliferation, but decreased cell adhesion (Kömüves *et al*., 2002), whereas overexpression of the *HOXA7* gene was reported to inhibit keratinocyte differentiation (La Celle and Polakowska, 2001). As discussed above, during mouse and chick CNS development, deletion of a particular *Hox* gene leads to shortening of the hindbrain, and loss or reduction of a certain compartment (Rossel and Capecchi, 1999; Barrow *et al*., 2000). Hence, *Hox* genes are implicated in cell cycling control, a subset of which may be involved in organ size determination, cell differentiation and carcinogenesis.

Important information also comes from the investigations of *Hox/HOX* genes in hematopoiesis, where *Hox* genes have been demonstrated to directly modulate cell proliferation, differentiation and phenotype. It seems clear that cells representing various stages of hematopoietic differentiation display differential overall patterns of *Hox/HOX* gene expression (Magli *et al*., 1991). More strikingly, genes located at the 3' end of the *B* cluster are expressed primarily in isolated cells that hold positions in the earlier precursor hierarchy, whereas the expression of genes close to the 5' region are progressively switched on along the differentiation course (Care *et al*., 1994; Quaranta *et al*., 1996). Furthermore, over-expression of some *Hox/HOX* genes, such as *HOXB4* and *HOXB6*, generally results in selective expansion of primitive hematopoietic cells (Sauvageau *et al*., 1995; Fischbach *et al*., 2005). In contrast, loss of function studies often lead to reduced size of animals and/or organs. Homozygous *Hoxa9-/-* mice show reduced numbers of peripheral blood granulocytes and lymphocytes, smaller spleens and thymuses (Izon *et al*., 2006); mice lacking *Hoxb4* exhibit significantly reduced cellularity in spleen and bone marrow (BM), as well as poor proliferation responses of several BM stem and progenitor cells (Bjornsson *et al*., 2003; Brun *et al*., 2004).

All together, the question arises as how *Hox* genes regulate the proliferation and differentiation of the various subtypes of cells. Convincing correlations between *Hox* genes and cell cycling machinery need to be demonstrated. Recent experiments suggest that in mice, homeodomain transcription factors *Pax6*, *Chx10*, and *Otx1* may serve as important regulators of cell proliferation by controlling population expansion on several levels, from setting the correct rate of proliferation to promoting cell cycle exit (Warren *et al*., 1999; Rhinn *et al*., 1999; Green *et al*., 2003). Alternatively, in *Drosophila*, the *homeobox* gene *Ultrabithorax* (*Ubx*) can regulate the size of haltere by restricting the transcription as well as the mobility of morphogen Decapentaplegic (Dpp), rather than directly impacting the cell cycling components. For clustered *Hox* genes, Yue and co-workers (2005) report the up-regulation of cyclin G1 and G2 genes in the uteri of *Hoxa10* mutant mice, indicating these genes act as downstream targets of *Hoxa10*, which might be involved in the negative control of uterine cell proliferation. More remarkably, one study clearly demonstrates the expression of *Hox* genes on the B cluster (except for *Hoxb1* and *Hoxb13*) exclusively requires cell proliferation, and the entire locus is

progressively activated within one cell cycle in P19 cells (Fisher and Mechali, 2003). According to this observation, deletion of a particular *HoxB* gene can disturb the defined pace of the cell cycling machine, thus resulting in reduced cell proliferation and smaller organ. However, it is important to point out that Fisher examined the activation of *Hox* genes at the RNA level by RT-PCR. Thus, the actual presence of these TFs at the protein level remains to be explored, since post-transcription modification, translation or post-translation events can also play key roles in controlling the progress of the cell cycling machine. In this model, a feedback effect may exist. Hence, at the protein level, a particular combination of *Hox* TFs at a defined time can alter the consequent transcription of *Hox* gene cluster, which then stops the cell proliferation drive, and results in cell fate determination or cell differentiation.

Hox genes have been implicated in stem/progenitor cell expansion, and regulating cell proliferation and differentiation (reviewed in Ford, 1998; Levine and Green, 2004; Del Bene and Wittbrodt, 2005). However, there are more questions left to be addressed. For example, how direct is the regulation of proliferation by these homeobox genes? Is there a hierarchy of homeobox genes that converges onto one or two critical *Hox* genes? Or does each homeobox gene contribute independently to ensuring the appropriate rate and extent of proliferation? In addition, is this proliferation regulation role of *Hox* genes common for various cell types during embryonic development? Or is it restricted to certain tissues, such as the neural system or the blood system? Thus, the discovery of *Hox* genes in OL-lineage cells requires further investigation.

2.3 Stable OL-lineage cell lines as useful tools to study oligodendrogenesis

While the *in vivo* system can provide a more reliable investigative model for researchers, the existence of multiple neural cell types and intricate environment can often lead to inconclusive results. Pure cultures of OPCs have the advantage of simplicity, and have been frequently used for investigating oligodendrogenesis. Primary OPCs can be isolated from embryonic mice/rat CNS and cultured *in vitro* with well established methods (Louis *et al*., 1992; Tang *et al*., 2000); however, it can be time consuming and resource intensive to obtain a homogeneous cell population in adequate quantity to conduct genetic manipulations. A number of immortalized CNS glial cell lines have been established, by transfection with simian virus 40 (SV40) large T mutant oncogene (Verity

et al., 1993; Bernard *et al*., 1994; Matsushita *et al*., 2005) or t-neu oncogene (Jung *et al*., 1995), from spontaneously transformed cells isolated from Wistar rat (Richter-Landsberg and Heinrich, 1996) or from cultures isolated from jimpy mouse brain (Feutz *et al*., 1995, 2001) (Table I). Although these attempts have succeeded in establishing permanent cell lines expressing OL-lineage markers, they either failed to fully retain characteristics of primary O-2A cells (Richter-Landsberg and Heinrich, 1996), or became heterogeneous after long periods of cultures (Barnett *et al*., 1993), or failed to differentiate into mature OLs *in vivo* (Barnett *et al*., 1993).

CG4 cells, a permanent cell line derived from primary cultures of O-2A progenitor cells isolated from rat cerebral cortex, is to date one of the two non-immortalized cell lines (Louis *et al*., 1992; Lagarde *et al*., 2007). As reported by Louis *et al*. (1992), during the preparation of primary O-2A cells, a group of O-2A cells (named CG4) escaped the usual fate and kept on proliferating for more than a year *in vitro* in defined medium, and thus was developed as a non-transformed O-2A cell line. The same research group also demonstrated that the phenotype, antigenic characteristics and bipotential differentiation of the CG4 cell line closely resemble those of cerebral O-2A cells (Louis *et al*., 1992). In addition, during maturation along the OL path, CG4 cells displayed morphological changes and corresponding cell markers that are consistent with that of primary OPCs, both *in vitro* and *in vivo* (Louis *et al*., 1992; Franklin *et al*., 1995). Since CG4 cells bear a normal genotype as primary O-2A cells, they have been frequently employed in *in vivo* transplantation studies to explore the potential therapeutic use of these cells in myelin-related disorders (Tontsch *et al*., 1994; Franklin *et al*., 1995, 1996; Tourbah *et al*., 1997; O'leary and Blakemore, 1997). The above mentioned features have made the CG4 cell line a good cell culture model to study OL development. Hence, the CG4 cells have been widely employed for studying gene function, cell myelination and signal transduction in oligodendrogenesis (reviewed in Franklin and Blakemore, 1997; Espinosa de los Monteros *et al*., 1997; Fatatis and Miller, 1997; Miskimins *et al*., 2002; Magy *et al*., 2003). In the present thesis, the CG4 cell line was used to establish stable cell lines where *Hoxa2* gene expression can be conditionally up- or down-regulated.

Table I. Permanent OL-lineage cell lines

Type of cell line	Name	Reference
Immortalized	N20.1	Verity *et al*., 1993
Immortalized	CR15 and CM8	Bernard *et al*., 1994
Immortalized	Oli-egfr-neu	Jung *et al*., 1995
Immortalized	Oli-neu	Jung *et al*., 1995
Immortalized	OLN93	Richter-Landsberg and Heinrich, 1996
Immortalized	OLP6	Matsushita *et al*., 2005
Immortalized	158N	Feutz *et al*., 1995, 2001
Immortalized	158JP	Feutz *et al*., 1995
non-immortalized	CG4	Louis *et al*., 1992
non-immortalized	O1	Lagarde *et al*., 206

III MATERIALS AND METHODS

3.1 Cell cultures

CG4 cells, an O-2A precursor cell line derived from new-born rat cortical cultures described previously (Louis *et al*., 1992), were used in this study. In all experiments, CG4 cells were cultured and maintained on Poly-D-Lysine (P-D-L) (Sigma) coated tissue culture dishes in growth medium (GM), composed of DMEM (Sigma), 50 μg/mL transferrin (Sigma), 5 μg/mL insulin (Sigma), 10 pg/mL biotin, 50 ng/mL selenium, and 30% B104 conditioned medium (B104CM).

For the production of B104CM, B104 neuroblastoma cells were plated at 5 $x10^5$ cells per 100 mm^2 dish and cultured in DMEM:F12 (1:1) with 10% of FBS until they reached 90% of confluence, after which the medium was changed to a defined medium comprised of DMEM (Dulbecco's Modified Eagle Medium) and 1% TPPS (1 mg of holo-transferrin bovine; 1.0 mM of putrescine; 2 mM of progesterone and 30 μM of sodium selenite). After 3-day incubation, the serum-free defined medium was collected, filtered and stored at -80 °C for storage.

To induce *in vitro* differentiation of the progenitor cells, growth medium was switched to differentiation medium (DM), which consisted of DMEM, 0.4% FBS, 50 μg/mL of transferrin, 2.5 μg/mL of insulin, 10 pg/mL of biotin and 50 ng/mL of selenium.

3.2 Genetic basis for the Tet-off gene expression system

The tetracyclin-based inducible systems (Tet-on/Tet-off) make use of the *E.coli* tetracyclin (Tet) resistance operon for regulating gene expression in a large collection of cell and animal models. The Tet-off expression system consists of two vectors (Figure 7), the first vector encodes a tetracycline-controlled transactivator protein (*t*TA), which is composed of the Tet repressor DNA binding domain (TetR) from the Tc resistance operon (Appendix I). The second vector contains multiple cloning sites (MCS) for inserting the target gene, which is under transcriptional control of a tetracycline-responsive promoter element (TRE) (Appendix II). The TRE is made up of Tet operator (tetO) sequence concatemers fused to a minimal promoter. In case of Tet-off, the presence of Tet or doxycycline (Dox) prevents *t*TA from binding to the TRE site and inhibits the transcription of target gene. Withdrawing Dox allows *t*TA binding to the TRE

site and activates the downstream transcription.

Figure 7 illustrates the processes to establish the two stable cell lines, where Hoxa2 can be either up- or down-regulated. The CG4 cells were firstly transfected with the pTet-off vector, which contains the *t*TA encoding region as well as a neomycin resistance allele, followed by G418 selection to select stable cell clones. Surviving clones were screened by transient transfections with control vector pTRE2hyg2-Myc-Luc for clones with low background and high Dox-dependent induction. A second transfection introduced the second vector which harbours sense-*Hoxa2* or anti-sense-*Hoxa2*, as well as the hygromycin resistance gene. Cells survived the hygromycin selection were then subjected to RT-PCR and western blot analysis.

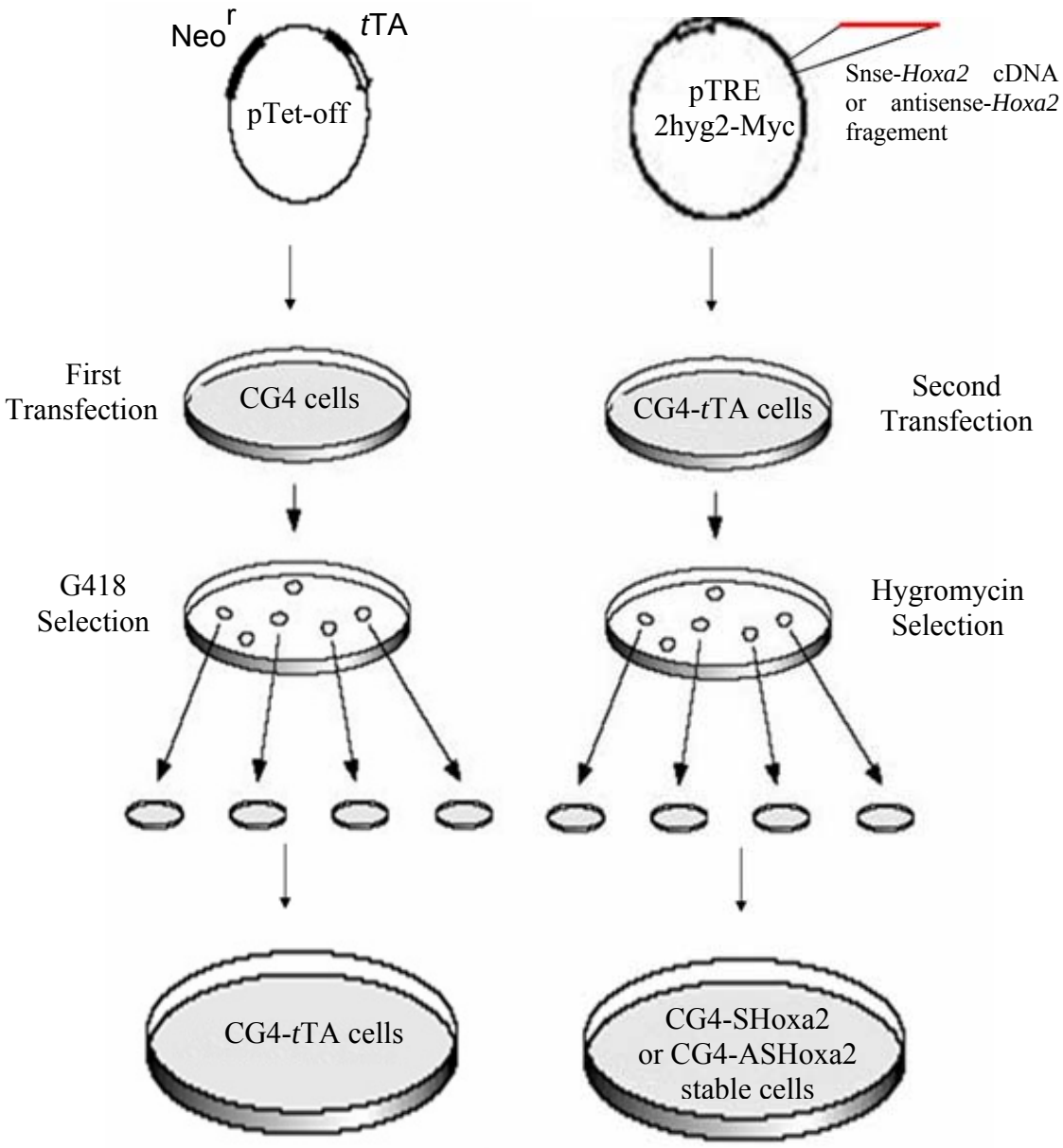

Neo^r
tTA
pTet-off
pTRE
2hyg2-Myc
Snse-Hoxa2 cDNA
or antisense-Hoxa2
fragement
First
Transfection
CG4 cells
CG4-tTA cells
Second
Transfection
G418
Selection
Hygromycin
Selection
CG4-tTA cells
CG4-SHoxa2
or CG4-ASHoxa2
stable cells

Figure 7. Schematic diagram depicts the procedures for developing the tetracycline (Tet) controlled gene expression system in CG4 cells. Initially the pTet-off vector (linearized with *ScaI*), which encodes the tetracycline-controlled transactivator (*t*TA), is introduced into the CG4 cells. Selection of stable clones that consistently express *t*TA was performed by G418 (300 μg/mL) screening. After selection, a control vector, pTRE2hyg2-Myc-Luc (Clontech), was introduced transiently into the G418-resistant clones to determine their inducibility by measuring the luciferase activity. Clones that gave the highest luciferase expression (Fig.12) were selected for subsequent experiments. A second stable transfection with the recombinant vectors, harboring the *Hoxa2* gene (sense or anti-sense), was carried out by using electroporation. The hygromycin resistant property of the pTRE2hyg2-Myc was utilized in the selection of double stable cell clones, which would either up-regulate or down-regulate the expression of *Hoxa2*.

3.3 Plasmids construction

The pTet-off vector (Appendix I) was a gift from Dr. Peter Dickie (University of Alberta). The pTRE2hyg2-Myc (Appendix II) and pTRE2hyg2-Myc-Luc vectors were purchased from BD Biosciences (Clontech, Mountain View, CA). Sense cDNA of *Hoxa2* was PCR amplified using forward primer 5'-CATCGATAGAAGGCCAT GAATTACG-3' and reverse primer 5'-TCGTCGACTTAGTAATTCAGATGC-3' from the pRSV-*Hoxa2* vector. Antisense cDNA of *Hoxa2* gene was amplified by PCR using forward primer 5'-GCTGTAATATCGATGTAATTCAGATGCTGTAGG-3' and reverse primer 5'-AGGAGGTCGACATGAATTACGAATTTGAGC-3' (restriction enzyme sites are underlined). The PCR products of sense *Hoxa2* cDNA and antisense *Hoxa2* cDNA were, respectively, double digested with *SalI* and *ClaI*, and subsequently cloned into the *SalI / ClaI* site of the pTRE2hyg2-Myc vector. All constructs were confirmed by sequencing (Appendix VIII shows the alignment of sequencing data of pTRE2hyg2-Myc-SenseHoxa2 with *Hoxa2* cDNA). The pTRE2hyg2-Myc-Luc vector (Clontech, Mountain View, CA) contains the Luciferase reporter and was used as a control for determining the expression profile of the Tet system in CG4 cells.

3.4 Titrating G418, hygromycin and doxycycline

Prior to transfection, kill curves were created for the antibiotics G418, hygromycin and doxycycline (Dox) to determine the optimal concentration for selection with CG4 wild-type cells. Wild-type CG4 cells were seeded at 2 x 10^5 per 100 mm tissue culture, and grown in 10 mL of GM with varying amounts of G418 (0, 100, 300, 500, 700 μg/mL) or hygromycin (0, 50, 100, 200, 400, 600 μg/mL). For Dox, this selection agent was tested at concentrations of 0, 1, 2, 4, 6 μg/mL. Selective medium was replaced every two days with fresh medium containing the same amount of antibiotic. According to Clontech protocol (PT3001-1), the lowest concentration that begins to cause massive cell destruction at day 5 and kills all the cells within two weeks was used for selecting stable transformants. For CG4 cells, 300 μg/mL of G418, 200 μg/mL of hygromycin and 2 μg/mL of Dox proved to be optimal. In addition, for G418, after stable transformants were selected and confirmed, a lower concentration (100 μg/mL) of this antibiotic was used to maintain a selection threshold (Clontech PT3001-1).

3.5 Transfections and engineering of double stable cell lines

Plasmids used for transfection were purified by using the EndoFree Plasmid Maxi Kit (Qiagen, Mississauga, Ontario). After DNA purification, 30 μg of pTet-off vector was linearized with *HindIII*, followed by phenol extraction and ethanol precipitation. Linearized pTet-off plasmid (10 μg) was added to 30 μL of 2.5M $CaCl_2$ and made up to a total volume of 150 μL in water. The DNA-$Ca_3(PO4)_2$ precipitate was prepared by adding the above mixture dropwise to an equal volume of 2XHBS (280 mM NaCL, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM glucose and 5 mM HEPES, pH7.0-7.1) while vortexing. This mixture was allowed to sit in the hood for 15 min before being slowly added to a 60 mm dish of 80% confluent CG4 cells, which was plated 24 h before transfection. The cells were incubated with the DNA precipitate in a humidified incubator in 5% CO_2 at 37 °C. After 12 h, the cells were rinsed with Puck's (200mM NaCl, 5mM KCl, 4mM $NaHCO_3$, 5.5mM glucose) and incubated with fresh GM for 48 h before G418 selection. Stable CG4-*t*TA clones were selected in the presence of 300 μg/mL of G418 (Sigma, St. Louis, MO) in 100 mm^2 dishes until 11 cell clones were obtained.

The CG4-*t*TA clones isolated were examined for their ability to induce gene expression by transient introduction of the control vector pTRE2hyg2-Myc-Luc into these cells using the calcium phosphate precipitation method. Luciferase activities were measured and a clone (clone 2) that gave the highest reading was subsequently used in the follow up experiments.

Electroporation was adopted in the following transfections with recombinant plasmids pTRE2hyg2-Myc-SHoxa2 and pTRE2hyg2-Myc-ASHoxa2, and was carried out using Gene Pulser and a 0.4 cm electroporation cuvette (Bio-Rad Laboratories, Hercules, CA). CG4-*t*TA cells were plated onto 100^2 mm dishes at 8 x 10^5 cells per dish two days before transfection. Fresh medium was supplied to the culture 5 h before the electroporation. The cells were then harvested and collected at a concentration of 5 x 10^6 cells per mL in electroporation medium (EM), a 1:1 mixture of HBS and GM (with twice the concentration of insulin described above). Twenty micrograms of DNA was diluted in 20 μL sterile water and added to a 780 μL cell suspension. Electroporations were performed at room temperature with a charge of 0.34 KV at a capacitance of 250 μF. After electroporation, the cells were allowed to sit undisturbed in the hood for 10 min,

and then transferred onto culture dishes in recovery medium (RM), comprised of DMEM 5%, insulin 5 μg/mL, pyruvate 2 mM and antibiotic/antimiotic 100 μg/mL. The medium was changed to the regular GM after 2 h incubation in RM. Hygromycin B (Sigma, St. Louis, MO) (200 μg/mL), G418 (100 μg/mL) and Dox (2 μg/mL) were added into the GM two days later for selection, and fresh GM with these drugs was fed every other day. Double-transfectant clones appeared after 3~4 weeks screening with 200 μg/mL of hygromycin B and 100 μg/mL of G418. Hygromycin resistant clones that were selected for the following proliferation and differentiation assay were named CG4-SHoxa2 (sense Hoxa2 up-regulation) and CG4-ASHoxa2 (anti-sense Hoxa2, down-regulation) cells, respectively. These clones were maintained in medium containing 100 μg/mL of G418 and 200 μg/mL of hygromycin B and kept under liquid nitrogen for long-term storage.

3.6 Luciferase assays

CG4-*t*TA cells were seeded at 2 x 10^5 on 35 mm^2 P-D-L coated dishes the day before transfection. Transfections were performed as described above. After growth for 48 h in the appropriate medium, cells were then washed twice with Puck's prior to harvesting for Luciferase assay using the Dual-Luciferase® Reporter assay kit (Promega Corp., Madison, WI). In brief, cells were subjected to 500 μL Passive Lysis Buffer (PLB), where 20 μL of the cell lysate were added to 100 μL of Luciferase assay reagent AR II and luminescence was measured. Luciferase activities were calculated as relative light units (rlu) /mg of total cellular protein.

3.7 Western blot analysis

For detection of the expression level of Hoxa2 protein, and to confirm the establishment of the double stable cell lines that contain the Tet-off system, CG4 cells and genetically modified CG4 cells were seeded at 3 x 10^5cells per well in a 6-well dish, and cultured in the presence or absence of 2 μg/mL of Dox. Following two days of growth in GM or DM, cells were harvested and lysed with RIPA buffer (150 mM NaCl, 0.5% SDS, 1% Triton-100, 0.1% deoxycholate, 10 mM Tris-HCl [pH 7.2], 5 mM EDTA). Protein quantification was completed using a Bio-Rad DC Protein Assay kit (Bio-Rad, Richmond, CA). Equivalent amounts of protein for each sample were electrophoresed on a 12% polyacrylamide/0.1% SDS gel and subsequently transferred to a PolyScreen® PDVF transfer membrane (PerkinElmer, Boston, MA). Transfer buffer

was composed of 10% methanol, 25 mM Tris-HCl [pH 7.2], and 199 mM glycine. The transfer was performed for 1.5 h at 30V/200 mAmp at room temperature (RT). After being blocked with 3% skim milk (SM) in phosphate-buffered saline (PBS) at 4°C overnight, the membrane was immunoblotted with a polyclonal rabbit antibody specific to Hoxa2 (Hao *et al*., 1999) at a dilution of 1:3000 in SM-PBS for 1 h at RT. Repeated washings (3 washes of 20 min each) were carried out in PBS with 0.08% Tween-20 (PBS-T), before the membrane was incubated with the secondary antibody, goat anti-rabbit IgG horseradish peroxidase conjugate (HRP) in SM-PBS at a 1:3000 dilution for 1 h at RT. After extensive washings and treatment with a chemiluminescence reagent (DuPont NEN®, Boston, MA), Hoxa2 protein expression was visualized on an X-ray film (Kodak Industrie, Cedex, France). The same membrane was blotted again with a monoclonal antibody specific to β-actin as an internal control (JLA20, 1:3000; Developmental Studies Hybridoma Bank, Iowa) for 1 h at RT, followed by a secondary anti-mouse IgM antibody (1:3000) for 1 h at RT.

3.8 Immunocytochemistry

For immunostaining, cells were plated onto P-D-L coated 15 mm^2 coverslips and grown as described above. The following primary antibodies were utilized: anti-Hoxa2 (1:800, Hao *et al*., 1999), A2B5 (1:100, ATCC), O4 (1:50, hybridoma; Sommer and Schachner, 1981), and anti-galactocerebroside (GalC) (1:100, hybridoma; Ranscht *et al*., 1982). A2B5, O4, GalC were used to mark cells at different stages of OL development (Fig.1). Double labelling of Hoxa2 with A2B5, O4, or GalC was conducted using a procedure described previously (Doucette and Devon, 1994) with the following modifications: live cells were incubated for 45 min at 37 ˚C in A2B5, O4, or anti-GalC monoclonal antibodies diluted in PBS; the blocking and permeabilization steps were combined with a 30-min incubation in 3% skim milk powder solution containing 0.1% Triton X (3% SM/0.1% TX); the cells were incubated for 4 h at RT in anti-Hoxa2 diluted in 1% SM/0.03% TX. The secondary antibodies utilized include anti-mouse IgM fluorescein isothiocyanate (FITC) (1:100, A2B5/O4, Sigma), donkey anti-mouse IgG FITC (1:50, GalC, Bio/CAN), and goat anti-rabbit IgG CY3 (1:200, Hoxa2/GFAP, Bio/CAN). Between incubations, cultures were washed twice for 5 min in PBS.

For the differentiation assay, cells were double labelled with O4 and anti-GalC

antibodies. Cells were fixed with 4% paraformaldehyde first, followed by incubation with primary antibodies of O4 and GalC at 37 ˚C for 45 min. After blocking with skim milk, coverslips were incubated with secondary antibodies which contained anti-mouse IgM Alexa Fluor 594 (1:500, O4, Sigma) for O4, and anti-mouse mouse IgG FITC for detection of GalC.

In all the stainings, nuclei were visualized by using Hoechst dye (Sigma, ON). The percentage of a particular population of OLs, positive for one of the following markers A2B5 (A2B5+ve), O4 (O4+ve), GalC (GalC+ve), and BrdU (BrdU+ve) markers was calculated from three independent experiments, with more than 500 cells counted in total for each coverslip.

3.9 Measure proliferation rate of cells by BrdU incorporation assay

CG4 cells were sub-cultured from the same passage and seeded onto 15mm^2 coverslips at 1x10^5 cells per well (2 coverslips per each well) in the 6-well dishes and maintained in GM, either supplemented with Dox or No Dox, two days before addition of BrdU. To induce cell differentiation, GM was changed to DM on the next day, and cells were subsequently cultured in DM for 2 days before the BrdU assay. BrdU was supplied for the last 12 h of incubation at a final concentration of 1 μM. Detection of BrdU was performed as follows: cells were fixed in ice-cold 4% paraformaldehyde for 15 min, followed by a 30 min incubation in 2 N HCl and a 20 min incubation in 3% skim milk/0.1%TritonX-100; cells were further incubated in anti-BrdU antibody (1:200, Sigma) in PBS for 1 h at RT. After several washes in PBS and incubation in secondary antibody (donkey anti-mouse IgG FITC [1:50] in PBS) for 30 min at RT, cells were counter stained with Hoechst dye.

To examine the developmental stage of BrdU+ve cells, either in GM or DM, double labelling of BrdU with either A2B5 or O4 antibodies was conducted. Cells were fixed first following a 12h-incubation with BrdU, and then subjected to the primary antibody (A2B5 or O4) for 45 min at 37 ˚C. After permeabilization, cell surface markers were detected using anti-mouse Alexa Fluor 594. A further fixation was performed before BrdU staining was carried out as described above.

3.10 Quantitative analysis of cell differentiation

For the quantitative differentiation analysis, $4x10^5$ cells of CG4 or CG4-SHoxa2 were seeded on each well of the 6-well dish (35 mm^2) (Fig. 7). Cells were further maintained in GM for overnight (16 h), since directly changing into DM resulted in poor attachment and a substantial amount of cell death. GM was then replaced with freshly made DM after thoroughly washing with Puck's saline. As illustrated in Figure 7, well 1 and 4 were incubated in DM for 2 days, the middle two wells (2 and 5) were maintained in DM for 3 days, whereas the last two (3 and 6) were kept for 4 days. Cells in places 1, 2 and 3 were continuously cultured in the presence of Dox (2μg/mL), while the bottom three wells (4, 5 and 6) were not subjected to Dox treatment. Each well contained two coverslips, one of which was utilized for double staining with anti-A2B5 and anti-GFAP, and the other used for detection of O4 and GalC.

3.11 Microscopy

We observed live cell cultures using an Olympus CKX41 light microscope, with objective lens magnification of either x10 or x40; pictures were taken by Nikon CoolPix4500 camera. Fluorescent images were examined with an Olympus BX40 microscope equipped with appropriate filter sets, and pictures were analyzed with the aid of Image-Pro Imaging software (Media Cybernetics).

3.12 RNA isolation and semiquantitative reverse transcription polymerase chain reaction (RT-PCR)

For detection of the mRNA of PDGFαR, CGT (Oligodendrocyte-specific ceramide galactosyltransferase), MBP and Hoxa2, CG4 and CG4-SHoxa2 cells were plated in 6-well dishes at $3x10^5$ cells per well and cultured in GM overnight either in the presence or absence of *Dox*. To induce cell differentiation, the medium was changed to DM the next day and cells then were incubated for either 2 or for 3 days. Cells were rinsed twice with Puck's before total RNA isolation, which was performed by using the RNeasy Protect Mini Kit (Qiagen, Mississauga, ON). The concentration of RNA was determined by OD (optimal density) measurements of aliquots at a wavelength of 260 nm. For detailed information on primers and PCR products please refer to Table II.

Subsequently, 200 ng of total RNA for each sample were reverse transcribed into cDNAs with the enzyme Superscript® reverse transcriptase (Invitrogen) using a random

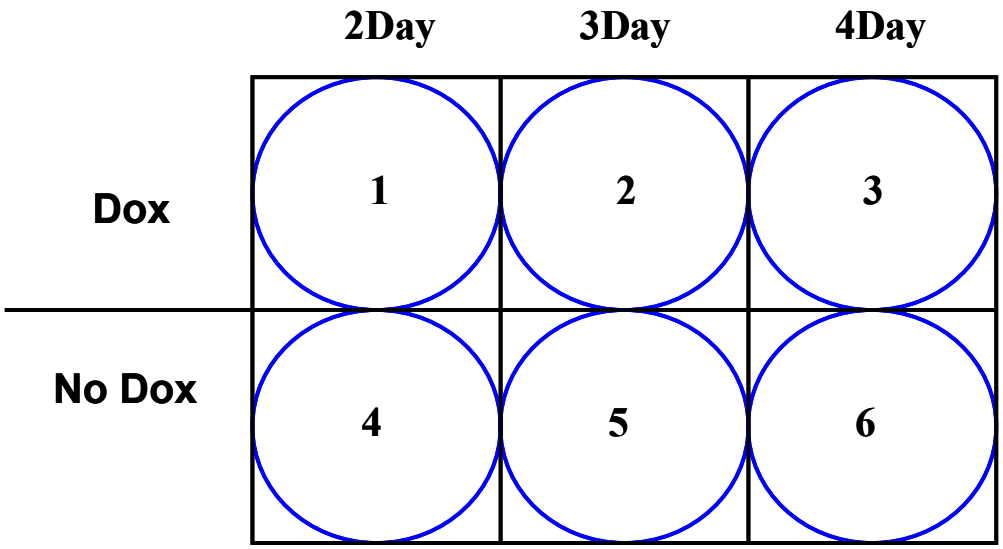

2Day
3Day
4Day
Dox
No Dox
1
2
3
4
5
6

Figure 8. Schematic illustration of the set up of the differentiation assay in culture dishes treated with doxycycline (Dox) or untreated (No Dox) at different time points (day 2, 3 and 4). Cells were sub-cultured from the same passage, and seeded onto a 35 mm^2 surface (one well) of the 6 multi-well plate ($4x10^5$ cells per well). Cells were allowed to recover in growth medium (GM) for 16 h after the split, with the upper three wells (1, 2 and 3, red color) supplemented with Dox, whereas cells in the bottom three wells did not receive Dox (4, 5 and 6, red color). GM was replaced with differentiation medium (DM) after 16 h, and the wells 1, 2, 3 remained treated with Dox. Each well contained two coverslips, with one coverslip subjected to A2B5/GFAP double immunostaining and the other one for double labelling of O4/GalC. Wells 1 and 4 were subjected to immunostaining after two days incubation in DM. Coverslips in wells 2 and 5 were used for the 3 DM study, and coverslips in wells 3 and 6 were subjected for the 4 DM study.

hexamer oligonucleotide primer in a standard RT-PCR reaction according to the manufacturer. The synthesized cDNAs were then used as templates for PCR amplification with primers specific for the genes of interest (Table II). PCR was carried out in a reaction of 50 μL containing 1 x PCR buffer (20 mM Tris.HCl [pH8.3], 25 mM KCl, 1.5 $MgCl_2$, 0.1% Tween 20), 200 μM concentrations of each of four deoxynucleotide triphosphates, 20 pmol of primers, 5 U of *Taq* DNA polymerase, and 2 μL of the reverse transcribed template. PCR was performed in a PTC-100™ thermal cycler (MJ Research, MA). The PCR program started with an initial denaturation at 95 °C for 1 min, followed by 40 cycles with each cycle consisting of 94 °C for 45 s, 53 °C for 1 min, and 72 °C for 1 min and ended with a final extension of 4 min at 72 °C. PCR products were analyzed on 1.5% agarose gel following electrophoresis and visualized by staining with ethidium bromide. All pictures were photographed and analyses were conducted with an AlphaImager™ Gel Imaging System.

TABLE II. Sequences of Primers and PCR Product Sizes Used in the RT-PCR

Gene	Primer	Sequence	Size (bp)	Reference
PDGFαR	Forward Reverse	5'-GCCAGGAGACGAGGTATCAA-3' 5'-TGTTCCCAATGCCAAGGTC-3'	426	(Hu *et al*., 2004)
CGT	Forward Reverse	5'-GGAGATTGCCTCAGAAAGT-3' 5'-GTCGTGGCGAAGAATGTAG-3'	428	(Hu *et al*., 2004)
MBP	Forward Reverse	5'-CTATAAATCGGCTCACAAGG-3' 5'-AGGCGGTTATATTAAGAAGC-3'	176	ATRC Reagent Bank*
β-Actin	Forward Reverse	5'-ATTGTAACCAACTGGGACG-3' 5'-TTGCCGATAGTGATGACCT-3'	553	(Hu *et al*., 2004)
Hoxa2	Forward Reverse	5'-GGGAAGGGTACACTTTTCAGC-3' 5'-TTAGGAACAGTGGGTGACTGG-3'	151	UniSTS:143365

* Harvard Center for Neurodegeneration & Repair, Advanced Tissue Resource Center.

3.13 Statistical analysis

For immunostaining experiments, the median was calculated for data from each cell group in each round of the study; data was further analyzed by ranks using a Kruskal-Wallis two-way nonparametric ANOVA (GraphPad Prism 4) followed by Dunn's multiple comparison test. For analyzing RT-PCR data of PDGFαR, CGT and MBP, the optical density ratio between each target band and internal control band (β-actin) was obtained using the AlphaImager^{TM} Gel Imaging Software. The ratios were subsequently analyzed by one-way ANOVA followed by Tukey's multiple comparison post hoc tests in GraphPad Prism if *p* value is significant. A *p* value of less than 0.05 was considered to be significant.

IV RESULTS

4.1 Phenotype of CG4 cells in culture during the course of differentiation

Previous work from our laboratory has identified for the first time the expression of *Hoxa2*, *Hoxb4* and *Hoxd1* genes *in vivo* throughout OL development (Hao *et al*, 1999; Nicolay *et al*., 2004a, b; Booth *et al*., 2007). In particular, *Hoxa2* expression is indicated to be down regulated at late stages during OL maturation. Although the specification and early development of OPCs appear to proceed normally in the *Hoxa2*-/- mice, data from our laboratory (unpublished) indicate that OPCs isolated from the mutant mice display more processes and are less efficient at myelinating axons in OL/neuron co-cultures. Since *Hoxa2* null mice die within the first 24h of birth and obtaining sufficiently pure primary cultures of OLs is difficult, we have been unable to investigate the potential role of *Hoxa2* during the late developmental stages of OL and to determine how OLs and neurons interact with each other in the *Hoxa2* null mice. The CG4 cells provide a suitable model to establish a tetracycline (Tet) regulated inducible *Hoxa2* expression system.

CG4 cells was established and regularly maintained in GM based on published protocols (Louis *et al*., 1992), described in the methods section (3.1). First, I carefully examined the morphological characteristics of CG4 cells, an O-2A precursor cell line, along their developmental pathway. At the progenitor stage, undifferentiated CG4 cells display a typical bipolar or tripolar phenotype with long unbranched processes (Fig. 9A), and produce progenitor stage cell surface markers A2B5 (Fig. 10A). To preserve this characteristic bipolar morphology, wild-type CG4 cells were maintained in GM and sub-cultured before reaching 80% of confluence according to previous studies (Louis *et al*., 1992; Raff *et al*., 1998). To initiate the OL-like differentiation of CG4 cells, the medium was changed from growth medium (GM) to differentiation medium (DM). After 2 days of incubation in DM, the developing network of membrane processes became visible and most cells acquired a multipolar phenotype, with 4-6 branched long processes and some thin, small unbranched processes (Fig. 9B). When the cultivation in DM was prolonged to 3 days, the cells enlarged their network of membrane processes and assumed an even more complex structure of membrane extensions (Fig. 9C).

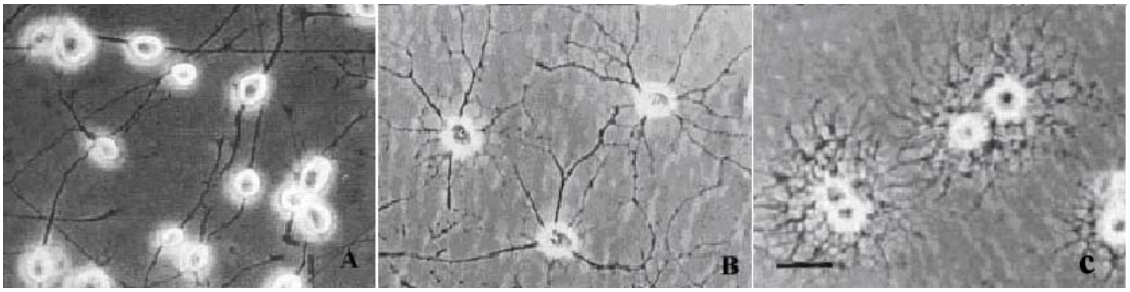

A
B
C

Figure 9. Morphological characteristics of the CG4 cells at progenitor stage (A), late-progenitor stage (B) and pre-myelinating stage (C). A. CG4 cells were grown in defined growth medium (GM). The majority of the cells (>99.9%) displayed bipolar or tripolar phenotype with long and smooth process extending from the nuclei. The cells maintained in differentiation medium (DM) for 2 days (B) or 3 days (C) acquired a much more advanced phenotype. Scale bar indicates 25 μm.

Unfortunately, cells detached from the culture plates and/or the coverslips when incubated in DM for longer than 3 days, impeding the study of OL cells at the later myelin basic protein (MBP) stage of development. In addition, during the differentiation of CG4 cells in DM, cells were examined for glial fibrillary acidic protein (GFAP) staining, since CG4 cells are also able to give rise to type 2 astrocytes (Louis *et al*., 1992). No GFAP positive cells were observed during the 3 day differentiation assay throughout the study (data not shown).

4.2 Hoxa2 is co-expressed with A2B5, O4 and GalC in CG4 cells

The expression of Hoxa2 protein was investigated in CG4 cells by immunocytochemistry (Fig. 10) and western blot analysis (Fig. 13). In agreement with a previous *in vivo* investigation (Nicolay *et al*., 2004), the presence of Hoxa2 was detected in the nuclei of CG4 cells expressing OL-lineage specific cell surface antigens, as revealed by the detection of A2B5 (Fig. 10A), O4 (Fig. 10D) and GalC (Fig. 10G) along their differentiation course. The nuclei were visualized using nuclear fluorescent Hoechst counter stains (Fig. 10 C, F, and I). Co-expression of Hoxa2 with OL-lineage markers A2B5, O4 and GalC was observed in over 98% of the total cell culture population.

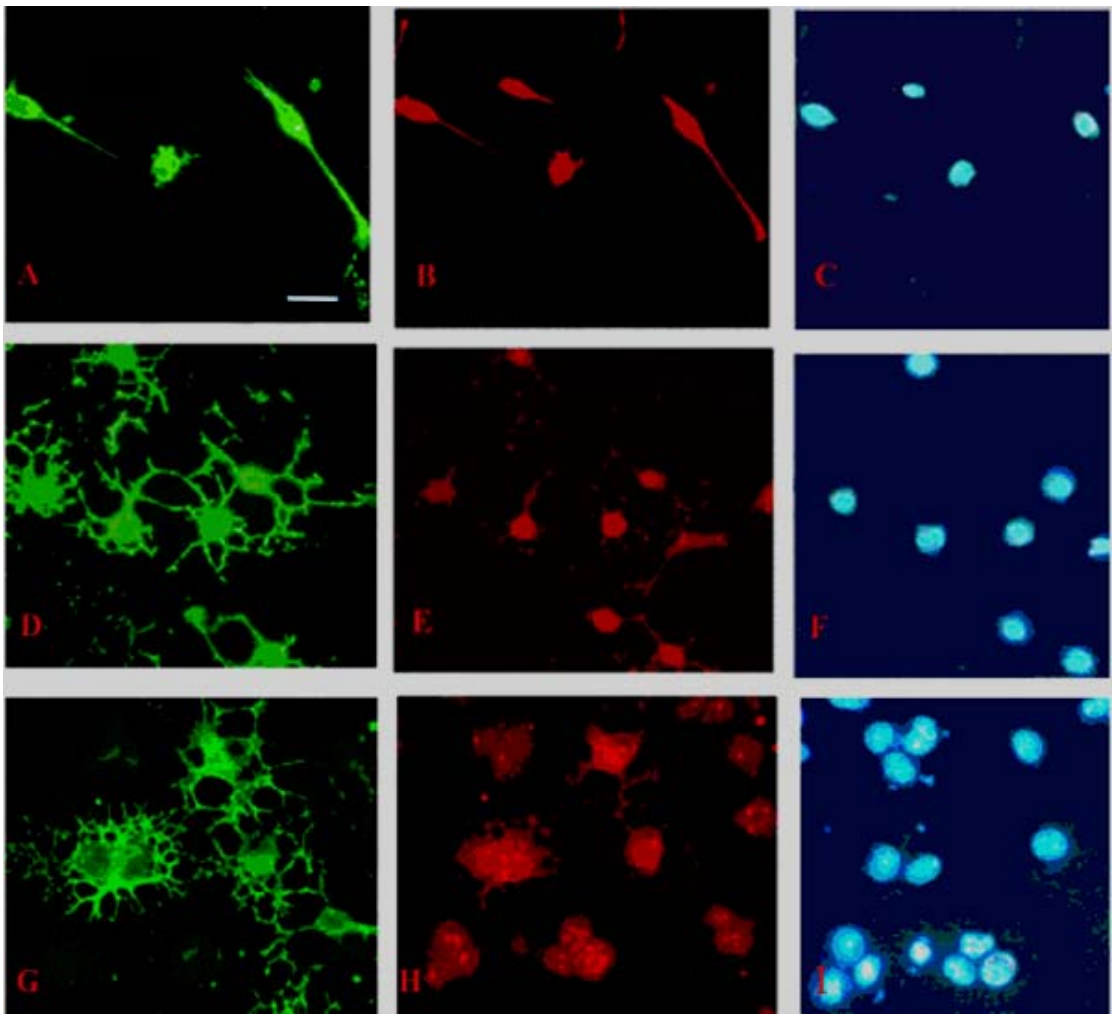

A
B
C
D
E
F
G
H
I

Figure 10. Double immunostaining of Hoxa2 (B, E, H) with A2B5 (A), O4 (D), and GalC (G), respectively, in CG4 cells cultured in DM for 3 days. Cell nuclei were visualized using nuclear fluorescent Hoechst counter stain (C, F, I) of the same field. Scale bar indicates 10 μm.

4.3 Construction of pTRE2hyg2-Myc-SHoxa2 and pTRE2hyg2-Myc-ASHoxa2 vectors

Control over the timing and level of gene expression is an important parameter to consider in the analysis of gene function or application in gene therapy. Several inducible gene expression systems have been developed for this purpose (reviewed in Zhu *et al*., 2002), and we adopted the Tet controlled system, or 'Tet-off', which can be successfully applied in CG4 cells with no toxic effects on endogenous cellular process (Magy *et al*., 2003).

The recombinant vectors, named pTRE2hyg2-Myc-SHoxa2 (containing sense *Hoxa2* in frame) and pTRE2hyg2-Myc-ASHoxa2 (containing anti-sense *Hoxa2* in frame), were confirmed by PCR, restriction enzyme digestion (Fig. 11) and DNA sequencing (Appendix IV).

4.4 Establishment of the CG4-SHoxa2 and CG4-ASHoxa2 cell lines

Two sequential stable transfections were required to engineer the Tet (Dox) modulated *Hoxa2* expression system in the CG4 cells, as shown in Figure 7 as a schematic representation (Materials and Methods 3.2). The vector, pTet-off expressing the tetracycline transcriptional activator (*t*TA), was first introduced into the CG4 cells by DNA-calcium-phosphate precipitation. Stable cell clones carrying the pTet-off plasmid were selected by screening with 300 μg/mL of active G418, a dosage determined from kill curve tests on wild-type CG4 cells (see Materials and Methods 3.4).

To ensure the Tet-off system was able to regulate gene expression in CG4 cells, and to select cells that exhibit the highest inducibility, eleven G418-resistant clones were examined for their functional inducibility by transient transfection with the pTRE2hyg2-Myc-Luc vector. This control vector contains an additional 1653 bp fragment encoding firefly luciferase inserted into the MCS, and was used as a reporter of induction efficiency. Among the 11 clones, clone 2 (CG4-*t*TA cells) showed the highest fold induction as indicated by measuring the luciferase activity in the absence of Dox (Fig.12), and therefore was selected for subsequent experiments. The vectors containing either the sense *Hoxa2* or anti-sense *Hoxa2* gene were transfected into the CG4-*t*TA cells, respectively, by electroporation; double-transfectant clones were selected after exposure to 200 μg/ml of hygromycin.

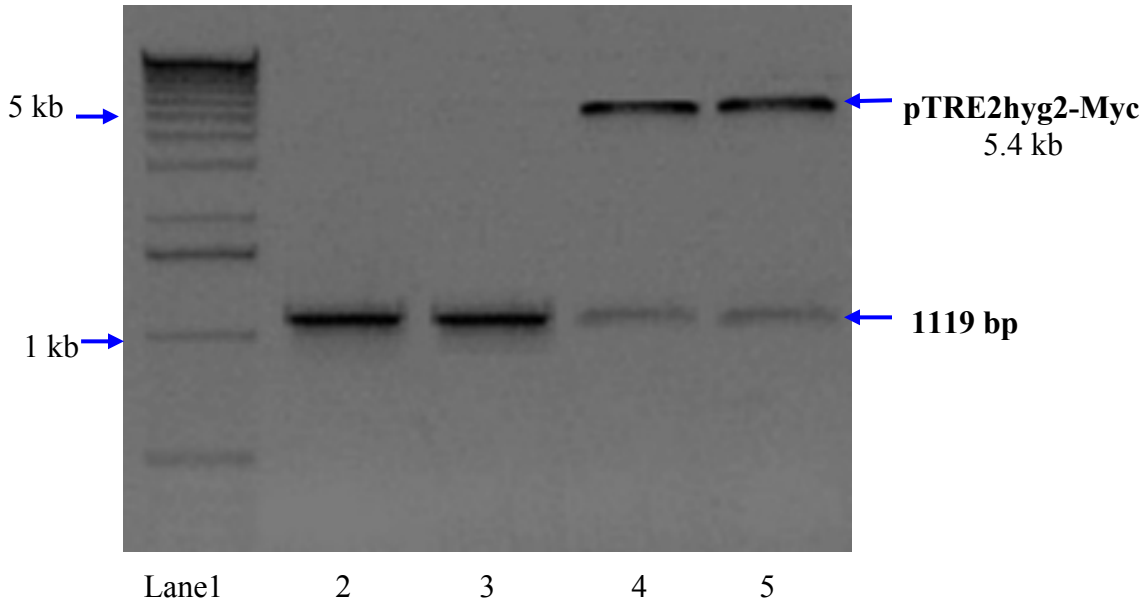

5 kb
1 kb
pTRE2hyg2-Myc
5.4 kb
1119 bp
Lane1
2
3
4
5

Figure 11. Confirmation of the recombinant vectors (pTRE2hyg2-Myc-SHoxa2 and pTRE2hyg2-Myc-ASHoxa2) by PCR amplification (Lane 2 & 3) and enzyme (*ClaI* / *Sa I*) digestion (Lane 4 & 5). Lane 2 and 3 are PCR amplification of Hoxa2 (either sense- or antisense-) insert using constructed vectors pTRE2hyg2-Myc-SHoxa2 and pTRE2hyg2-Myc-ASHoxa2 as template, respectively. Both vectors were subjected to *ClaI* / *SalI* double digestion. In all cases a fragment of 1119 bp was obtained, which represents either sense *Hoxa2* (Lane 4) or anti-sense *Hoxa2* (Lane 5). The larger bands in lane 4 and lane 5 are linearized vector pTRE2hyg2-Myc after digestion. Lane 1 is 1kb DNA ladder (Invitrogen).

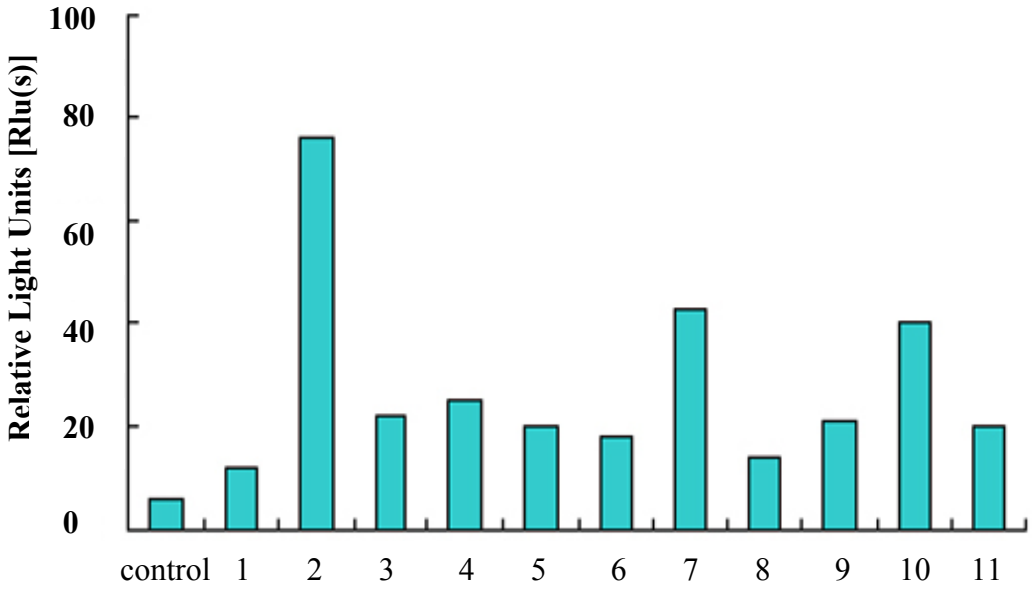

Relative Light Units [Rlu(s)]
100
80
60
40
20
0
control
1
2
3
4
5
6
7
8
9
10
11

Figure 12. Measurement of the luciferase activities of the eleven G418-resistant cell clones as indication of induction efficiency of individual clone. Cells were grown in GM in the absence of Dox and G418, and were transiently transfected with the luciferase control vector pTRE2hyg2-Myc-Luc. The first bar indicates background luciferase activity in wild-type CG4 cells. The numerical number underneath each bar stands for each CG4-*t*TA clone. Luciferase activity was expressed as relative light unit [Rlu(s)]. Data represents a mean of two repeated experiments.

4.5 Regulation of the expression of *Hoxa2* in CG4-SHoxa2 and CG4-ASHoxa2

The "Tet-off" system is a transcription activation system with a pTet-off regulator plasmid that is controlled by tetracycline (Tet) or its analogue doxycycline (Dox). In this binary transgenic system, expression of *Hoxa2* or anti-sense *Hoxa2* by CG4 cells is repressed when Tet or Dox is added to the culture medium and is induced upon withdrawal of Tet or Dox.

The expression of Hoxa2 was detected in cells cultured in GM either in the presence or absence of Dox (2 μg/ml) by western blot analysis (Fig. 13B) after transfection with plasmid pTRE2hyg2-Myc-SHoxa2 or pTRE2hyg2-Myc-ASHoxa2 and hygromycin selection. The transfected cells were maintained in GM supplemented with G418 (100 μg/mL), hygromycin (200 μg/mL) and Dox (2 μg/mL) for over 4 weeks (Material and Methods, 3.3 and 3.4). In total, ten double stable clones containing both pTet-off and pTRE2hyg2-Myc-SHoxa2 plasmids survived and were then transferred to new culture area using cloning cylinders. These ten double stable clones were referred to as clones I through X. Individual RNA samples, isolated from each clone cultured in GM without Dox supplement, were prepared for RT-PCR to determine whether the *Hoxa2* gene was up- or down-regulated at the RNA level (Fig. 13A) (PCR primers described in section 3. 11). Clone X exhibited the highest level of mRNA of *Hoxa2*, and was named the CG4-SHoxa2 cell line. Descendants of clone X were subjected to western blot analysis to examine the expression level of Hoxa2 protein (Fig. 13B). In comparison, only four antisense-Hoxa2 containing double stable clones (clone i, ii, iii, iv) were obtained after transfection and antibiotic screening, due to poor attachment and low viability of the antisense-Hoxa2 harbouring cells. Antisense-clone-i, namely CG4-ASHoxa2 was used for western blot analysis (Fig. 13B), because this cell clone exhibited a relatively faster proliferation rate and higher viability after subculture.

Wild-type CG4 cells treated with or without Dox were used as the controls, and as shown in Figure 13 the presence of Dox does not seem to exert any impact on endogenous Hoxa2 expression level. In summary, a higher level of Hoxa2 protein was observed from the sample harvested from the CG4-SHoxa2 No-Dox group, compared with CG4 control groups (Dox and No-Dox) or with Dox treated CG4-SHoxa2 group. In contrast, Hoxa2 dramatically decreased in CG4-ASHoxa2 when cultured without Dox.

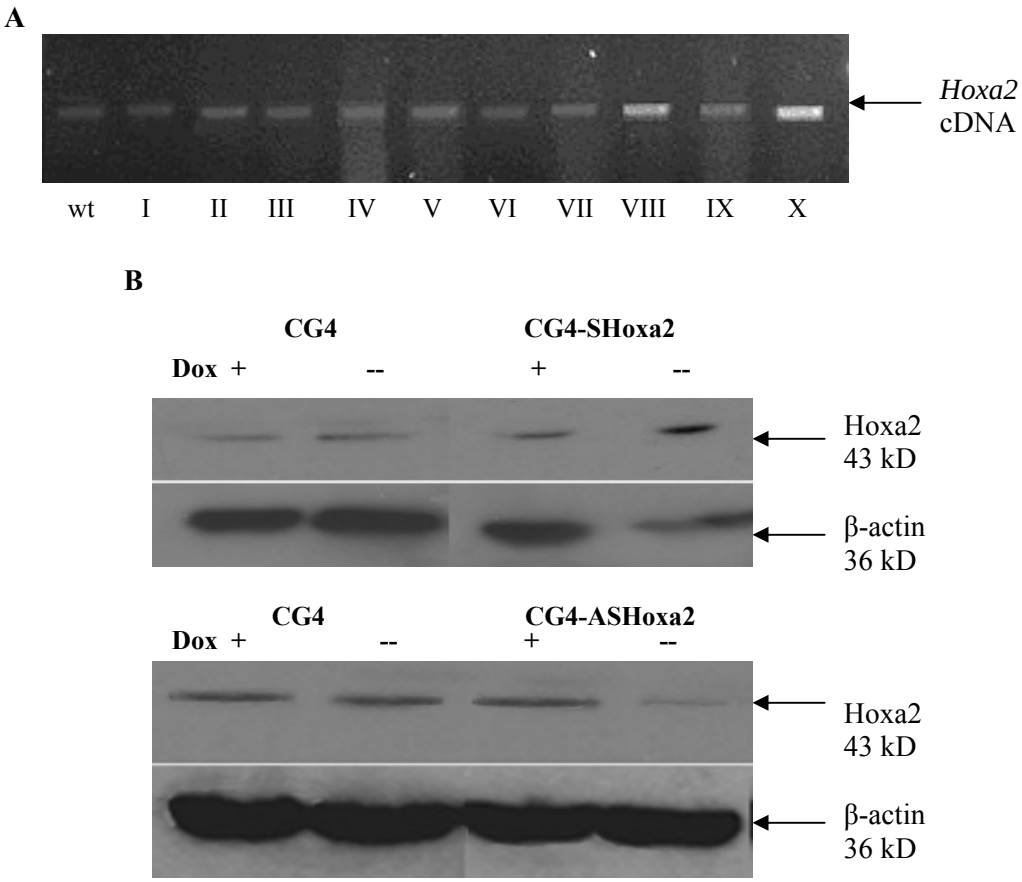

A
Hoxa2
cDNA
wt I II III IV V VI VII VIII IX X
B
CG4
CG4-SHoxa2
Dox + -- + --
Hoxa2
43 kD
β-actin
36 kD
CG4
CG4-ASHoxa2
Dox + -- + --
Hoxa2
43 kD
β-actin
36 kD

Figure 13. RT-PCR and western blot analysis demonstrate the successful development of the two double stable cell lines. A, represents RT-PCR results from the ten sense double stable cell clones (I to X) that survived 5 weeks of selection against hygromycin (see section 3.11 for RT-PCR procedures). Five micro liters of the PCR products of each sample were loaded on a 1% agarose gal. Lane1 wt is CG4-*t*TA cells, which were used as wild-type control. Lane 11, cDNA of Hoxa2 in clone X, was demonstrated to have the highest *Hoxa2* RNA transcription. B, represents the expression of Hoxa2 at the protein level in genetically modified cells. In CG4-SHoxa2 cells, withdrawing Dox led to increased level of Hoxa2 expression, whereas CG4-ASHoxa2 cells cultured in the absence of Dox showed reduced-Hoxa2 expression.

The antibiotic resistance test, RT-PCR, and western blot analysis confirmed the successful generation of the double stable cell lines.

4.6 Morphological characteristics of the genetically modified cells

CG4 cells are known to exhibit morphology typical of oligodendrocyte progenitors (OPCs) (Louis *et al*., 1992), which display a bipolar or tri-polar phenotype. After the two double stable cell lines were generated, microscopy was utilized to examine whether any morphological alterations had occurred in these genetically modified CG4 cell lines**.**

Following transfection of the second plasmid, cells were plated at low density (1 x $10^3/cm^2$) to perform the antibiotic screening. Within 3 weeks, cells that were not successfully transfected would not survive, and only those that consistently express hygromycin and neomycin resistant genes would show growth. Due to the low density of initial cell populations for drug screening, a single cell proliferates, expands and eventually grows into a small cell population without any inter-crossing with other cells and their descendants. By doing so, cells with a homogeneous genetic background could be sub-cultured. We noticed that cell clones containing either sense *Hoxa2* (Fig. 14A) or anti-sense *Hoxa2* (Fig. 14B) exhibited different morphological characteristics during drug selection. Cells carrying the sense *Hoxa2* containing vector exhibited a bipolar phenotype in GM, with long and unbranched processes, similar to that of the wild-type cells. In contrast, most of the antisense-*Hoxa2* expressing CG4 cells appeared to have round nuclei with few processes extending out, even though they were cultured under the same selection conditions as sense *Hoxa2* expressing cells.

As the cell clones developed into a size ready for transfer, individual cell clones were then passed onto a new culture surface in a 6-well dish to expand the total cell population. For CG4 wild-type cells, no obvious phenotype alteration was observed when Dox was incorporated in the medium. To further assess cell morphology changes in the newly engineered cell lines, cells were cultured at $3x10^4$ per $35mm^2$ in GM without the addition of any selection antibiotics or Dox. Both wild-type CG4 cells (Fig. 14C) and

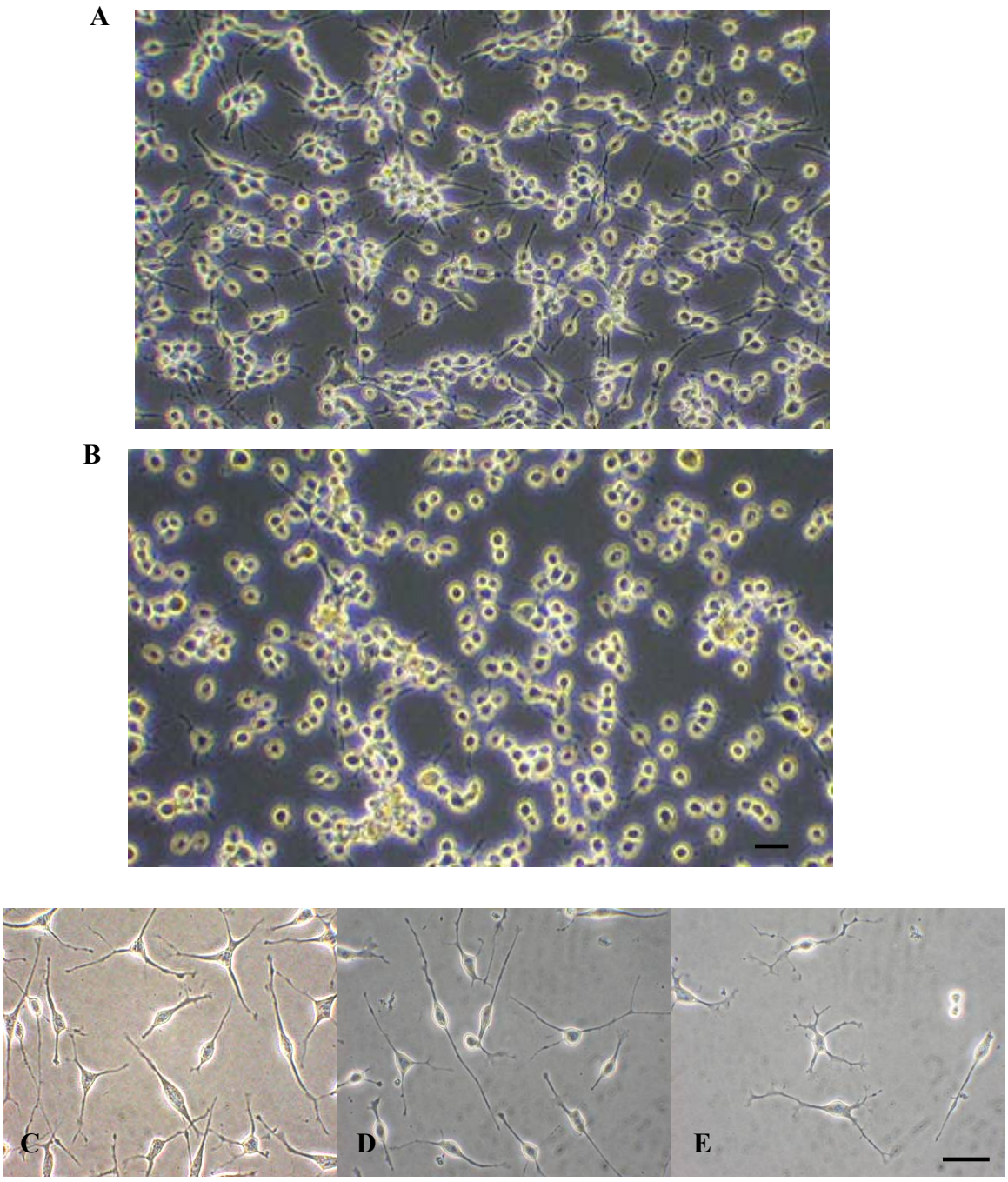

A
B
C
D
E

Figure 14. Phenotype of CG4-SHoxa2 (A, D), CG4 wild-type (C) and CG4-ASHoxa2 (B, E) cells. When performing antibiotic screening, individual cells that contained the hygromycin resistant gene survived and gave rise to a cell clone. Images A and B were taken three weeks after transfection during the hygromycin selection. After confirming the establishment of CG4-SHoxa2 and CG4-ASHoxa2 cell lines, CG4 wild-type (C), CG4-SHoxa2 (D) and CG4-ASHoxa2 (E) cells were cultured under the same condition in GM. Images C, D and E were taken after cells were plated and cultured in GM (No Dox, No hygromycin) for 1 day. Scale bar indicates 25 μm.

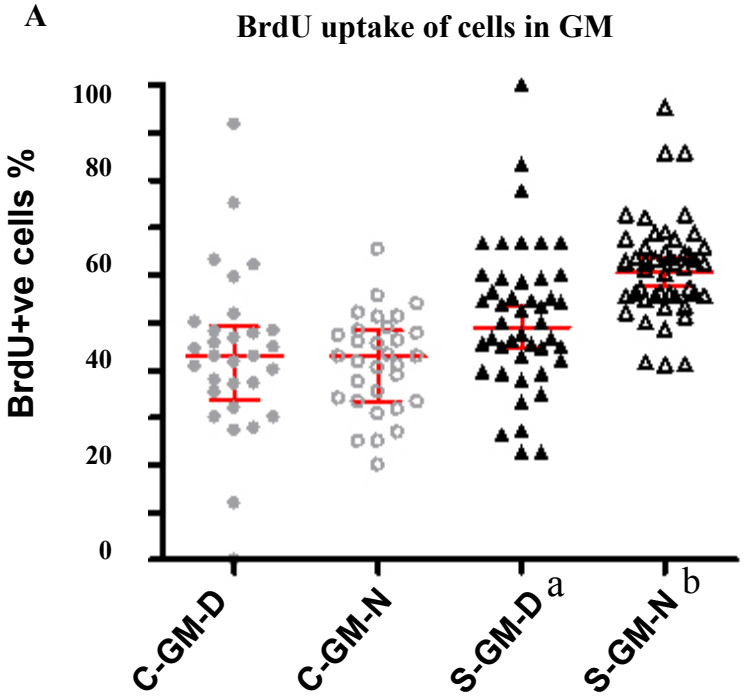
sense *Hoxa2* expressing cells (Fig. 14D) displayed a typical phenotype known for O-2A progenitor cells. In comparison, the cells that carried the anti-sense *Hoxa2* vector (Fig. 14E) exhibited multiple short and branched processes, which are distinguishable from the long and bipolar processes of the other two cell lines (Fig. 14C, D). These observed phenotype alterations are consistent with *in vivo* findings, where primary OPCs isolated from *Hoxa2* null mice exhibit multiple relatively shorter and branched processes (unpublished data from Dr.Nazarali's Lab).

4.7 Over-expression of *Hoxa2* promotes the proliferation of CG4-SHoxa2 cells

Because of limited time, I decided to focus on the impact of up-regulating *Hoxa2* expression on the proliferation and differentiation of CG4 cells, in contrast to the loss-of-function study (Nicolay *et al*., 2004b). BrdU uptake assay was employed to investigate whether over-expression of *Hoxa2* would affect the proliferation of genetically manipulated CG4 cells. Both CG4 and CG4-SHoxa2 cells were cultured either with or without Dox for 2 or 3 days in appropriate medium, and BrdU was added for the last 2 h of incubation. Both CG4 (Dox), CG4 (No Dox) and CG4-SHoxa2 (Dox) cells served as controls. Interestingly, CG4-SHoxa2 cells exhibited BrdU uptake at ~49% in the presence of Dox and ~59% in the absence of Dox (Fig. 15A). In comparison, the proliferation rate of wild-type CG4 cells was not significantly different between Dox and No Dox groups (Fig. 15A). Data were collected from 3 repeated studies with evaluation of at least 500 cells or 10 scoring fields per experiment. The percent of BrdU+ve cells in each field was quantified and subjected to ranking to obtain the median, which was then analyzed using nonparametric Kruskal-Wallis method followed by Dunn's multiple comparison test.

The proliferation rate for CG4-SHoxa2 cells in the absence of Dox was significantly higher compared to the three control groups ($p<0.001$), indicating that over-expression of *Hoxa2* promotes the proliferation rate of CG4 cells. Even with the presence of Dox, these genetically modified CG4-SHoxa2 cells exhibited greater BrdU uptake capability ($p<0.05$) compared with two wild-type CG4 cell groups. Although the presence of Dox did not appear to alter the level of Hoxa2 protein in CG4-SHoxa2 cells, whether the introduction of the 'Tet-off' system affects the molecular and cycling events of CG4 cell is yet to be determined. Another potential factor is the homogeneity of the

A


BrdU uptake of cells in GM
BrdU+ve cells %
100
80
60
40
20
0
C-GM-D
C-GM-N
S-GM-D a
S-GM-N b

B

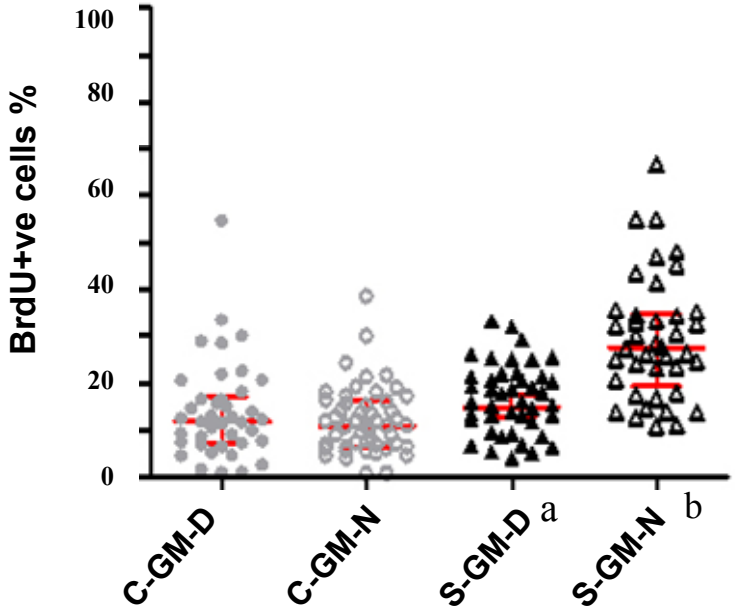

BrdU uptake of cells in 2DM
BrdU+ve cells %
100
80
60
40
20
0
C-GM-D
C-GM-N
S-GM-D a
S-GM-N b

Figure 15. Analysis of proliferation rate by BrdU incorporation assay. (A) For CG4 wild-type cells, when cultured in GM, no significant difference in proliferation rates was observed for groups with or without Dox. In contrast, CG4-SHoxa2 cells over-expressing *Hoxa2* (No Dox) displayed a significantly higher percentage of BrdU uptakes than the wild-type CG4 cells and CG4-SHoxa2 cells cultured in the presence of Dox. a, $p<0.05$, compared to C-GM-D and C-GM-N; b, $p<0.001$, compared to all other groups. (B) The cells were then induced to differentiate to examine whether the increased cell proliferation continued during maturation. As shown here, the proliferation rate for all four groups decreased. Regardless of this decline, CG4-Shoxa2 cells in the absence of Dox exhibit a substantially higher proliferation rate than cells subjected to Dox. a, $p<0.05$, compared to C-2DM-D and C-2DM-N; *b*, $p<0.001$, compared to all other cell groups; C-wild-type CG4 cells, S-sense *Hoxa2* carrying CG4 cells, GM-growth medium, 2DM-cells grown in differentiation medium for 2 days, D-exposed to Dox, N-not exposed to Dox. Experiments were repeated three times, and 10-12 fields were selected each time. Each data point represents one field from the total collected fields. The red bar in the middle indicates median of the data, and symbol ┬ stands for interquartile range.

CG4 cell line used in this project. Due to previous manipulation and sub-cultures, the pool of CG4 cells might already contain group of cells at more advanced developmental stage; therefore, these sub-group of cells may age faster and be less proliferative. In comparison, the CG4-SHoxa2 cells were recently derived from one single progenitor cell, thus, each daughter cell would possess the same genetic properties and be at the same developmental phase. This assay was repeated on all groups of cells cultured in DM for 2 days to determine whether the increased proliferation would continue when cells differentiated. After two days of incubation to induce cell differentiation, BrdU uptake was dramatically reduced for all cell groups, although the CG4-SHoxa2 No-Dox treated group retained the highest uptake of BrdU molecule (Fig. 15B).

4.8 Cells at early developmental stages are proliferative

To further investigate the identity of BrdU+ve cells in the proliferation study, cells were double labelled with BrdU/A2B5, BrdU/O4, or BrdU/GalC. As mentioned previously, no GFAP staining was observed here, although O-2A progenitor cells have been reported to co-express A2B5 and GFAP antigens in some cases (Wang *et al*., 1994). Thus, further GFAP staining was not carried out for this aspect of the study. Initially, each of the four cell groups (CG4-Dox, CG4-No Dox, CG4-SHoxa2-Dox, CG4-SHoxa2-No Dox) was cultured in GM in two wells of a 6-well dish, with two coverslips per well for staining. The two coverslips in the first well were subjected to BrdU/A2B5 and BrdU/O4 staining, respectively, whereas the second set of two coverslips were stained with BrdU/GalC. GalC staining was not observed in any of the four cell groups when they were cultured in GM (data not shown). For CG4 cells, a majority of the BrdU+ve cells were also positive for A2B5 labelling, and only a small number of CG4 cells were positive for the O4 antibody. Both A2B5 and O4 positive cells were seen to co-localize with BrdU staining (O4 data not shown). In comparison, virtually all the CG4-SHoxa2 cells (either treated with or without Dox) were A2B5+, with only very few scattered O4+ve cells detected. Additionally, all the BrdU+ve cells were co-localized with A2B5 in the CG4-SHoxa2 group cells (Dox, No-Dox) (Fig. 16C, G).

Cells (CG4, CG4-SHoxa2) were allowed to differentiate in DM for two days before being subjected to double immunolabelling with anti-BrdU antibody and OL cell lineage maturation markers (A2B5, O4 or GalC). Upon examination, the percent of

CG4-SHoxa2 Cells

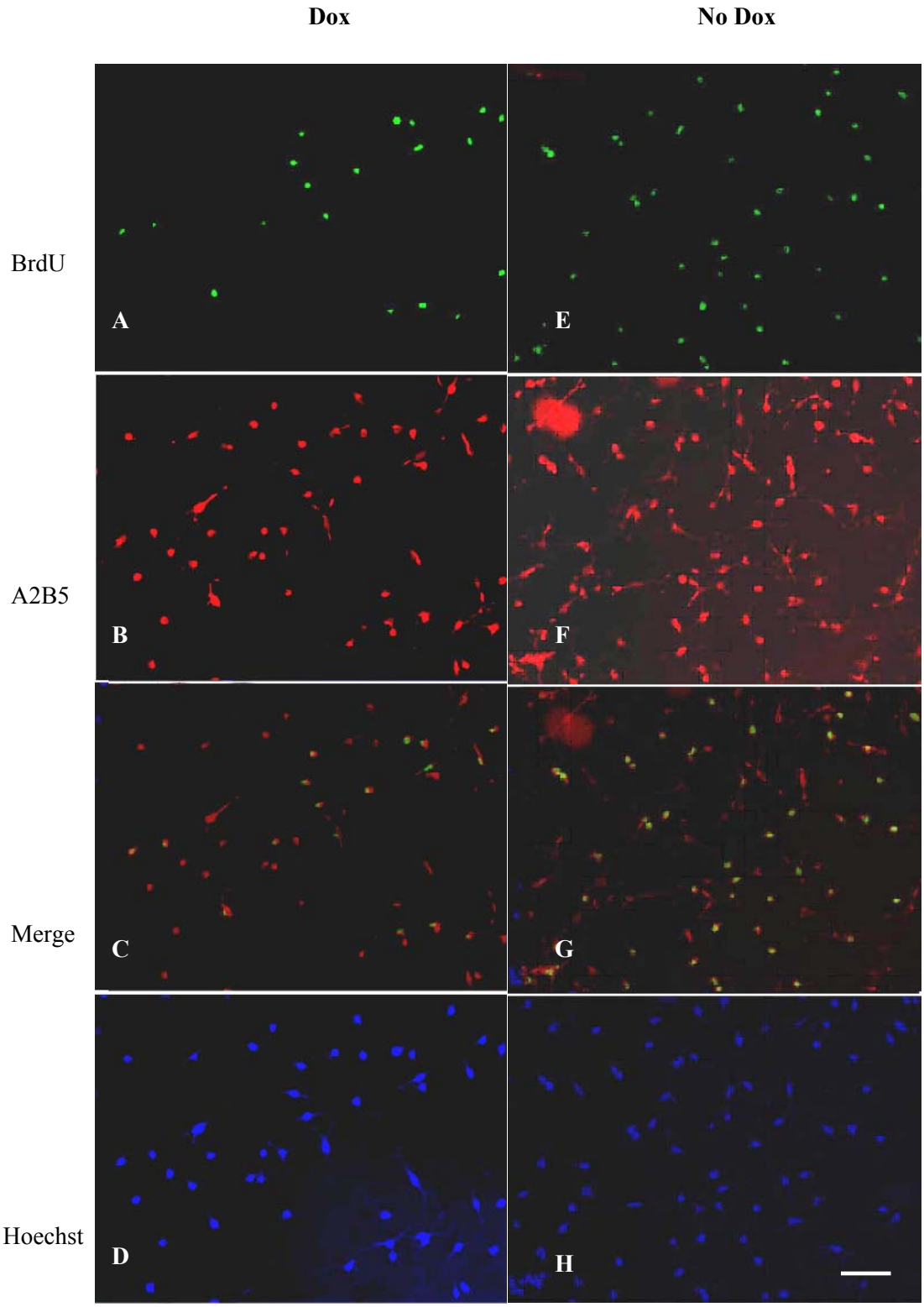


Figure 16. Double labelling of BrdU (A, E) with A2B5 (B, F) antibody for CG4-Shoxa2 cells (Dox and No Dox treated). Cells were cultured in GM overnight, and then subjected to BrdU incubation. The cells were fixed first, followed by A2B5 staining. BrdU immunocytochemistry was performed afterwards. Co-localization of BrdU positive cells and A2B5 expressing cells was demonstrated by merging the A and B or E and F images, respectively, indicated by yellow (C, G). D and H was the Hoechst staining of the same field. Scale bar indicates 100μm.

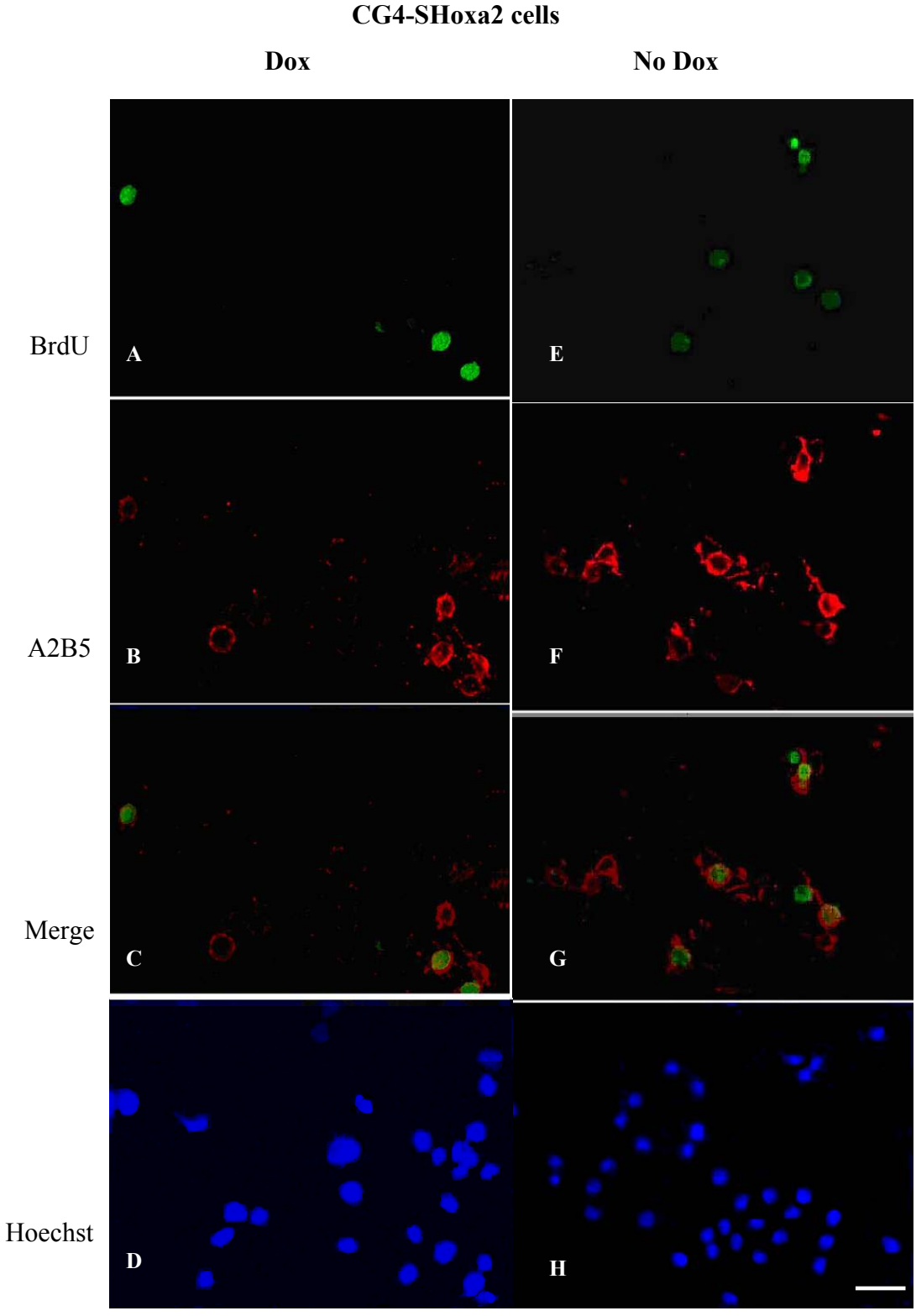

CG4-SHoxa2 cells
Dox
No Dox
BrdU
A2B5
Merge
Hoechst
A
B
C
D
E
F
G
H

Figure 17. Double labelling of BrdU (A, E) with A2B5 (B, F) in 2DM cultures. After two days incubation in DM, one coverslip was subjected to double labelling with anti-BrdU and anti-O4, and the other one was stained with anti-BrdU and anti-A2B5 (or anti-GalC). The number of BrdU labelled cells (A, E) decreased significantly compared to the cell groups cultured in GM (Fig. 16A, E), either in the presence or absence of Dox. In addition, a large portion oF the cells may stop expressing antigen A2B5, as immunocytochemical staining revealed substantially fewer A2B5+ve cells (B, F). Scale bar indicates 25 μm.

CG4-SHoxa2 cells

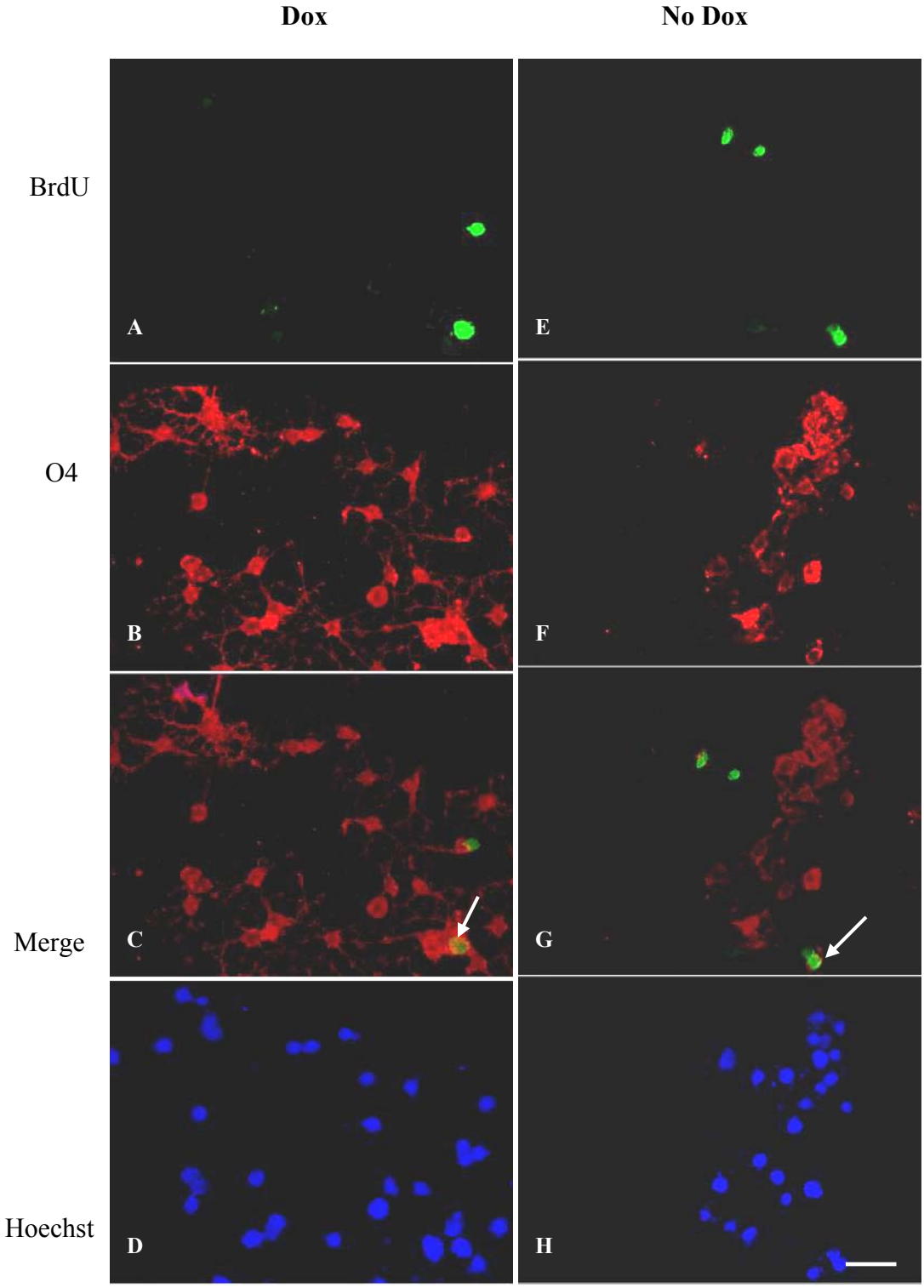


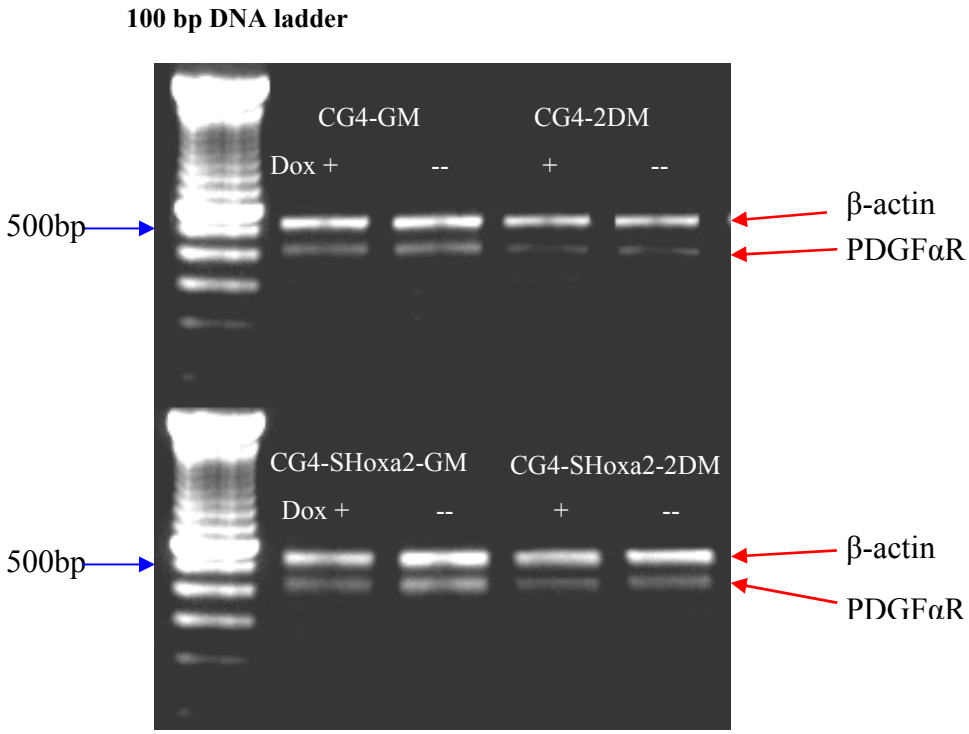
Figure 18. Double labelling of CG4-SHoxa2 cells in 2DM cultures with anti-BrdU (A, E) and anti-O4 (B, F) antibodies. Immunostaining of OL-cell lineage maturation markers revealed that a significant portion of cells may stop producing A2B5 antigen (Fig. 17B, F) but start to express O4 (B, F). Scale bar indicates 25 μm.

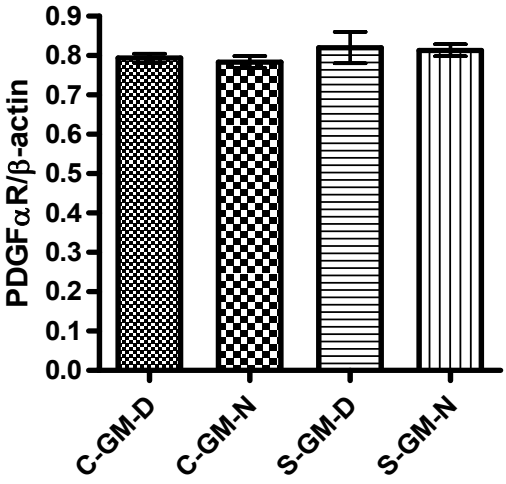
A2B5+ve cells was reduced significantly in all four cell groups, [compare Figure 16 with Figure 17 (data only represent CG4-Shoxa2 cells)]. Regardless of the Dox condition, no GalC staining was detected in either of the two CG4-SHoxa2 groups. In contrast, a few patches of GalC+ve cells were observed in the two wild-type CG4 cell groups, but no BrdU+ve staining was seen colocalized with GalC+ve cells. In agreement with previous findings (Fig. 15B), much fewer BrdU+ve cells were observed after 2 days incubation in DM. Again, a majority of the BrdU positive staining was found colocalized with A2B5+ve cells (Fig. 17C, G), whereas only a small percentage of O4+ cells incorporated the BrdU (Fig. 18C, G), supporting previous findings reported by Tang *et al*. (2000).

4.9 Over-expression of *Hoxa2* does not impact the mRNA level of PDGFαR

Wild-type CG4 cells, in the presence or absence of Dox, demonstrated a comparable percentage of BrdU+ve cells, confirming that Dox, by itself, does not impact cell proliferation (Fig. 15). Therefore, an up-regulation of *Hoxa2* expression in the no Dox-treated CG4-Shoxa2 cells likely contributes to the enhanced proliferation rate. Since PDGF is a known mitogen for OPCs, and has been shown to increase cell proliferation (Engel and Wolswijk, 1996; Baron *et al*., 2002), we then examined whether the expression of PDGFαR in cells is altered when the expression of *Hoxa2* gene is up-regulated. For this purpose, I used RT-PCR to determine the mRNA level of PDGFαR in cells at two distinct developmental stages, using β-actin as an internal standard.

For RNA isolation and preparation, parallel experiments to the BrdU incorporation assay were conducted. Cells were subjected to Dox or No Dox, either in GM or DM. RT-PCR products were visualized on a 1.5% agarose gel, and the expression levels of PDGFαR and beta-actin were quantified with the AlphaImager™ Gel Imaging Software. PDGFαR mRNA signals were normalized to β-actin and the ratios were calculated from three repeated RT-PCR experiments. As shown in Fig. 19, the PDGFαR receptor gene is expressed at high levels in early progenitors, although no significant differences were observed in the Mrna level of PDGFαR among the four examined cell groups. After 2 days in DM, the mRNA of PDGFαR expression in more advanced cells were slightly reduced (Fig. 19), in agreement with previous findings (Ellison and de Vellis, 1994; Garcion *et al*., 2001). However, the RT-PCR analysis did not reveal any statistically significant differences in the mRNA of PDGFαR among four groups of cells.


100 bp DNA ladder
CG4-GM
CG4-2DM
Dox +
--
+
--
500bp
β-actin
PDGFαR
CG4-SHoxa2-GM
CG4-SHoxa2-2DM
Dox +
--
+
--
500bp
β-actin
PDGFαR


PDGFαR/β-actin
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
C-GM-D
C-GM-N
S-GM-D
S-GM-N

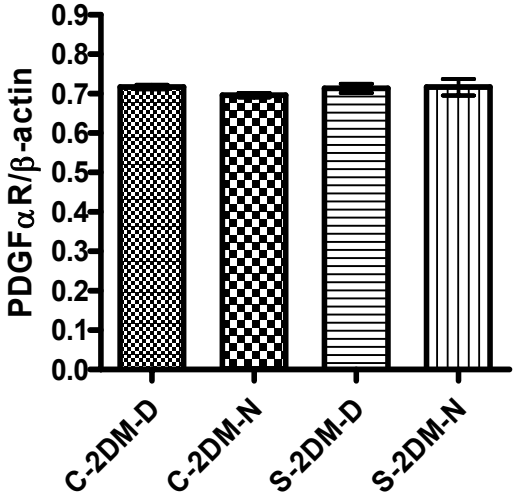

PDGFαR/β-actin
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
C-2DM-D
C-2DM-N
S-2DM-D
S-2DM-N

Figure 19. RT-PCR analysis of PDGFαR mRNA expression in CG4 and CG4-SHoxa2 cells. For RNA preparation, all cell groups followed the same protocol as used in the proliferation assay. C-GM-D, CG4 cells were cultured in GM treated with Dox; C-GM-N, CG4 cells were cultured in GM without Dox; 2DM, cells were grown in DM for 2 days. Studies were based on three independent batches of RNA preparations. AlphaImager™ Gel Imaging Software was utilized to obtain the intensity of each band on 1.5% agarose gel. The ratio between PDGFαR and β-actin was then calculated and subjected to one-way ANOVA with Tukey's multiple comparison post hoc tests in GraphPad Prism. Histogram bars represent the mean value from three repeated experiments with standard diviation error.

4.10 Effect of enhanced *Hoxa2* level on the differentiation of CG4-SHoxa2 cells

4.10.1 Immunocytochemistry study and quantitative analysis

My next aim was to examine if up-regulated *Hoxa2* would impact on the developmental course of CG4 cells. Although immunohistochemical studies of the OL lineage in the developing spinal cord suggest OPCs progress normally in the absence of *Hoxa2* (Nicolay *et al*., 2004b), a quantitative analysis has not been carried out before. Thus, I decided to monitor the differentiation of CG4 and CG4-SHoxa2 cells by precisely determining the percentage of specific subpopulations - A2B5-expressing cell subgroup, O4-expressing cell subgroup and GalC-expressing cell subgroup - at distinct developmental stages in differentiation cultures.

To stimulate cell differentiation, cells were seeded at $4x10^5$ cells per well and incubated in DM after an initial 16-h culture in GM (see Materials and Methods 3.1). For cells in wells 1, 2 and 3 (Fig. 7), the concentration of Dox was sustained at 2 μg/mL, but the cells in wells 4, 5 and 6 were kept Dox free after seeding. By removing Dox, CG4-SHoxa2 cells cultured in the wells 4, 5 and 6 were allowed to over express *Hoxa2* during the differentiation study. Regardless of the presence or absence of Dox, both groups of CG4-SHoxa2 cells underwent differentiation toward mature OL-like morphology, and began to express late stage OL-lineage markers O4 and GalC (Fig. 20), similar to that observed in wild-type CG4 cells (Fig. 8 and Fig. 9). Thus, I came to the conclusion that over-expression of *Hoxa2* does not block the OL-like maturation of CG4-SHoxa2 cells in DM.

To quantify A2B5+ve, O4+ve and GalC+ve cell populations in each cell group as the cells differentiated, ten to twelve fields of each coverslip were selected to best represent the staining results. In each field, the percentage of positive cells (e.g. A2B5+ve) was calculated based on Hoechst counterstain. The data collected from three repeated experiments were subjected to Kruskal-Willis ranking test followed by Dunn's multiple comparison tests.

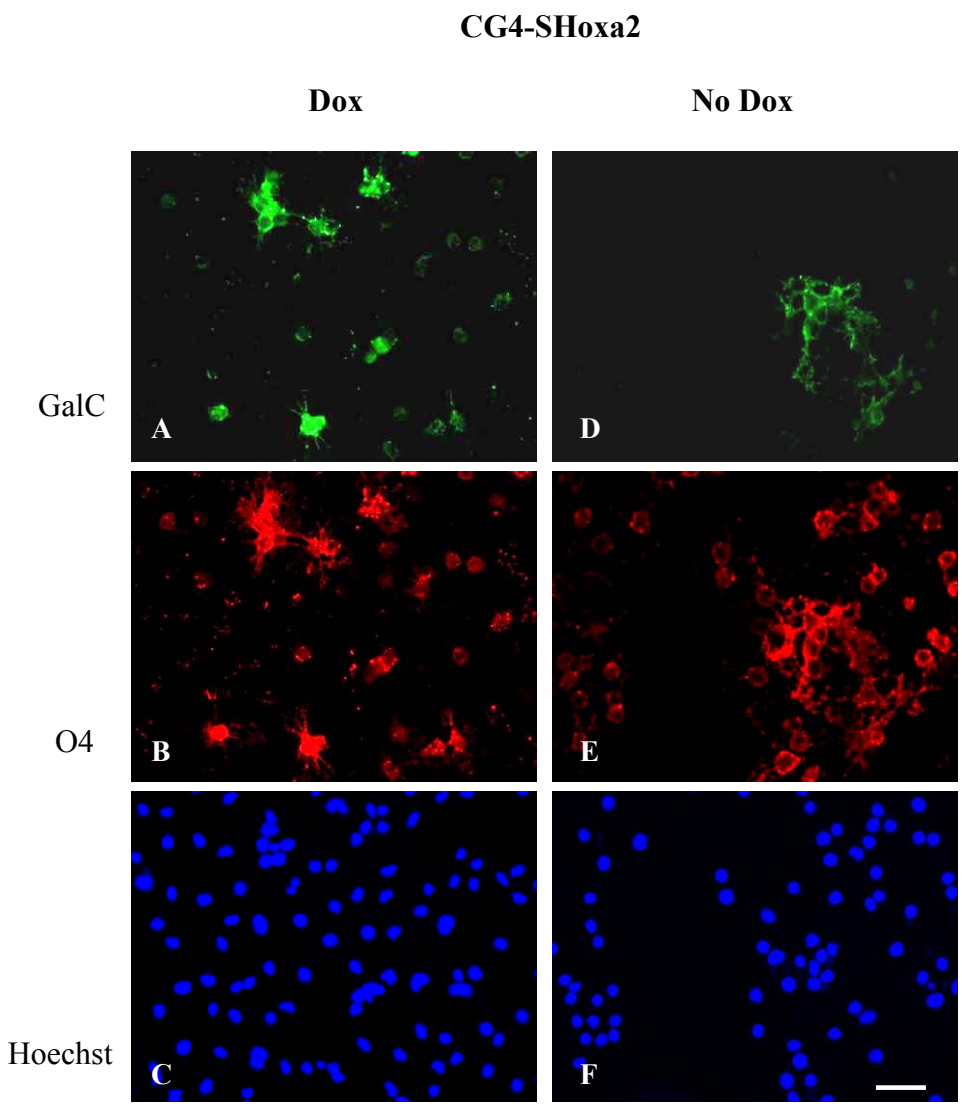
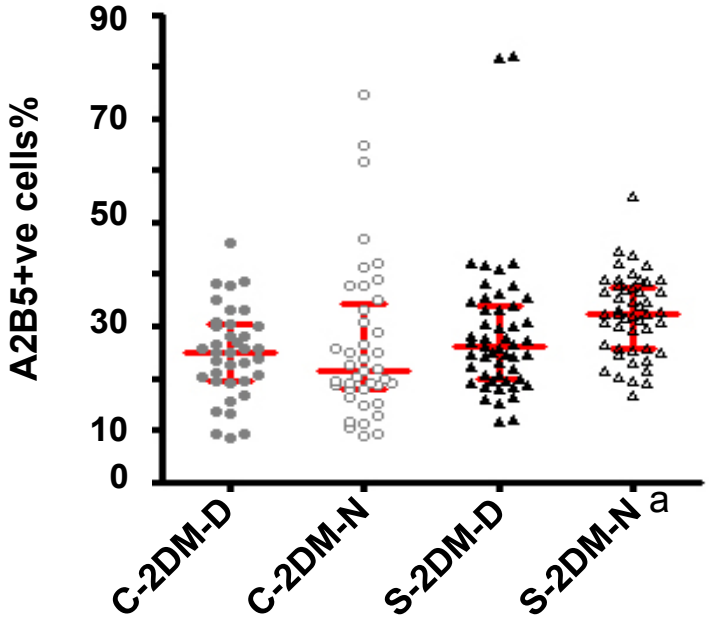

CG4-SHoxa2
Dox
No Dox
GalC
O4
Hoechst
A
B
C
D
E
F

Figure 20. Differentiation of CG4-SHoxa2 cells occurred in the presence (A, B, C) and absence of Dox (D, E, F). Similar to wild-type CG4 cells (Fig. 9), genetically modified cells were induced to differentiate by switching the GM to DM. After three days in DM, GalC+ve (A, D) and O4+ve cells (B, E) were observed in both cell groups, supplemented with or without Dox. C and F are the Hoechst nuclear staining of the same field. Scale bar indicates 25 μm.

In the 2DM cultures, very few GalC+ve cells were observed in CG4-SHoxa2 cell groups, either in the presence or absence of Dox (data not shown). In contrast, several patches of GalC+ve staining were seen in CG4 cell groups. Therefore, I only assessed the proportion of A2B5+ve and O4+ve cells in these four cell groups. Similar to what had been demonstrated in the proliferation assays, the CG4-Dox group and CG4-No Dox group displayed a comparable percentage of cells expressing OL cell markers, A2B5 and O4. For CG4-SHoxa2 cells, the *Hoxa2* up-regulated cell group (No Dox) showed significantly higher percentage of A2B5+ve cells (>30%, $p<0.05$) than those in the wild-type CG4 cells and in the Dox-treated CG4-SHoxa2 group (Fig. 21A), whereas the median of A2B5+ve% in Dox treated CG4-SHoxa2 cells was not statistically different from those in the wild-type CG4 groups. As for the percentages of O4+ve cell population, no statistically significant differences were observed among the four cell groups.

After one additional day in DM (3DM), CG4-SHoxa2 cells, in the absence of Dox, exhibited similar amounts of A2B5+ve%, O4+ve% and GalC+ve% cells as the Dox-treated CG4-SHoxa2 cell group (Fig. 22). The two CG4-SHoxa2 cell groups were now noticeably at a less advanced stage, since they displayed a significantly higher percentage of A2B5+ve cells (Fig. 22A) and much lower percentage of GalC+ve cells (Fig. 22C) when compared to wild-type CG4 cells. However, no statistically significant differences in the percentage of O4+ve cells were found among these four cell groups (Fig. 22B).

A


A2B5+ve cells%
90
70
50
30
10
0
C-2DM-D
C-2DM-N
S-2DM-D
S-2DM-N
a

B

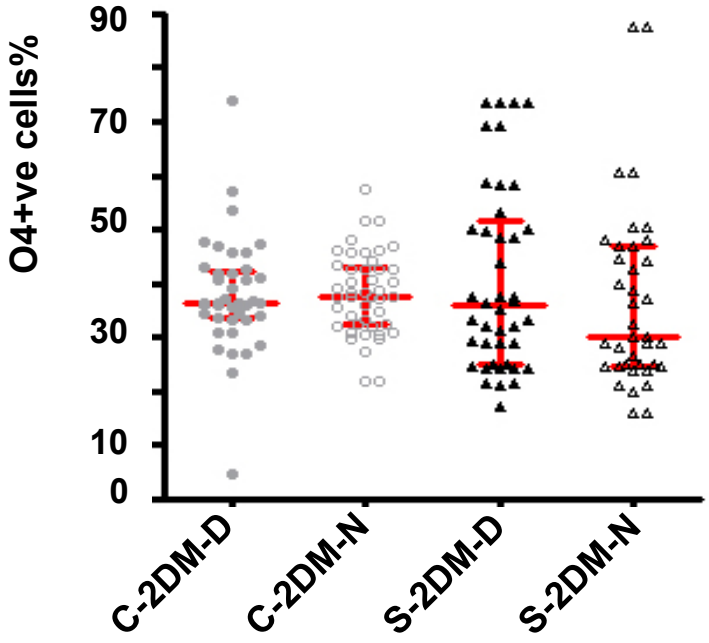
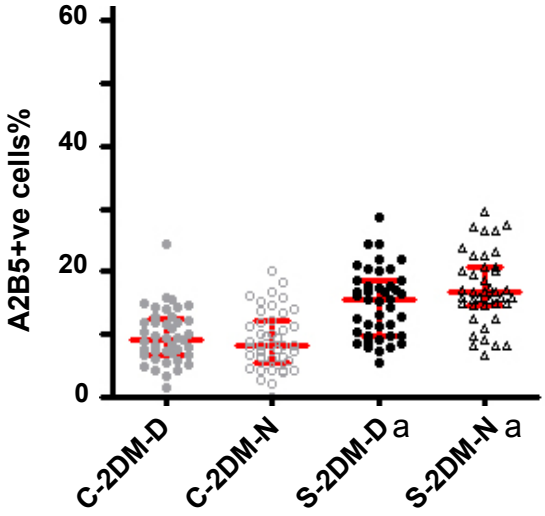
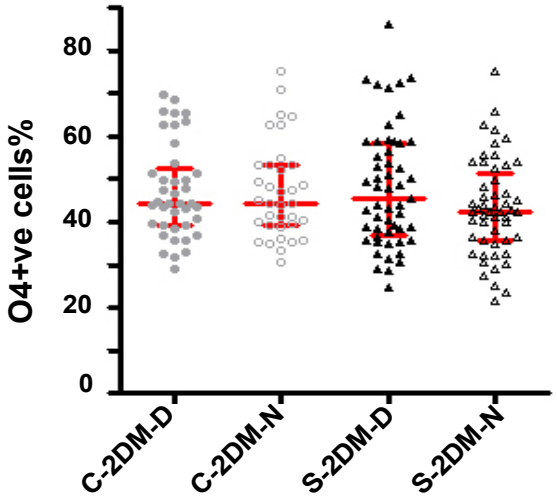

O4+ve cells%
90
70
50
30
10
0
C-2DM-D
C-2DM-N
S-2DM-D
S-2DM-N

Figure 21. Characterization of the portion of A2B5+ve and O4+ve subpopulations in each of the four cell groups at 2 DM. Twelve fields were selected for each coverslip to represent total cell population on the coverslip and total cell number for each field was established by counting Hoechst-stained nuclei. In each field, cells that were A2B5+ or O4+ were summarized in percentage and results were analyzed for statistical significance by using non-parametric Kruskal-Willis ranking followed by Dunn's multiple comparison tests with the aid of GraphPad Prism Software. C, CG4 cells; S, CG4-SHoxa2 cells; 2DM, indicates 2 days of culture in DM. D, cells were exposed to Dox; N, cells were cultured in the absence of Dox. a, $p<0.05$ when compared to all other groups of cells. Experiments were repeated three times, and 10-12 fields were selected each time. Each data point represents for one field from the total collected fields. The red line in the middle indicates median of the data, and symbol ┬ stands for interquartile range.

A


A2B5+ve cells%
60
40
20
0
C-2DM-D
C-2DM-N
S-2DM-D a
S-2DM-N a

B


O4+ve cells%
80
60
40
20
0
C-2DM-D
C-2DM-N
S-2DM-D
S-2DM-N

C

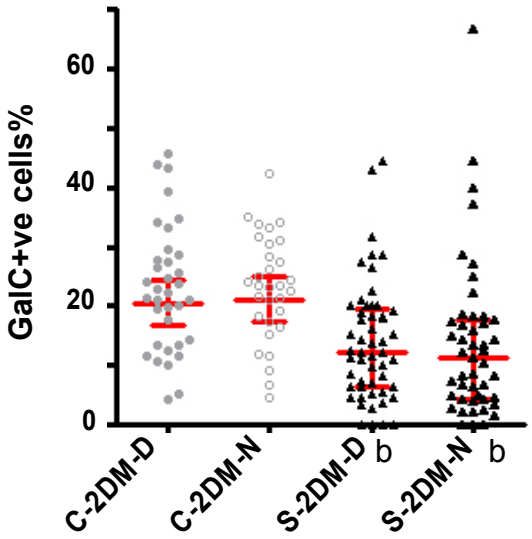

GalC+ve cells%
60
40
20
0
C-2DM-D
C-2DM-N
S-2DM-D b
S-2DM-N b

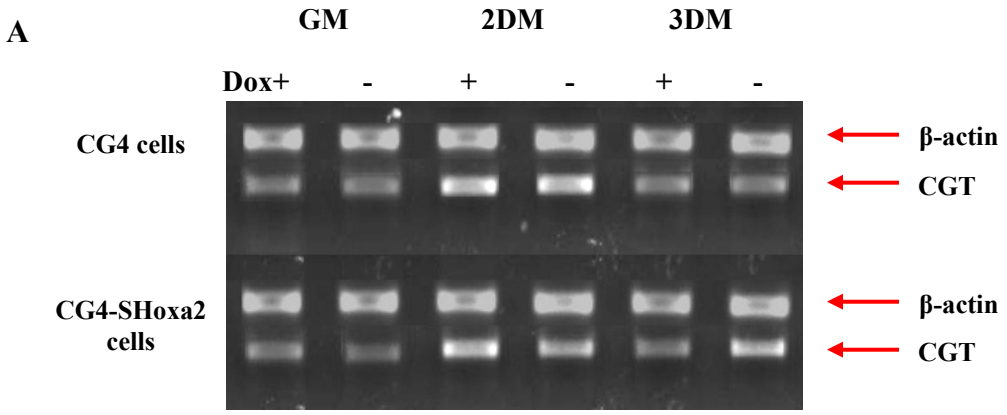
Figure 22. The proportion of each OL-lineage marker labelled subpopulation in four cell groups at 3DM. The data of each cell group was compared using Kruskal-Wallis ranking, followed by Dunn's multiple comparison tests. CG4 cells either treated with or without Dox displayed nearly identical percentages for each marker labelled subpopulation. At this time point, the proportion of A2B5, O4 and GalC labelled subgroup cells in No Dox-treated CG4-SHoxa2 did not significantly different from those of the Dox-treated CG4-SHoxa2 group. However, when compared with wild-type CG4 groups, both two CG4-SHoxa2 cell groups showed statistically higher percentage of A2B5+ve cells and lower percentage of GalC+ve cells. C or S, indicates CG4 or CG4-SHoxa2 cell lines. 3DM represents 3 days in DM culture. D, Dox-treated; N, No Dox-treated. Superscripts a, b indicate statistical significance when compared with C-3DM-D and C-3DM-N groups, $p<0.05$. Experiments were repeated three times, and 10-12 fields were selected each time. Each data point represents for one field from the total collected fields.

4.10.2 Semi-quantitative RT-PCR

Immunocytochemistry reveals the presence of gene products at the protein level. Conversely, it can also produce false results if many parameters are not adequately controlled for and tested (Gordon and Rekhter, 1993). In addition, background noise may interfere with cell counting and quantifying obtained data may be difficult. In comparison, recent techniques highlight the advantages of RT-PCR over immunocytochemistry with respect to quantification and sensitivity. Therefore, semi-quantitative RT-PCR analysis was used as an alternative technique for further investigating the impact of up-regulated *Hoxa2* on the differentiation of CG4-SHoxa2 cells, by examining the expression of two OL-lineage late stage markers, ceramide galactosyltransferase (CGT) (Fig. 23) and myelin basic protein (MBP) (Fig. 24). To validate the performance of RT-PCR in each reaction, β-actin was utilized as an internal control.

CGT is a key enzyme that catalyzes the final step in the biosynthesis of GalC and is frequently used as late stage marker for OLs (Gokhan *et al*., 2005). CGT has been reported to be first expressed around the time that myelination begins (Schulte and Stoffel, 1993). Although Tokumoto *et al*. (1999) have reported the presence of CGT mRNA and protein in OPCs isolated from the optic nerve of newborn rats, adding to the confusion, Gokhan and coworkers (2005) have suggested that the transcription of CGT begins only after the O4 marker is expressed in OL-lineage cells derived from dorsal forebrain of embryonic mice. In this study, we characterized the expression profile of the mRNA of CGT in a dynamic manner (Fig. 23A). CGT mRNA were readily detected at the early progenitor stage when the four groups of cells were maintained in GM, but the CGT Mrna level in *Hoxa2* over-expression cell group was significantly lower ($p<0.05$, Fig. 23B). A considerable increase in the mRNA of CGT was observed in wild-type CG4 cells (Dox- and No Dox-treated groups) after two days in DM, but the mRNA levels detected at 3DM subsequently declined to the level observed in GM (Fig. 23). Similarly, CG4-SHoxa2 cells in the presence of Dox reacted in the same manner as wild-type CG4 groups, exhibiting comparable CGT mRNA level and change during the differentiation study. In contrast to the other three cell groups, CG4-SHoxa2 cells in the absence of Dox,

A


GM
2DM
3DM
Dox+
-
+
-
+
-
CG4 cells
CG4-SHoxa2 cells
β-actin
CGT
β-actin
CGT

B

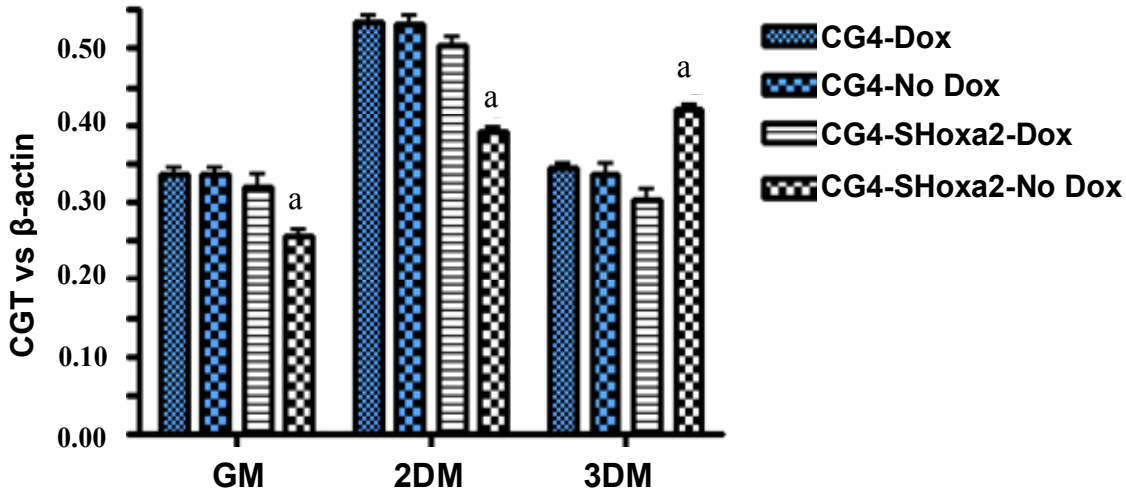

CGT vs β-actin
0.50
0.40
0.30
0.20
0.10
0.00
a
a
a
GM
2DM
3DM
CG4-Dox
CG4-No Dox
CG4-SHoxa2-Dox
CG4-SHoxa2-No Dox

Figure 23. Expression profile of CGT mRNA in CG4 and CG4-SHoxa2 cells. Mean value of CGT-mRNA/β-actin-mRNA ratio with an error bar of standard deviation (n=3) is presented. A is one of the three RT-PCR results evident on a 1.5% agarose gel. B. The density ratios between CGT and β-actin in the four cell groups at each time point were subjected to one-way ANOVA followed by Tukey's multiple comparison post tests. +, Dox-treated; -, No Dox-treated. a, statistical significance when compared with C-3DM-D, C-3DM-N and CG4-SHoxa2-Dox cell groups, $p<0.05$.

where exogenous *Hoxa2* expression is allowed, displayed statistically less CGT mRNA both in GM and at day 2 in DM. In addition, the presence of CGT mRNA in the *Hoxa2* up-regulated cell group continued to increase and reached the highest level in DM at day 3.

Although MBP is recognized as a late stage marker for OL and the presence of the MBP protein, in general, announces terminal maturation, several lines of evidence demonstrated that the mRNA of MBP is present in OPCs (Ye *et al*., 2003; Gokhan *et al*., 2005). Our data lend support to previous findings, where the mRNA of MBP was observed in both CG4 cells and CG4-SHoxa2 cells when cultured in GM (Fig. 24A). In addition, it appears that the mRNA level of MBP in CG4 cell groups (Dox-treated, No Dox-treated) increased steadily as cell differentiation advanced, similar to the pattern of MBP mRNA detected in lineage-restricted neuronal-OL cells (Gokhan *et al*., 2005). In the CG4-SHoxa2 cell line, a gradual increase of MBP mRNA level was observed as cell differentiates, either in the presence or absence of Dox. Notably, *Hoxa2* up-regulated CG4-SHoxa2 cells displayed significantly lower MBP transcripts than those shown in the other three groups ($p<0.001$), and they in the presence of Dox still contained less MBP mRNA than the two wild-type CG4 cell groups ($p<0.05$).

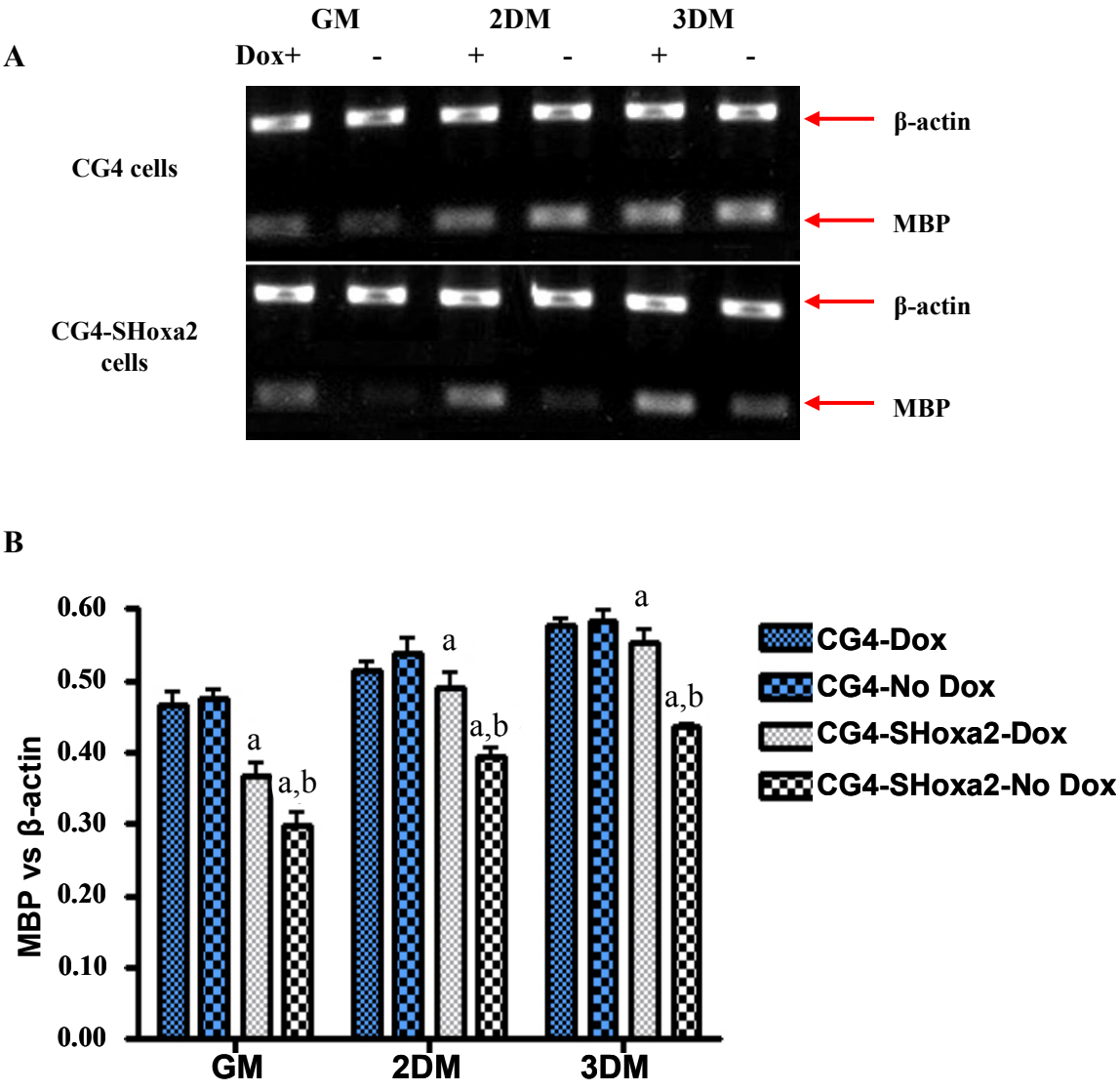

A
GM
2DM
3DM
Dox+
-
+
-
+
-
CG4 cells
β-actin
MBP
CG4-SHoxa2 cells
β-actin
MBP
B
MBP vs β-actin
0.60
0.50
0.40
0.30
0.20
0.10
0.00
a
a,b
a
a,b
a
a,b
GM
2DM
3DM
CG4-Dox
CG4-No Dox
CG4-SHoxa2-Dox
CG4-SHoxa2-No Dox

Figure 24. Expression profile of MBP mRNA in CG4 and CG4-SHoxa2 cells. Mean value of CGT-mRNA/β-actin-mRNA ratio with an error bar of standard deviation (n=3) is presented. A is one of the three RT-PCR results evident on a 1.5% agarose gel. B. The density ratios between MBP and β-actin in the four cell groups at each time point were subjected to one-way ANOVA followed by Tukey's multiple comparison post tests. +, Dox treated; -, No Dox treated. a, significance of $p \leq 0.05$ compared with CG4-Dox and CG4-No Dox groups; b, significance of $p \leq 0.001$ compared to all other groups.

V DISCUSSION

One of the most common control mechanisms underlying the development of OLs involves the nuclear DNA-binding TFs, which regulate the transcription of downstream genes at the level of initiation. Therefore, identifying key TFs and illustrating their role during oligodendrogenesis will greatly advance our knowledge in understanding cell fate specification and the programming of OL differentiation. Clustered *Hox* genes are TFs known to play a central role in current models of both the establishment of body axes, as well as cell fate determination in multiple organs (reviewed in Keynes and Krumlauf, 1994; Akin and Nazarali, 2005). In recent studies, our laboratory reported that *Hoxa2*, *Hoxb4*, and *Hoxd1* genes, members of the *Hox* transcription family, are expressed in OLs (Hao *et al*., 1999; Nicolay *et al*., 2004a, b; Booth *et al*., 2007). Arenkiel and co-workers (2003, 2004) revealed that majority of r4 originated *Hoxb1*-expressing neural crest cells give rise to myelinating cells in the peripheral nervous system. Together, the above findings raise the following question: do *Hox* genes, or particularly *Hoxa2*, play a role during oligodendrocyte development? And if so, what is the nature of this role? In this context, the aim of this research project was to investigate the role of *Hoxa2* gene in regulating the development of the OL-lineage cells.

In the course of this work, I established the Tetracycline regulated inducible expression systems in CG4 cells, where Hoxa2 protein can be elevated (CG4-SHoxa2) or reduced (CG4-ASHoxa2). We showed that CG4-SHoxa2 cells, either in the presence or absence of Dox, displayed a typical morphology of O-2A progenitor cells, as the CG4 cell line does. The CG4-ASHoxa2 cells, on the contrary, exhibited somewhat altered morphology under the same culture condition as CG4 and CG4-SHoxa2 cells, showing multiple shorter processes instead of long and smooth processes. To complement the loss-of-function study carried out by Nicolay *et al*. (2004b), I focused on analyzing the impact of up-regulated Hoxa2 level on the proliferation and differentiation of CG4-SHoxa2 cells. The BrdU uptake data suggests a positive correlation between up-regulated *Hoxa2* expression and increased cell proliferation. Further, we demonstrated that overexpression of *Hoxa2* does not block the differentiation of CG4-SHoxa2 cells, but the mRNA expression pattern of CGT and MBP, two OLs late stages markers, indicated that the transcriptions of CGT and MBP were delayed in No Dox-treated CG4-SHoxa2 cells.

5.1 Evaluation of the Tet system

Exogenous and switchable control of the synthesis of target genes has become a popular requirement for both studies of gene function and therapeutic purposes. One particular attempt reported the development of a two vector-based tetracycline (Tet, or its analogue Dox) regulated system for on-off switching of a transgene (Harding *et al*., 1998), which can be utilized in a tissue/cell specific, dose dependent manner. Recently, the 'tet-off' has been successfully applied in a variety of models (Miyazaki *et al*., 2005) including the CG4 cell line (Ralph *et al*., 2000; Magy *et al*., 2003). The key step in establishing the 'tet-off' system was selection of two sequential stable cell clones which ensured long-term control of gene expression since the transgene was integrated into the chromosomes of the host.

The first vector, pTet-off, contains a neomycin-resistance (G418) cassette for selection in eukaryotic cells; the second vector encoding the target insert carries a hygromycin resistance marker. Individual kill curves for antibiotics Dox, G418 and hygromycin, were established for the CG4 cells and a suitable concentration was determined before application to the new cell line. For Dox, a final concentration 2 μg/mL was chosen based on cytotoxicity evaluation (Materials and Methods 3.3). In addition, the western results (Fig. 13) obtained for both CG4-SHoxa2 cells and CG4-ASHoxa2 cells confirmed the proper dosage of Dox, thus avoiding any significant leaking of expression or cytotoxic effects on cells.

To further eliminate any impact from Dox and to exclude any positional effects from gene integration into the host genome, I divided each cell line into two groups, one cultured with Dox and the other without, as shown in Fig. 8. Therefore, in addition to wild-type CG4 cells, Dox-treated CG4-SHoxa2 cell group also serve as a control. In all the proliferation and differentiation assays, wild-type CG4 cells treated with Dox or without Dox behaved in the same manner, as expected. In theory, CG4-SHoxa2 cells should behave similarly to wild-type CG4 cells when grown in the presence of Dox, since the level of Hoxa2 remains the same as that in wild-type CG4 cells (Fig. 13). Interestingly, our data shows that Dox-treated CG4-SHoxa2 cells displayed statistically significant differences from the wild-type CG4 cells, in the BrdU uptake study and the differentiation investigations. The observed differences might be due to position effects

from the integration site. Alternatively, it may be because the engineered cell line contains a much purer population with highly proliferative progenitor cells after cloning selections than the wild-type CG4 cell line.

During the selection of double stable cell clones, both G418 (100μg/mL) and hygromycin (200μg/mL) were present in the culture. In contrast, neither of these drugs was added for the BrdU assay or differentiation assays, to eliminate any cytotoxic effects on CG4-SHoxa2 cells. Due to a short half-life and light sensitivity of doxycycline, all Dox-treated cell groups were fed every other day with fresh medium containing newly added Dox to ensure proper inhibition of *Hoxa2* transcription.

5.2 Homogeneity and stability of selected cell line

Stable cell lines offer a number of desirable attributes over a transiently transfected test system, including an inherent degree of homogeneity, which is particularly important for cell based function assays. In the protocol here, a low cell density was combined with suitable selection drugs; thus, individual cell clones which appeared after 10~15 days selection were derived from virtually the same single cells. In comparison, the wild-type CG4 cells used in this study included a small portion of more mature-looking cells, which possibly resulted from frequent manipulations and exhausted passages. Additionally, during the construction of CG4-SHoxa2 and CG4-ASHoxa2 cell lines, Dox was not added to the medium until two days after the vector was introduced into the cells, which might have an impact on the cycling and differentiation of the cells. Consequently, one of the limitations of my experimental work is the potentially inherent stage-related difference among wild-type CG4, CG4-SHoxa2 and CG4-ASHoxa2 cell lines. Nevertheless, it is of interest to compare the results regarding cell morphology and viability from the CG4-ASHoxa2 cell line with that observed in CG4-SHoxa2 cell line. These two cell lines went through similar genetic manipulations differing only with different insert integrated into the host genome. The CG4-SHoxa2 cells maintained progenitor-like morphology and actively proliferated in the GM, whereas CG4-ASHoxa2 cells were comparatively slow in cell proliferation, poor in attachment, and featured a more mature cell phenotype. Given the above data, I conclude that *Hoxa2* participates in regulating the maturation of CG4 cells.

Stability is another issue in stable cell line construction. The cells endured

selective pressure from both G418 and hygromycin after the second transfection until they proliferated from one single cell to sufficient amounts, which generally took approximately 4 weeks. The western blot analysis was performed after 6 weeks selection (Fig. 13). In principle, a stable cell line should be able to express a transgene at a constant level indefinitely. However, stable expression often can be "silenced" or cease after the cells has been cultured for some time, because cells can recognize and silence multiple copies of genes, presumably as part of a defense mechanism against mass replication by viruses (Garrick *et al*., 1998). This suppression phenomenon can be a potential limitation for application of these constructed cell lines over the long term.

5.3 Effect of over-expression of *Hoxa2* on cell proliferation

Previous data from our lab has revealed the presence of *Hoxa2*, *Hoxb4* and *Hoxd1* in OL-lineage cells *in vivo* (Nicolay *et al*., 2004a, b; Booth *et al*., 2007). The study I present here shows *Hoxa2* expression throughout the development course of CG4 cells, a derivative of O-2A cells isolated from rat brain. CG4 cells proliferate and remain in the progenitor stage in the presence of mitogens secreted by the neuronal B104 cell line. Potentially, the immortalizing mitogens for CG4 cells can be bFGF and PDGF, the combination of which has been demonstrated to arrest O-2A cells at the early stage (Bögler and Noble, 1994). This cell line has served as a useful model to study the many aspects of OLs without introducing immortalizing oncogenes. Thus, the detection of *Hox* genes in CG4 cells is not due to any tumorigenic transformation, but rather reflects the genetic property of OLs.

The BrdU uptake experiments demonstrated that overexpression of *Hoxa2* elevates the proliferation rate of CG4-SHoxa2 cells compared with the Dox-treated CG4-SHoxa2 cell group and wild-type CG4 cells. Consistent with this BrdU uptake assay, CG4-SHoxa2 cells with up-regulated *Hoxa2* level contained a significantly higher percentage of A2B5+ve cells at 2DM when the four groups of cells were triggered to differentiate (Fig. 21A). It is, therefore, of interest to investigate by what mechanism *Hoxa2* increases the proliferation of cells. Mitogen PDGF is one of the key factors known to promote the survival and proliferation of OLs, possibly, by increasing cyclin D1 and repressing the synthesis of p27/kip1 (Winston *et al*., 1996; Agrawal *et al*., 1996). Since the mitogens presented in the medium were of the same concentration in four cell groups,

I wondered if the level of receptor for mitogen PDGF expressed on CG4-SHoxa2 cells was elevated by overexpressing *Hoxa2*, rendering the cells more sensitivity to PDGF. Two isoforms (α and β) of PDGF receptor have been identified (Claesson-Welsh, 1994), but only PDGFαR is expressed in O-2A progenitor cells (McKinnon *et al*., 1990; Ellison and de Vellis, 1994). Thus, I only examined the mRNA of PDGFαR in the RT-PCR study. In The RT-PCR results showed that as the cells differentiate, the transcription of PDGFαR is down-regulated in all cell groups, but no significant difference was detected among No Dox-treated CG4-SHoxa2 cells and the other three groups regarding the transcription level of PDGFαR, indicating that *Hoxa2*-regulated cell proliferation may be independent of the PDGF signalling pathway. Nevertheless, further investigations of PDGFαR at the protein level are required to make a firm conclusion.

Alternatively, a question to be asked is whether *Hoxa2* directly interacts with the cell cycling machinery. Recently, *Hox/HOX* genes have been shown to contribute to tumorigenesis or function as indicators of tumor when aberrantly expressed (Del Bene and Wittbrodt, 2005). This has led to recent progress made in understanding and explaining the complex interactions between Hox/HOX TFs and components of the cell cycle machinery. One of the best examples for clustered Hox genes is the identification of p21/kip1, a cyclin-dependent kinase inhibitor (CKI), as the direct target of HOXA10 (Bromleigh and Freedman, 2000) in differentiating myelomonocytic cells. Bromleigh and Freedman (2000) further demonstrate that overexpression of *HOXA10* in U293 cells leads to arrest in G1 phase and subsequent differentiation to the monocytic cell type, probably via activation of p21 transcription by HOXA10 protein. Similarly, Gabellini and co-workers (2003) suggest that HOXC10 has the potential to influence mitotic progression through ubiquitin mediated proteolytic destruction. Furthermore, geminin, a cell-cycle regulator during neural differentiation has been shown to interact with *Hox* proteins in Xenopus, preventing Hox proteins from binding to DNA, and thus inhibiting *Hox*-dependent transcriptional activation (Kroll *et al*., 1998). Although no direct evidence suggesting a direct interplay between the *Hoxa2* gene and cell cycling machine has been shown, it is important to test this hypothesis in vertebrate cell models, including OLs from rodents.

At 3DM, to our surprise, the differences seen at 2DM between Dox-treated and

No Dox-treated CG4-SHoxa2 cells diminished, particularly regarding the percentage of A2B5+ve cells. Is it because the cells with up-regulated *Hoxa2* level became less proliferative after 2DM? Or had some other factors began to play a role in No Dox-treated CG4-SHoxa2 cell group, suppressing cell proliferation in favor of cell differentiation? Since the percentages of GalC+ve cells in two CG4-SHoxa2 cell groups appear the same, it is more likely that the proliferation rate in No Dox-treated CG4-SHoxa2 cells slowed down. One of the possible factors that slowed down the elevated proliferation rate of CG4-SHoxa2 cells (No-Dox) could be the cell density within the culture dish. Zhang and Miller (1996) observed that when primary OPCs cultured at higher concentration displayed more advanced morphology. In my study, with enhanced proliferation in CG4-SHoxa2 (No-Dox) cells, the cell density in this group was higher than the other groups, which may have limited cell proliferation and promoted the differentiation of precursor cells in this particular cell group.

5.4 Impacts of up-regulated *Hoxa2* level on cell differentiation

Whether the intrinsic timer in OPCs primarily controls the cessation of proliferation, the onset of differentiation, or both, is not certain. After demonstrating *Hoxa2* promotes the proliferation of CG4-SHoxa2 cells, the next objective was to examine if *Hoxa2* impacts the cell maturation course after cell cycle exit. By utilizing immunocytochemistry, I attempted to determine the pace of cell advancement in both Dox-treated and No Dox-treated CG4 and CG4-SHoxa2 cells. If the differentiation in all four cell groups proceeded at the same pace, they would have comparable proportions of cells expressing a particular staged marker (A2B5, O4, and GalC).

Notably, not all the cells exit cell cycling and start to differentiate immediately upon medium change, as a significant amount of A2B5+ve cells were detected at both 2DM and 3DM in all four cell groups (Fig. 21 and 22). This might be due to the fact that not all the cells are at the G_1. Additionally, during progression from progenitor cells (A2B5-stage) to the late-progenitor cells (O4-stage), cells remain proliferative, as O4+ve cells are still able to incorporate BrdU molecules (Fig. 18). Although CG4-SHoxa2 cells with up-regulated Hoxa2 showed higher percentage of A2B5+ve cells at 2DM than the other three cell groups, this might be due to elevated proliferation rate of these cells. Moreover, no statistically significant differences were observed among the four cell

groups regarding the percentage of O4+ve cells, and the Dox-treated and No Dox-treated CG4-SHoxa2 cells displayed comparable cell counts at day 3 in DM. Thus, it appears that *Hoxa2* does not affect the maturation programming of the CG4-SHoxa2 cells.

On the other hand, two potential factors may have confounded the immunocytochemistry study. Firstly, the increased cell proliferation resulted in a higher cell density in No Dox-treated CG4-SHoxa2 cell group, which might suppress the expansion of OPCs and lead to accelerated cell maturation. Another factor to consider is the nature of the O4-expressing cells. Although the generation of cell surface antigens follows a temporal order, there are overlaps between each stage, as shown in Figure 1. Consequently, the O4-expressing cell population can consist of cells at very distinct stage, and only observing the percentage of O4+ve cells leaves detailed but important differences uncovered.

To overcome the drawbacks discussed in the previous paragraph, I decided to employ RT-PCR analysis to examine the expression pattern of CGT and MBP, which are generally used as late OL developmental markers. The advantage of utilizing semi-quantitative RT-PCR is that by measuring the mRNA level, we can define the developmental stage of the whole cell group without having to consider the stage of individual cells. Interestingly, RT-PCR analysis of the expression of MBP and CGT strongly indicated that cell differentiation may be hindered when *Hoxa2* is over-expressed in CG4-SHoxa2 cells. The transcription of CGT in the current study displayed a dynamic pattern in CG4 and Dox-treated CG4-SHoxa2 cells, which lends support to previous findings suggesting a similar dynamic transcription pattern of CGT in rodent brain (Garbay and Cassagne, 1994; Yaghootfam *et al*., 2005). Intriguingly, the CG4-SHoxa2 cells (No-Dox) displayed significantly lower level CGT mRNA at GM and 2DM, and a much different pattern of CGT expression during the same study period in the *Hoxa2* up-regulated CG4-SHoxa2 cell group.

The CGT enzyme is responsible for catalyzing the final step in GalC synthesis; therefore, it is possible that a dramatic increase of the CGT mRNA level is required before terminal differentiation in OL-lineage cells. The distinct pattern of CGT mRNA in *Hoxa2* up-regulated CG4-SHoxa2 cells indicates a delay in the differentiation of this group of cells. What was surprising is that I did not observe any substantial variance in

the GalC staining between the two CG4-SHoxa2 groups. Further, Dox-treated CG4-SHoxa2 cells contained much lower percentage of GalC+ve cells compared to wild-type CG4 cell groups, despite the fact that these three groups showed similar CGT mRNA expression levels and pattern. It will be important to investigate whether CGT mRNA level correlate with its enzyme activity regarding the synthesis of GalC protein. Furthermore, to fully interpret the data it will be necessary to continue monitoring the transcription of CGT in No Dox-treated CG4-SHoxa2 cells, and to examine the proportion of GalC+ve cells in two CG4-SHoxa2 cell groups after 3DM.

The MBPs, which function as membrane adhesion molecules, constitute 30% of all myelin proteins in the CNS. Rodent MBP is encoded by a single gene composed of seven exons. Varied RNA slicing is known to result in different MBP isoforms in rodents (de Ferra *et al*., 1985): 21.5Kd, which contains all seven exons; 18.5kD, the most predominant isoform where the exon II is deleted; 17kD, where exon VI is missing; and 14kD, which lacks both exons II and VI. The primers used in my study detect a 176 fragment that spans exons 4 and exon 5, and are able to detect all the isoforms described above. Previous studies have demonstrated the presence of MBP mRNA and proteins in cultured rat OLs obtained from various tissues (Mirsky *et al*., 1980; Zeller *et al*., 1985), and have revealed a developmental regulation of MBP in dispersed brain cultures in the absence of myelin formation (Zeller *et al*., 1985). Although MBP proteins are produced immediately before the onset of myelination, several lines of evidence indicate MBP can be detected in OPCs at mRNA level, preceding the synthesis of the protein (Ye *et al*., 2003; Gokhan *et al*., 2005; Wei *et al*., 2005). This could explain why results from RT-PCR reveal MBP mRNA in undifferentiated CG4 and CG4-SHoxa2 cells, while few MBP+ve cells were observed in the immunostaining studies. As the differentiation progresses, the mRNA of MBP gradually increased in all four cell groups, in agreement with the findings reported by other groups (Gokhan *et al*., 2005; Wei *et al*., 2005). However, the *Hoxa2* up-regulated CG4-SHoxa2 cell group consistently displayed significantly lower levels of MBP mRNA among the four cell groups (GM, 2DM, 3DM), further implying a delay of cell maturation in this particular cell group. It is, therefore, concluded that *Hoxa2* does play a role in both proliferation and differentiation of the CG4-SHoxa2 cell line.

5.5 Future directions

In the present work, I used the gain-of-function approach to investigate the role of *Hoxa2* in CG4-SHoxa2 cells particularly focusing on the impacts of elevated *Hoxa2* level on the proliferation and differentiation of cells. The data collected by immunocytochemistry and RT-PCR in the differentiation study will be useful for interpreting the role of *Hoxa2* in OL-lineage cells, however, limitations exist in these studies. The first concern is the time length of the differentiation study. The cells were only allowed to differentiate for 3 days in DM till they reached the early GalC stage. The experiments did not go beyond three days because of poor cell attachment. It is important to overcome the culture problem in order to examine the differentiation of CG4-SHoxa2 cells at much later stages, to fully describe the expression patterns of GalC and MBP proteins during terminal maturation. In addition, quantitative real-time RT-PCR is suggested to provide more convincing data in the study of the transcriptions of PDGFαR, CGT and MBP.

Since recent evidence implies a link between some *Hox* genes and cell cycling machinery, it is of significant interest to investigate if any components of the cell cycling machinery acts as the downstream target of *Hoxa2* gene or participates in *Hoxa2*-induced transcriptional cascade. It may be promising to focus on investigating the relationship between *Hoxa2* and CDK inhibitors, member of which has been shown to be directly regulated by other *Hox* gene (Bromleigh and Freedman, 2000), and can activate the promoter of *mbp* gene in CG4 cells (Miskimins *et al*., 2002). Increasing evidence from the study of neoplasm and hematopoiesis has suggested a pivotal link between the processes of cell proliferation and differentiation and *Hox* genes, as discussed in the literature review section (II). It may, therefore, be worthwhile to explore this relationship in OL-lineage cells. Another rationale for this idea came from the studies of retinoic acid (RA). It is well known that RA influences the expression of many *Hox* genes in multiple developing systems (Marshall *et al*., 1992, 1996). Recently, RA is also shown to increase the expression of two CDK inhibitors, p15 and p21, at both the mRNA and protein level in murine neural cells (Wang *et al*., 2005), and to regulate the proliferation and differentiation of OL-lineage cells (Noll and Miller, 1994). It is possible that there are molecular links between RA, *Hox* genes and cell cycling components, as has been

proposed by Martinez-Ceballos *et al*. (2005), where they suggest Hoxa1 protein acts as a repressor of RA-induced endodermal differentiation of ES cells *in vitro*. Given these findings, one interesting topic to investigate in the future is whether *Hox* genes, in particular *Hoxa2*, play a role in regulating OLs development by participating in the RA-induced signalling pathway.

In addition, further experiments that characterize migration, apoptosis and myelination of CG4-SHoxa2 cells are needed. After examination of CG4-SHoxa2 cells, parallel investigations on CG4-ASHoxa2 cells, where the expression of *Hoxa2* is down-regulated, need to be carried out. Besides *Hoxa2* gene, *Hoxb4* and *Hoxd1* are also reported to be expressed by OL-lineage cells *in vivo* (Nicolay *et al*., 2004a; Booth *et al*., 2007). Do they have overlapping roles in OLs to ensure the proper progress of the cell development? Or are the expressions of *Hox* genes temporally regulated along with the course of a cell intrinsic timer, as suggested for the HOXB cluster genes in lymphocytes (Care *et al*., 1994; Quaranta *et al*., 1996)? Detailed examinations of the expression profile of each *Hox* gene in oligodendrogenesis are required to answer the above questions.

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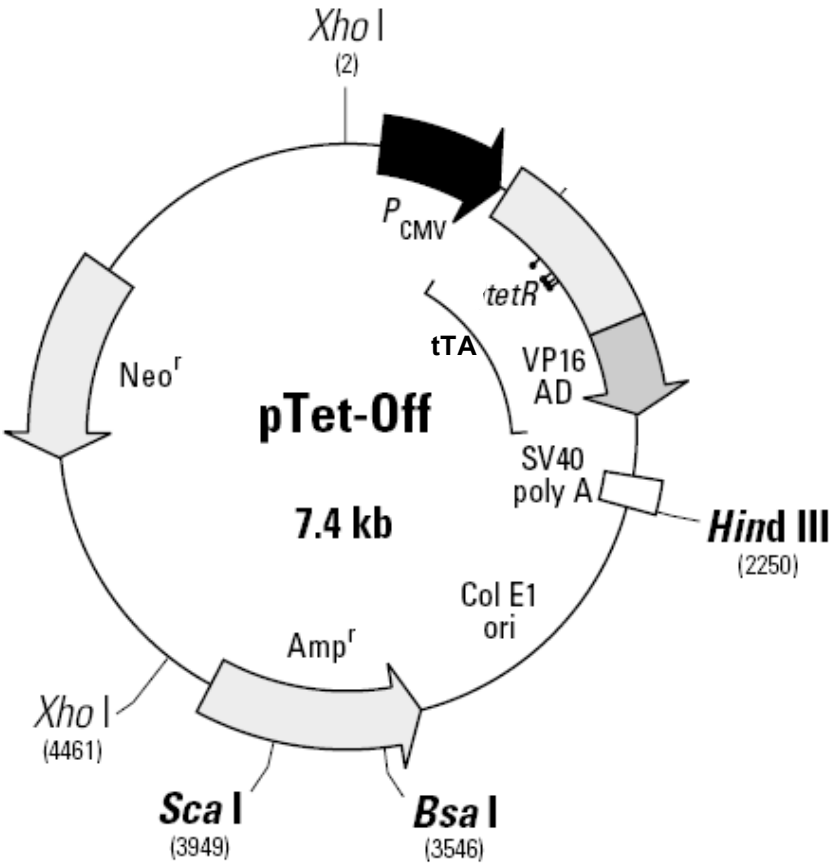
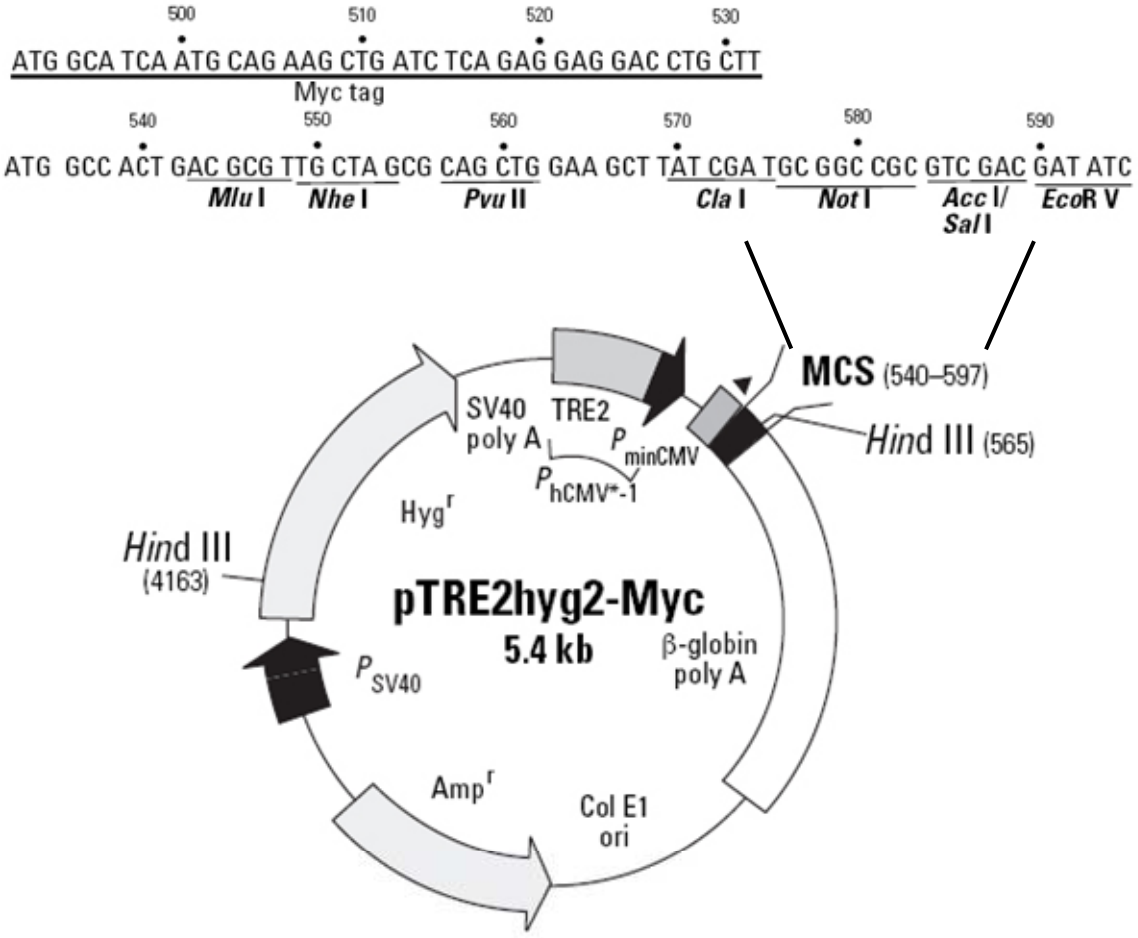
Appendix I MAP OF THE pTet-off VECTOR (Clontech)



Appendix II MAP OF THE pTRE2hyg2-Myc VECTOR (Clontech)



Appendix III RESULTS OF NON-PARAMETRIC TEST FOR

BrdU UPTAKE DATA IN GM AND 2DM

Kruskal-Wallis test of BrdU+ve% in GM		
P value	P<0.0001	
Exact or approximate P value?	Gaussian Approximation	
P value summary	***	
Do the medians vary significant (P < 0.05)	Yes	
Number of groups	4	
Kruskal-Wallis statistic	52.45	
Dunn's multiple comparison test	Difference in rank sum	P value
C-GM-D vs C-GM-N	6.654	P > 0.05
C-GM-D vs S-GM-D	-23.17	P > 0.05
C-GM-D vs S-GM-N	-58.72	P < 0.001
C-GM-N vs S-GM-D	-29.82	P < 0.05
C-GM-N vs S-GM-N	-65.38	P < 0.001
S-GM-D vs S-GM-N	-35.56	P < 0.001

Kruskal-Wallis test of BrdU+ve% in 2DM		
P value	P<0.0001	
Exact or approximate P value?	Gaussian Approximation	
P value summary	***	
Do the medians vary significant(P < 0.05)	Yes	
Number of groups	4	
Kruskal-Wallis statistic	52.45	
Dunn's multiple comparison test	Difference in rank sum	P value
C-GM-D vs C-GM-N	6.654	P > 0.05
C-GM-D vs S-GM-D	-23.17	P > 0.05
C-GM-D vs S-GM-N	-58.72	P < 0.001
C-GM-N vs S-GM-D	-29.82	P < 0.05
C-GM-N vs S-GM-N	-65.38	P < 0.001
S-GM-D vs S-GM-N	-35.56	P < 0.001

Appendix IV RESULTS OF ONE WAY ANOVA TEST FOR RT-PCR DATA OF PDGFαR TRANSCRIPTION

One-way ANOVA test for RT-PCR data of PDGFαR in GM			
P value	0.2662		
Are means significant different? (P < 0.05)	No		
Number of groups	4		
F	1.591		
R squared	0.3737		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.002625	3	0.000875
Residual (within columns)	0.0044	8	0.00055
Total	0.007025	11	
No post tests. P > 0.05			

One-way ANOVA test for RT-PCR data of PDGFαR in 2DM			
P value	0.2366		
Are means significant different? (P < 0.05)	No		
Number of groups	4		
F	1.737		
R squared	0.3944		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.000825	3	0.000275
Residual (within columns)	0.001267	8	0.0001583
Total	0.002092	11	
No post tests. P > 0.05			

Appendix V RESULTS OF NON-PARAMETRIC TEST FOR IMMUNOSTAINING DATA OF A2B5 AND O4 AT 2DM

Kruskal-Wallis test of A2B5+ve% in 2DM		
P value	P<0.0001	
Exact or approximate P value?	Gaussian Approximation	
Do the medians vary significant (P < 0.05)	Yes	
Number of groups	4	
Kruskal-Wallis statistic	21.22	
Dunn's multiple comparison test	Difference in rank sum	P value
C-2DM-D vs C-2DM-N	6.276	P > 0.05
C-2DM-D vs S-2DM-D	-7.187	P > 0.05
C-2DM-D vs S-2DM-N	-39.12	P < 0.01
C-2DM-N vs S-2DM-D	-13.46	P > 0.05
C-2DM-N vs S-2DM-N	-45.39	P < 0.001
S-2DM-D vs S-2DM-N	-31.93	P < 0.05

Kruskal-Wallis test of O4+ve% in 2DM	
P value	0.3526
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Do the medians vary significant (P < 0.05)	No
Number of groups	4
Kruskal-Wallis statistic	3.264
No post tests. P > 0.05	

Appendix VI RESULTS OF NON-PARAMETRIC TEST FOR IMMUNOSTAINING DATA OF A2B5, O4 AND GalC AT 3DM

Kruskal-Wallis test of A2B5+ve% at 3DM		
P value	P<0.0001	
Exact or approximate P value?	Gaussian Approximation	
P value summary	***	
Do the medians vary significant (P < 0.05)	Yes	
Number of groups	4	
Kruskal-Wallis statistic	56.93	
Dunn's multiple comparison test	Difference in rank sum	P value
C-3DM-D vs C-3DM-N	4.831	P > 0.05
C-3DM-D vs S-3DM-D	-46.55	P < 0.001
C-3DM-D vs S-3DM-N	-60.4	P < 0.001
C-3DM-N vs S-3DM-D	-51.38	P < 0.001
C-3DM-N vs S-3DM-N	-65.23	P < 0.001
S-3DM-D vs S-3DM-N	-13.86	P > 0.05

Kruskal-Wallis test of O4+ve% at 3DM	
P value	0.3325
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Do the medians vary significant (P < 0.05)	No
Number of groups	4
Kruskal-Wallis statistic	3.411
No post tests. P > 0.05	

Kruskal-Wallis test of GalC+ve% at 3DM		
P value	P<0.0001	
Exact or approximate P value?	Gaussian Approximation	
P value summary	***	
Do the medians vary significant (P < 0.05)	Yes	
Number of groups	4	
Kruskal-Wallis statistic	35.07	
Dunn's multiple comparison test	Difference in rank sum	P value
C-3DM-D vs C-3DM-N	-3.319	P > 0.05
C-3DM-D vs S-3DM-D	39.94	P < 0.001
C-3DM-D vs S-3DM-N	47.11	P < 0.001
C-3DM-N vs S-3DM-D	43.26	P < 0.001
C-3DM-N vs S-3DM-N	50.43	P < 0.001
S-3DM-D vs S-3DM-N	7.171	P > 0.05

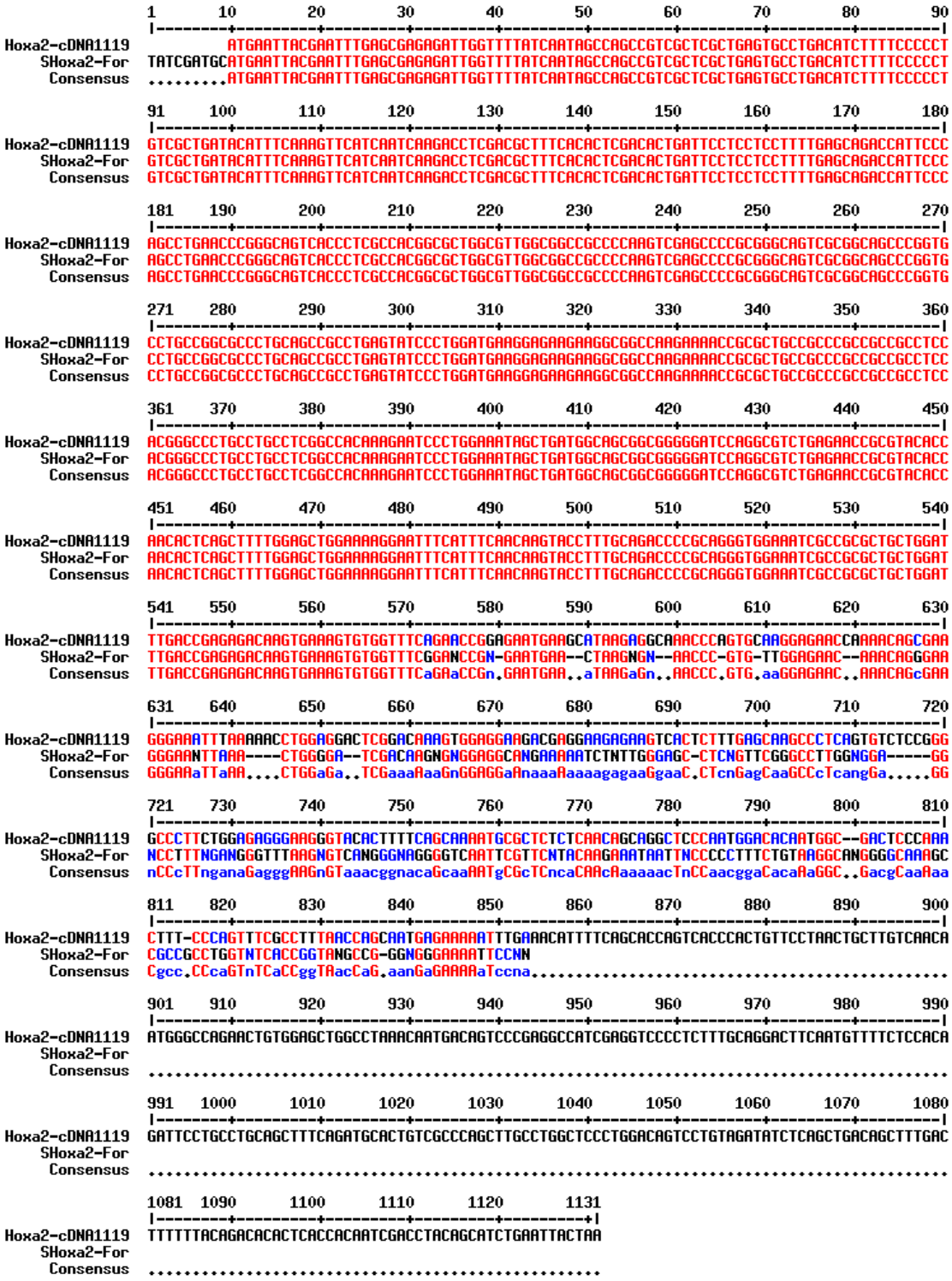
Appendix VII RESULTS OF ONE WAY ANOVA TEST FOR RT-PCR DATA OF CGT mRNA

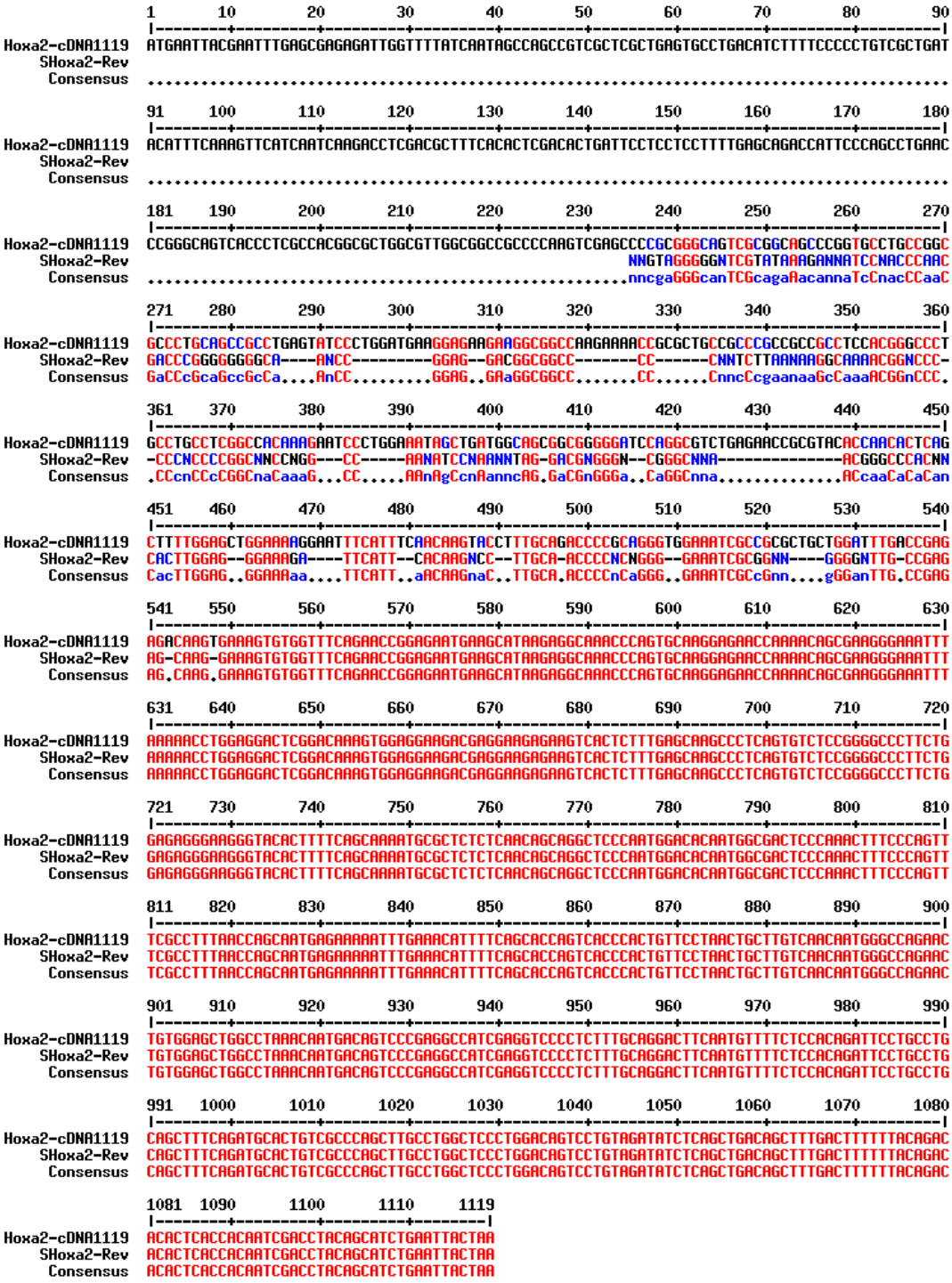
One-way analysis of variance of CGT transcription at GM		
P value	0.0018	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	4	
Tukey's Multiple Comparison Test	Mean Diff.	P value
CG4-Dox vs CG4-No Dox	0	P > 0.05
CG4-Dox vs CG4-SHoxa2-Dox	0.01667	P > 0.05
CG4-Dox vs CG4-SHoxa2-No Dox	0.08	P < 0.01
CG4-No Dox vs CG4-SHoxa2-Dox	0.01667	P > 0.05
CG4-No Dox vs CG4-SHoxa2-No Dox	0.08	P < 0.01
CG4-SHoxa2-Dox vs CG4-SHoxa2-No Dox	0.06333	P < 0.05
One-way analysis of variance of CGT transcription at 2DM		
P value	P<0.0001	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	4	
Tukey's Multiple Comparison Test	Mean Diff.	P value
CG4-Dox vs CG4-No Dox	0.003333	P > 0.05
CG4-Dox vs CG4-SHoxa2-Dox	0.03	P > 0.05
CG4-Dox vs CG4-SHoxa2-No Dox	0.1433	P < 0.001
CG4-No Dox vs CG4-SHoxa2-Dox	0.02667	P > 0.05
CG4-No Dox vs CG4-SHoxa2-No Dox	0.14	P < 0.001
CG4-SHoxa2-Dox vs CG4-SHoxa2-No Dox	0.1133	P < 0.001
One-way analysis of variance of CGT transcription at 3DM		
P value	0.0002	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	4	
Tukey's Multiple Comparison Test	Mean Diff.	P value
CG4-Dox vs CG4-No Dox	0.006667	P > 0.05
CG4-Dox vs CG4-SHoxa2-Dox	0.04	P > 0.05
CG4-Dox vs CG4-SHoxa2-No Dox	-0.07667	P < 0.01
CG4-No Dox vs CG4-SHoxa2-Dox	0.03333	P > 0.05
CG4-No Dox vs CG4-SHoxa2-No Dox	-0.08333	P < 0.01
CG4-SHoxa2-Dox vs CG4-SHoxa2-No Dox	-0.1167	P < 0.001

Appendix VIII RESULTS OF ONE WAY ANOVA TEST FOR RT-PCR DATA OF MBP mRNA

One-way analysis of variance of MBP transcription at GM		
P value	0.0002	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	4	
Tukey's Multiple Comparison Test	Mean Diff.	P value
CG4-Dox vs CG4-No Dox	-0.006667	P > 0.05
CG4-Dox vs CG4-SHoxa2-Dox	0.1	P < 0.05
CG4-Dox vs CG4-SHoxa2-No Dox	0.17	P < 0.001
CG4-No Dox vs CG4-SHoxa2-Dox	0.1067	P < 0.01
CG4-No Dox vs CG4-SHoxa2-No Dox	0.1767	P < 0.001
CG4-SHoxa2-Dox vs CG4-SHoxa2-No Dox	0.07	P > 0.05
One-way analysis of variance of MBP transcription at 2DM		
P value	0.002	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	4	
Tukey's Multiple Comparison Test	Mean Diff.	P value
CG4-Dox vs CG4-No Dox	-0.02333	P > 0.05
CG4-Dox vs CG4-SHoxa2-Dox	0.02333	P > 0.05
CG4-Dox vs CG4-SHoxa2-No Dox	0.12	P < 0.01
CG4-No Dox vs CG4-SHoxa2-Dox	0.04667	P > 0.05
CG4-No Dox vs CG4-SHoxa2-No Dox	0.1433	P < 0.01
CG4-SHoxa2-Dox vs CG4-SHoxa2-No Dox	0.09667	P < 0.05
One-way analysis of variance of MBP transcription at 3DM		
P value	0.0007	
Are means signif. different? (P < 0.05)	Yes	
Number of groups	4	
Tukey's Multiple Comparison Test	Mean Diff.	P value
CG4-Dox vs CG4-No Dox	-0.006667	P > 0.05
CG4-Dox vs CG4-SHoxa2-Dox	0.02333	P > 0.05
CG4-Dox vs CG4-SHoxa2-No Dox	0.1417	P < 0.001
CG4-No Dox vs CG4-SHoxa2-Dox	0.03	P > 0.05
CG4-No Dox vs CG4-SHoxa2-No Dox	0.1483	P < 0.001
CG4-SHoxa2-Dox vs CG4-SHoxa2-No Dox	0.1183	P < 0.01

APPENDIX IX ALIGNMENT OF SEQUENCING DATA OF pTRE2hyg2-Myc-SHoxa2 PLASMID WITH *Hoxa2* cDNA





Note: Hoxa2-cDNA119 sequence information was obtained from GenBank (NM 010451). SHoxa2-For represents sequence data from the 5' end, whereas SHoxa2-Rev represents sequence data from the 3' end.