

**The relationship between proliferation and  
differentiation during oligodendrocyte  
development**

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## **Abstract**

How do precursor cells know when it is time to stop dividing and differentiate? The phenomenon of lineage-specific progenitor cells undergoing a limited period of proliferation prior to terminal differentiation is a common theme in multicellular development. Despite this, little is understood about how these two events are co-ordinated during the normal schedule of development.

I have studied the question of how proliferation and differentiation are co-regulated in the oligodendrocyte lineage in the rodent optic nerve. Oligodendrocytes are post-mitotic cells that myelinate axons in the vertebrate central nervous system. They develop from precursor cells whose maturation is controlled by a timer, which is an intrinsic property of the cells, that limits proliferation. The timer seems not to control the number of divisions the cell can undergo but rather the length of time during which divisions can occur.

Significant effort has been devoted to understanding how the intracellular timer regulates oligodendrocyte development. The timer consists of two components that are modulated by distinct kinds of extracellular signals. Mitogens drive a timing component whose value increases as precursor cells continue to divide. Once this value exceeds a critical threshold, it signals that the proliferative period has elapsed, and hydrophobic signalling molecules trigger an effector component that elicits cell-cycle arrest and differentiation.

The value of the timing component is determined by several intracellular molecules whose activities change as the timer runs. One of these molecules is the cell-cycle inhibitor p27: it accumulates in oligodendrocyte precursor cells as they proliferate in culture. When p27 expression is high the

precursor cells are more likely to stop dividing and differentiate than when it is low. In oligodendrocyte precursor cells derived from mice that lack p27, the timer runs aberrantly and cell-cycle arrest and terminal differentiation are delayed.

It is not understood how the molecular mechanics of the timer control oligodendrocyte development. Does the timer serve to arrest the cell-cycle, with differentiation following by default, or is cell-cycle arrest subordinate to the programme of terminal differentiation? These questions remain unanswered, largely because of a persistent inability to experimentally manipulate the genome of oligodendrocyte precursor cells.

The present study was an attempt to overcome these problems and had two aims - first, to devise a reliable system for transfecting oligodendrocyte precursor cells and second, to determine whether the timer primarily controls the timing of cell-cycle arrest. I developed a new retroviral vector that co-expresses p27 and green fluorescent protein (GFP) in precursor cells. The use of GFP allows the identification of living precursor cells that over-express p27, which can then be followed over many days in culture.

My findings support previous work showing that p27 plays a role in governing the timing of oligodendrocyte differentiation. They show that over-expression of p27 promotes oligodendrocyte differentiation by advancing the value of the timing component, although it does not promote differentiation if the effector component is inoperative. The cell-cycle time of precursor cells that over-express p27 is dramatically extended, but not stopped. It appears that a firm cell-cycle arrest and entry into a quiescent state may be required to elicit terminal differentiation.

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## **Abbreviations**

<b>bFGF</b>	basic fibroblast growth factor
<b>bp</b>	base pairs
<b>B-S</b>	Bottenstein-Sato
<b>BSA</b>	bovine serum albumin
<b>CDK</b>	cyclin-dependent kinase
<b>CKI</b>	cyclin-dependent kinase inhibitor
<b>CMV</b>	cytomegalovirus
<b>CNS</b>	central nervous system
<b>DAB</b>	3,3'-diaminobenzidine
<b>DMEM</b>	Dulbecco's modified Eagle's medium
<b>DMSO</b>	dimethylsulphoxide
<b>E</b>	embryonic day
<b>EMCV</b>	encephalomyocarditis virus
<b>EtBr</b>	ethidium bromide
<b>FCS</b>	foetal calf serum
<b>FITC</b>	fluorescein isothiocyanate
<b>GFP</b>	green fluorescent protein
<b>HRP</b>	horseradish peroxidase
<b>IRES</b>	internal ribosome entry site
<b>LTR</b>	long terminal repeat
<b>MBT</b>	mid-blastula transition
<b>MCS</b>	multiple cloning site
<b>MEM-Hepes</b>	Hepes-buffered minimal Eagle's medium

NT-3	neurotrophin-3
OD <sub>600</sub>	optical density at 600nm
P	postnatal day
p27	p27 <sup>Kip1</sup>
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDL	poly-D-lysine
PFA	paraformaldehyde
pRb	<i>retinoblastoma</i> gene product
PSM	presomitic mesoderm
SV40	simian virus 40
TBLS	Tris-buffered saline containing BSA and lysine
TH	thyroid hormone

# **1 General Introduction**

The ultimate goal of developmental biology is to explain how a multicellular body is built from a fertilised egg. This is not a trivial ambition: from a single cell, the concerted events of cell proliferation, cell movement, cell differentiation and cell death serve to form an adult organism that may contain trillions of cells. How then, given the bewildering complexity of this phenomenon, can we ever hope to understand it?

Considerable effort has been devoted to the study of developmental biology and we are now in a position where many of the important principles involved are understood (Wolpert, 1996). However, despite a wealth of knowledge concerning the spatial regulation of development, our understanding of how events are temporally co-ordinated is comparatively poor.

The normal development of all individuals of an animal species follows a precise and almost invariant schedule. This schedule may simply reflect the time required to complete successive steps in a cascade of contingent gene activations and biochemical reactions. Alternatively, each cell of the animal may measure elapsed time autonomously in the form of a programme that dictates the possible activities that the cell may undertake, whether or not it is destined to do so.

Several examples are known where cells appear to monitor the passage of time before initiating specific developmental changes. During muscle formation in *Xenopus laevis*, muscle-specific actin genes are activated at the mid-gastrula stage of development, in response to signals transmitted from vegetal pole cells to animal pole cells. Although animal pole cells are



competent to respond to these inductive signals throughout the seven-hour period of the morula and blastula stages they only need to be exposed to the signals for two hours for muscle determination to occur. Irrespective of when the signal is received during the responsive period, actin-gene transcription always begins at the mid-gastrula stage. Thus, if the signal is given early in the blastula stage, the delay before actin expression occurs is nine hours. If, however, the signal is made to occur at the late blastula/early gastrula stage, the delay is only five hours (Gurdon *et al.*, 1985). These experiments suggest that embryonic cells are capable of measuring the time elapsed since fertilisation and initiate muscle development according to a timetable of events that is somehow hardwired into their physiology.

A cell intrinsic timer has been proposed to operate during erythroid development. In primates and some ruminants, the pattern of globin gene expression changes from a foetal form ( $\alpha_2\gamma_2$ ) to a adult form ( $\alpha_2\beta_2$ ) shortly after birth (Bard, 1975). The switch occurs at every site of erythropoiesis (Wood and Weatherall, 1973) with identical chronology in all individuals of the same species, and independently of the time of parturition (Wood *et al.*, 1976; Wintour *et al.*, 1985). This strongly suggests that the timing of the switch is measured relative to the time of conception, or to some other event that is triggered at a specific stage during embryogenesis.

Further studies have revealed that the timing of the foetal-to-adult switch of globin gene expression is governed by an autonomous mechanism that operates in erythroid progenitor cells. If haematopoietic stem cells are transplanted from foetal sheep into irradiated adult recipients, the transplanted cells switch gene expression at a time relative to the age of the donor animal,

not to the age of the host (Zanjani *et al.*, 1979). *In vitro* clonal analysis of hybrid cells, formed by fusion of foetal liver cells and mouse erythroleukaemia cells, shows that the timing of the switch in globin gene expression depends solely upon the age of the animal from which the liver cells were obtained (Papayannopoulou *et al.*, 1986; Melis *et al.*, 1987). It is now understood that the switch in globin gene expression is mediated by a large region of DNA sequence proximal to the  $\beta$ -globin gene cluster, termed the locus control region (Blom van Assendelft *et al.*, 1989; Enver *et al.*, 1990). Despite much progress in understanding the molecular basis for globin-gene switching (Townes and Behringer, 1990; Patient *et al.*, 1987), the nature of the timer that triggers the switch remains undetermined, largely because of problems in experimentally manipulating the switching event.

These examples show that during development cells record the passage of time to initiate developmental events on schedule. How is time measured by biological systems? Man-made chronometers are classified into two groups, depending on the type of timing mechanism that they employ. Oscillators, such as the pendulum of a grandfather clock, operate by maintaining a cyclical period of fixed duration. Hourglass timers, on the other hand, measure time using a rate-limited process that reaches a threshold after a defined interval.

Biological chronometers can also be classified as clocks or timers, respectively, depending upon whether they employ oscillators or rate-limiting processes as the driving force for the counting mechanism (Pourquie, 1998; Cooke and Smith, 1990). I will now summarise some examples of biological

chronometry where studies have uncovered details of the molecular basis of the timing process.

Cells are the unit of existence for all life on Earth, and new cells arise only by the division of existing ones. The cell division cycle is an intracellular biochemical oscillator, based upon the serial activation of a family of protein kinases that depend upon the periodic expression of cyclins for their activity. These so-called cyclin-dependent kinases (CDKs) drive the cycle by phosphorylating important targets, such as the *retinoblastoma* gene product (pRb; Weinberg, 1995) and replication licensing factors (Coue *et al.*, 1996). The cycle is reset by dephosphorylation and ubiquitin-dependent protein degradation (Morgan, 1995; King *et al.*, 1996). Early developmental cell cycles proceed without gap phases, and consist only of rapidly alternating S and M phases, which are controlled by the degradation and synthesis of cyclins A and B (Newport and Kirschner, 1984; King *et al.*, 1994). In principle, this fundamental biological clock could be used by cells to measure the passage of time, with events being scheduled after the completion of a given number of cell divisions.

Some developmental processes use the cell cycle oscillator to measure elapsed time. During the early embryogenesis of insects and vertebrates, zygotic gene transcription begins at a defined time, which is known as the mid-blastula transition (MBT; Newport and Kirschner, 1982b; Newport and Kirschner, 1982a). In many animal species, in which the egg contains a large quantity of maternal material, cell divisions occur initially without much macromolecular synthesis, thus the ratio of DNA to cytoplasm increases

following each cell cycle. The MBT is thought to be triggered when specific maternal factors are titrated below a critical amount as a result of successive cell divisions and the concomitant rise in the nucleo-cytoplasmic ratio (Sibon *et al.*, 1997; Pritchard and Schubiger, 1996; Prioleau *et al.*, 1994).

Another developmental process that is thought to use the cell cycle as a device for measuring time is the progressive opening of *Hox* gene clusters during organogenesis (Duboule, 1994a; Duboule, 1994b). *Hox* genes encode transcription factors, which define the positional identity of embryonic segments along both the antero-posterior axis of the body and the proximo-distal axis of the limbs. Their spatial expression pattern is collinear with their arrangement along the chromosome, such that a gene located more 3' is expressed anterior to its 5' neighbour. The expression pattern of *Hox* genes also exhibits temporal collinearity, with genes located at the 3' end of the cluster being expressed earlier than genes located more 5'.

From studies on the developing limb, it has been proposed that the progressive and unidirectional opening of the *Hox* gene cluster, which dictates the patterns of collinearity, depends upon successive cell divisions. The rate at which cells are proliferating determines how much of the *Hox* cluster is opened, starting from the 3' end, before they stop dividing and differentiate. Cells at the distal tip of the limb maintain a higher rate of proliferation and therefore express more 5' genes than cells from the proximal zone. Once cells stop proliferating the state of the *Hox* cluster is frozen so that the pattern of gene expression is maintained. During the putative opening of the cluster, it is proposed that certain proteins, which occlude the DNA from the transcriptional machinery, bind with a higher affinity to the more 5' genes. Successive cell

divisions titrate out these proteins resulting in their progressive depletion proceeding from the 3' to 5' genes (Kondo *et al.*, 1998).

The timing of the MBT and the temporal regulation of *Hox* gene expression appears to be mediated by a mechanism that depends upon the cell cycle clock. There is an obligate relationship between the timing of these processes and continued cell division. However, many examples of biological timing devices use alternative methods of metering time.

Circadian rhythms are changes in the biochemistry and behaviour of organisms that occur with a period of about one day (Menaker *et al.*, 1978). They are ubiquitous in nature, being found up and down the phylogenetic tree, from humans to cyanobacteria (Dunlap, 1999). These rhythms are generated by an intracellular timekeeping mechanism referred to as the circadian clock. The circadian clock consists of an intracellular oscillator in which transcription factors, most notably the products of the *period* and *timeless* genes, once synthesised, feedback to inhibit their own expression (Rosbash *et al.*, 1996; Rosbash, 1995). Subsequently, transcription of the *period* and *timeless* genes is re-activated by positively acting factors, including another transcription factor encoded by the gene *clock* (Wilsbacher and Takahashi, 1998). This cycle forms the kernel of the circadian clock, which is proposed to operate in all cells of an organism (Rosbash, 1998), and has a period of approximately 24 hours.

In principle, the circadian clock could be involved in the timing of events during development and postnatal maturation. However, this seems unlikely, at least in animals, as mutations that disable the circadian clock in *Drosophila* and mice have no discernible effect on development.

An intracellular clock operating during development that does not count cell cycles has been discovered recently, and is associated with vertebrate somitogenesis (reviewed in Cooke, 1998). Remarkably, the existence of such an clock had been predicted by theoretical models some decades earlier (Meinhardt, 1986; Cooke, 1975).

Somites are the most obvious manifestation of segmentation during vertebrate development, and ultimately give rise to the vertebral column and the skeletal muscle of the trunk. They consist of small balls of epithelial cells that form by the progressive bilateral budding of presomitic mesoderm (PSM). In the developing chick, a new pair of somites buds from the PSM every 90 minutes. *In situ* hybridisation analysis of *c-hairy1*, a chick homologue of the *Drosophila* pair-rule gene *hairy*, revealed that its mRNA was expressed dynamically, with a period of exactly 90 minutes, appearing as a wavefront that swept through the PSM (Palmeirim *et al.*, 1997). In the time between passing through the blastopore lip and budding into a somite, every cell of the PSM undergoes 12 cycles of *c-hairy1* expression. The cycles of expression are not a result of cell movement; neither are they propagated by signalling through the PSM. Rather, they are an intrinsic property of the constituent cells. Blocking protein synthesis does not effect the cycling of *c-hairy1* expression, suggesting that the segmentation clock does not rely on unstable proteins or transcriptional cycles. It is likely that *c-hairy1* is not a core component of the oscillator but, instead, an output of that mechanism. Since these initial observations, the expression of several other genes has been found to oscillate during somitogenesis in chicken and mouse (McGrew *et al.*, 1998; Forsberg *et al.*,

1998). However, the mechanism by which the output of the clock controls the timing of somite budding remains unclear (McGrew and Pourquie, 1998).

In the nematode *Caenorhabditis elegans* a set of genes has been identified that controls the temporal pattern of development of several lineages in the larva (Chalfie *et al.*, 1981; Ambros and Horvitz, 1984). Mutations in these genes produce a heterochronic phenotype: events that usually occur at specific developmental stages occur either earlier or later than normal. Just as homeotic mutations cause cells to adopt fates normally expressed in different positions (Lewis, 1978), heterochronic mutations cause cells to adopt fates normally expressed at different times. The heterochronic genes determine the temporal fate of cells that have equivalent developmental potential but are generated at successive stages. Two of the heterochronic genes encode intracellular proteins whose expression progressively decreases during larval development. The decrease is regulated by a third heterochronic gene that encodes two small antisense RNAs. These RNAs inhibit the expression of the other heterochronic genes by binding to the 3' untranslated regions of their messenger RNAs, blocking translation (reviewed in Ambros and Moss, 1994). It is unclear what controls expression of the antisense RNAs during development. Heterochronic genes have been identified in many organisms, including plants (Dudley and Poethig, 1991), *Dictyostelium* (Simon *et al.*, 1992), and fungi (Mirabito *et al.*, 1989). In *Drosophila*, the *anachronism* locus is responsible for the timing of neuroblast proliferation and differentiation. While certain neuroblasts divide precociously in *anachronism* mutants, other developmental timing events appear normal (Ebens *et al.*, 1993). In humans, where most post-partum timing events are controlled by hormones, gain-of-

function mutations in the luteinising hormone receptor results in precocious male puberty (Laue *et al.*, 1995). Conversely, loss-of-function mutations result in pseudohermaphroditism and failure to develop secondary sexual characteristics, which can be interpreted as a retarded expression of pubescent cell fates (Kremer *et al.*, 1995). It remains a mystery how the timing of the luteinising hormone receptor is controlled to regulate the timing of puberty.

During development, the precursor cells of many lineages divide a limited number of times before they exit the cell cycle and terminally differentiate. What determines when these cells stop dividing, often in the continued presence of mitogens? The simplest explanation for this phenomenon is that cells count successive cell divisions and stop dividing after a certain number (Temple and Raff, 1986; Quinn *et al.*, 1985). However, despite this attractively straightforward explanation, experimental data supporting the theory is lacking. For example, blocking the early cleavage divisions in ascidians or *C. elegans* does not stop the appearance of certain differentiation markers at the appropriate times (Sato, 1979; Laufer *et al.*, 1980). Similarly, degradation of maternal cyclin A and E messenger RNAs prior to gastrulation in *Xenopus* is a cell-intrinsic event that is timed independently of the cell cycle and macromolecular synthesis (Howe and Newport, 1996; Howe *et al.*, 1995). These results suggests the existence of a developmental timer that measures the time elapsed since fertilisation, and then activates maternal mRNA degradation at the onset of gastrulation. More remarkably, *Xenopus* embryos treated with DNA synthesis inhibitors at the onset of gastrulation proceed normally through neurulation, neural tube closure



and early neuronal determination in absence of any further cell division (Harris and Hartenstein, 1991).

Early studies by Raff and colleagues provided the first evidence for the existence of cell-intrinsic timers in the developing vertebrate central nervous system (CNS; Williams *et al.*, 1985; Abney *et al.*, 1981). If embryonic day 13 (E13) rat brains are dissociated and the cells cultured *in vitro*, one finds that the three major classes of macroglial cells develop three days earlier than when cultures are prepared from E10 brains. Moreover, the timing of the first appearance of the various cell types in culture matches the time when these cells are first detected *in vivo*. These findings suggest that the timing of glial differentiation depends on mechanisms that operate independently of CNS morphogenesis, enabling the study of the timing mechanisms involved in culture.

The vertebrate brain is an exceptionally complicated organ, containing billions of cells, including a plethora of different neuronal cell types and the glia that support them. To simplify the problem, studies were continued in one of the simplest parts of the CNS, the optic nerve, which develops from an extension of the neural tube called the optic stalk (Raff, 1989). The optic nerve is simple because it does not contain any neuronal cell bodies or synapses (figure 1-1). It contains the axons of retinal ganglion cells, that project from the retina to the brain, and two major types of macroglial cell, astrocytes and oligodendrocytes, which support the axons in the nerve.

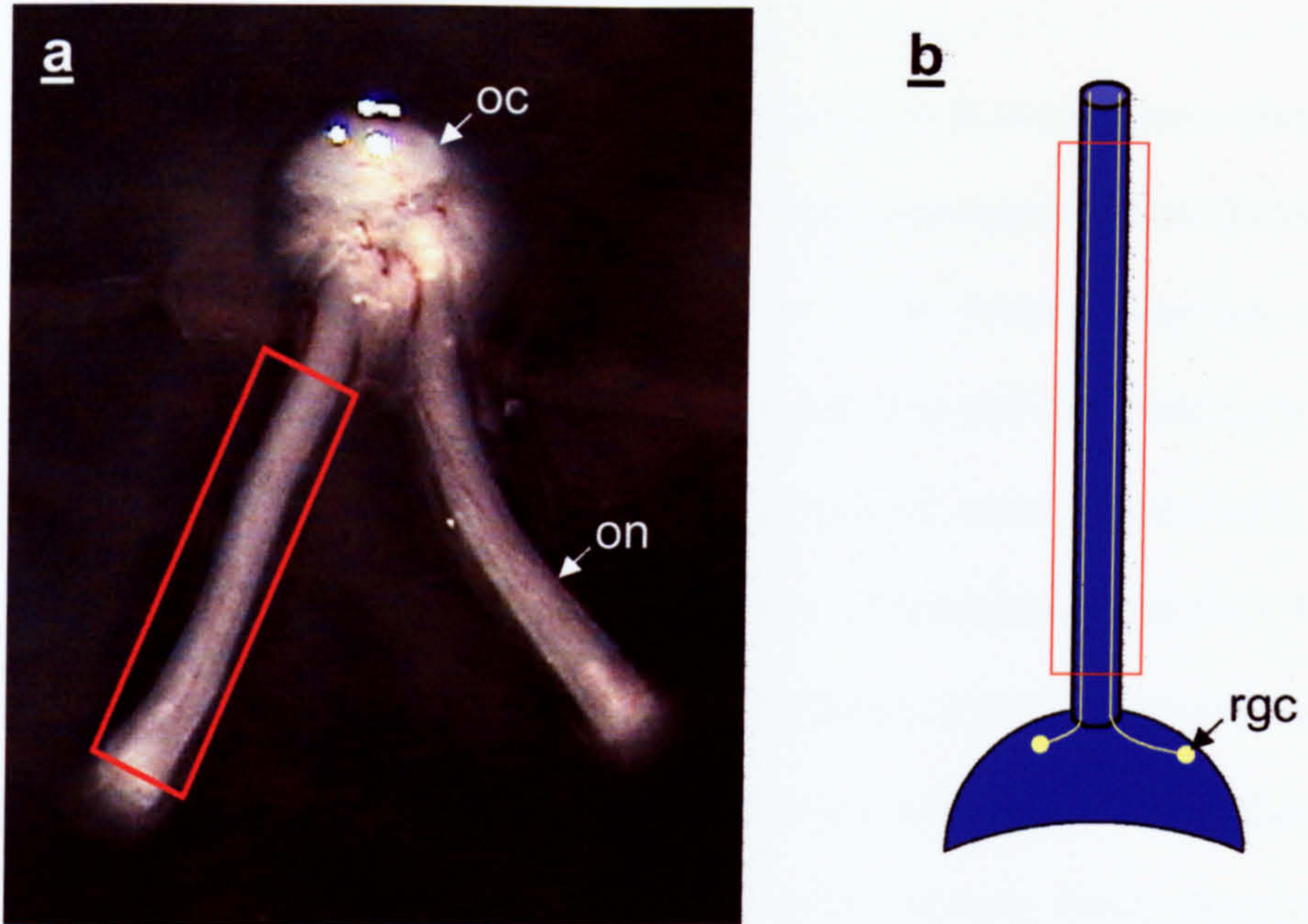


Figure 1-1: The optic nerve. (a) Two post-natal day seven optic nerves (on), joined at the optic chiasm (oc), are shown. (b) A schematic representation of a single optic nerve with retina attached. The optic nerve consists of the axons of retinal ganglion cells (rgc) that project from the retina to the brain, as well as the glial cells that support them. The images are oriented such that the brain would be at the top.

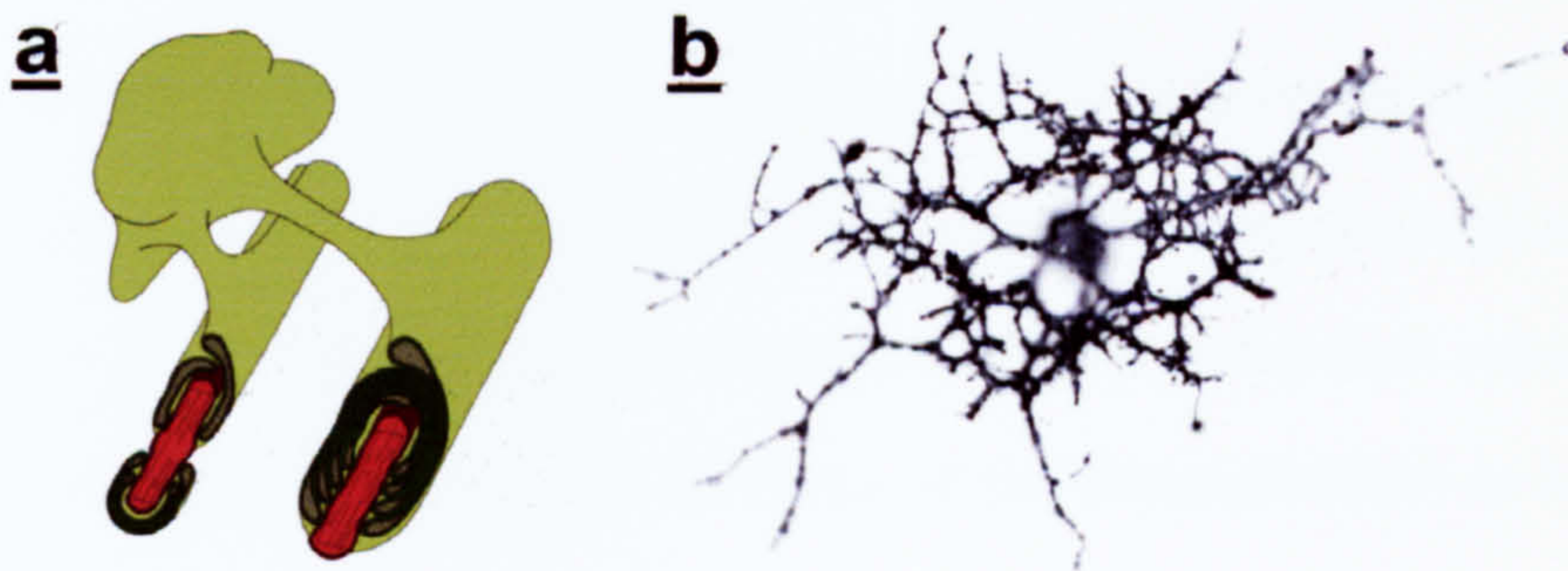


Figure 1-2: Oligodendrocytes are the myelinating cells of the CNS. (a) A cartoon oligodendrocyte is shown ensheathing two axons. (b) An micrograph of an oligodendrocyte growing in culture.

The only known function of oligodendrocytes is to myelinate axons in the vertebrate CNS, ensuring rapid and efficient neurotransmission (Peters *et al.*, 1990; Peters and Vaughn, 1970; Bunge, 1968). Unlike Schwann cells, which are responsible for myelination in the periphery and myelinate only one axon each, oligodendrocytes often ensheath multiple axons (figure 1-2). The role of astrocytes is less clear, although many different functions have been proposed, such as guiding migrating neurones (Rakic, 1971) and growth cones (Silver and Sapiro, 1981; Silver *et al.*, 1982) during development, the induction of the formation of the blood brain barrier (Janzer and Raff, 1987), the control of extracellular ion composition (Hertz, 1981), the production of mitogens during development (Raff *et al.*, 1988), and the formation of glial scars following injury (Maxwell and Kruger, 1965; Vaughn and Pease, 1970).

Commitment to the oligodendrocyte lineage occurs in the ventricular zone of the neuroepithelium (Ono *et al.*, 1995). The earliest oligodendrocyte precursor cells, which can be identified by expression of platelet-derived growth factor (PDGF)  $\alpha$ -receptors, originate in a discrete region of the neural tube (Pringle and Richardson, 1993). This specialised domain comprises two narrow, longitudinal columns of neuroepithelial cells along each side of the ventral midline. Induction of this region of the neuroepithelium to become oligodendrocyte precursor cells appears to be mediated by signals from the notochord, including Sonic hedgehog (Orentas *et al.*, 1999; Orentas and Miller, 1996; Pringle *et al.*, 1996). After their first appearance, at around E14 in the rat spinal cord, the PDGF $\alpha$ -receptor<sup>+</sup> cells proliferate and migrate away from the ventricular zone into developing white matter, dispersing throughout the spinal

cord between E16 and E18 (Pringle and Richardson, 1993). Oligodendrocyte precursor cells that are destined to populate the optic nerve arise in a restricted region of the ventral ventricular zone of the third ventricle, which overlies the optic chiasm, first appearing at stage 26-27 in the developing chick and migrating to the chiasm-end of the nerve by stage 29 (Ono *et al.*, 1997).

In the rat, oligodendrocyte precursor cells actively migrate into the developing optic nerve, from the brain, beginning before birth, but do not become evenly distributed along the nerve until the beginning of the second post-natal week (Small *et al.*, 1987). Precursor cells are prevented from entering the retina by a dense plug of astrocytes at the boundary with the sclera termed the *lamina cribosa* (Ffrench-Constant *et al.*, 1988; Berliner, 1931).

The first oligodendrocytes appear in the rat optic nerve on a predictable schedule, beginning on the day of birth (Miller *et al.*, 1985) and increasing in number for the following six weeks (Skoff *et al.*, 1976; Barres *et al.*, 1992a). The adult rat optic nerve contains about 100,000 axons (Lam *et al.*, 1982) and about 300,000 oligodendrocytes (Barres *et al.*, 1992a). The final number of oligodendrocytes in the nerve depends upon the number of precursor cells that migrate into the nerve, the number of times the precursor cells divide before they terminally differentiate, and the number of oligodendrocytes and precursors that undergo programmed cell death (Barres *et al.*, 1992a).

What determines the timing of oligodendrocyte development in the optic nerve? Oligodendrocytes develop from proliferating precursor cells,

previously known as O-2A progenitor cells<sup>1</sup> (Raff *et al.*, 1983). When oligodendrocyte precursor cells are cultured in serum-free medium in the presence of survival signals, but in the absence of mitogens, they immediately stop dividing and differentiate into oligodendrocytes (Temple and Raff, 1985; Barres *et al.*, 1994). They can be stimulated to divide by several mitogens, but PDGF is the most important, both *in vitro* (Noble *et al.*, 1988; Raff *et al.*, 1988) and *in vivo* (Calver *et al.*, 1998). In mice in which the *PDGFA* gene has been inactivated, oligodendrocyte precursor cells do not proliferate and very few oligodendrocytes develop (Fruttiger *et al.*, 1999). Both astrocytes (Noble *et al.*, 1988; Raff *et al.*, 1988) and retinal ganglion cells (Mudhar *et al.*, 1993) synthesise PDGF-AA, although the astrocytes seem to be the major source the developing optic nerve (Calver *et al.*, 1998). In the embryonic mouse spinal cord, the total number of oligodendrocyte precursor cells is controlled largely by competition for a limiting amount of PDGF (Fruttiger *et al.*, 1999).

The normal timing of oligodendrocyte development can be reconstituted in cultures of dissociated embryonic or perinatal optic nerve cells, as long as the precursor cells are stimulated to proliferate by either astrocytes (Raff *et al.*, 1985) or PDGF (Raff *et al.*, 1988). The reason why oligodendrocyte precursor cells ultimately stop dividing and differentiate in such cultures is not because PDGF becomes limiting or that PDGF receptors

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<sup>1</sup> They were given this name because, under certain conditions, oligodendrocyte precursor cells can give rise to so-called type-2 astrocytes. However, there is little evidence to suggest that the precursor cells normally develop into type-2 astrocytes *in vivo* (Grove *et al.*, 1993; Fulton *et al.*, 1992; Williams and Price, 1992) and, to avoid confusion, I will refer to O-2A progenitor cells as oligodendrocyte precursor cells.

disappear or can no longer be activated. Adding excess PDGF to the cultures does not change the timing of oligodendrocyte differentiation (Raff *et al.*, 1988), and newly formed oligodendrocytes still express PDGF receptors (Hart *et al.*, 1989b), which can induce both an increase in cytosolic  $\text{Ca}^{2+}$  (Hart *et al.*, 1989a) and immediate-early gene expression (Hart *et al.*, 1992).

Clonal analysis of single (Temple and Raff, 1986) or purified (Barres *et al.*, 1994) oligodendrocyte precursor cells suggest that a cell-intrinsic timer is important for governing when a precursor cell will stop dividing and differentiate, at least in culture. Precursor cells isolated from postnatal day seven (P7) optic nerves divide between zero and eight times before they stop dividing and terminally differentiate into post-mitotic oligodendrocytes (Barres *et al.*, 1994). Moreover, if the two daughter cells of an individual precursor cell are sub-cultured into separate microwells, they tend to undergo the same number of divisions before they stop dividing and differentiate into oligodendrocytes (Temple and Raff, 1986). These findings suggest that a timing mechanism operates in the precursor cells to limit their proliferation, and that the timer is an intrinsic property of individual cells. The heterogeneity in the number of divisions that different precursor cells undergo before differentiating is believed to reflect a variation in the maturation of precursor cells that occurred *in vivo* prior to isolation, as embryonic precursor cells divide many more times on average than do postnatal precursors (Gao and Raff, 1997). The finding that precursor cells cultured at 33°C divide more slowly but stop dividing and differentiate earlier, after fewer divisions, than at 37°C suggests that the timer does not operate by counting cell divisions but instead measures time in some other way (Gao *et al.*, 1997).

The intrinsic timer consists of at least two components – a timing component that measures the elapsed proliferative time, and an effector component that triggers differentiation once the timing component signals that ‘time is up’ (figure 1-3; Barres *et al.*, 1994; Bogler and Noble, 1994). Mitogens, such as PDGF, drive the timing component of the timer and hydrophobic signals, such as thyroid hormone (TH), are required for the effector mechanism to operate normally. For example, if oligodendrocyte precursor cells are purified from the optic nerve of P8 rats, and are cultured at clonal density in the presence of mitogens and TH, they divide up to eight times before they differentiate into oligodendrocytes. Differentiation occurs synchronously within clones, such that after a few days, some clones will be entirely composed of post-mitotic oligodendrocytes whereas others, in the same culture dish, contain only proliferating precursor cells. By contrast, if the cells are cultured in the presence of mitogens but in the absence of TH most of them continue to divide more than eight times and few differentiate into oligodendrocytes. However, if TH is added to these cultures after eight days, by which time almost all of the cells would have differentiated had TH been present all along, then most of the cells stop dividing and differentiate within four days, suggesting that TH is not required for the timing component of the timer to operate (Barres *et al.*, 1994). Bögler and Noble provided independent evidence for distinct timing and effector components, using a combination of PDGF and basic fibroblast growth factor (bFGF), rather than an absence of TH, to inhibit differentiation (Bogler and Noble, 1994).

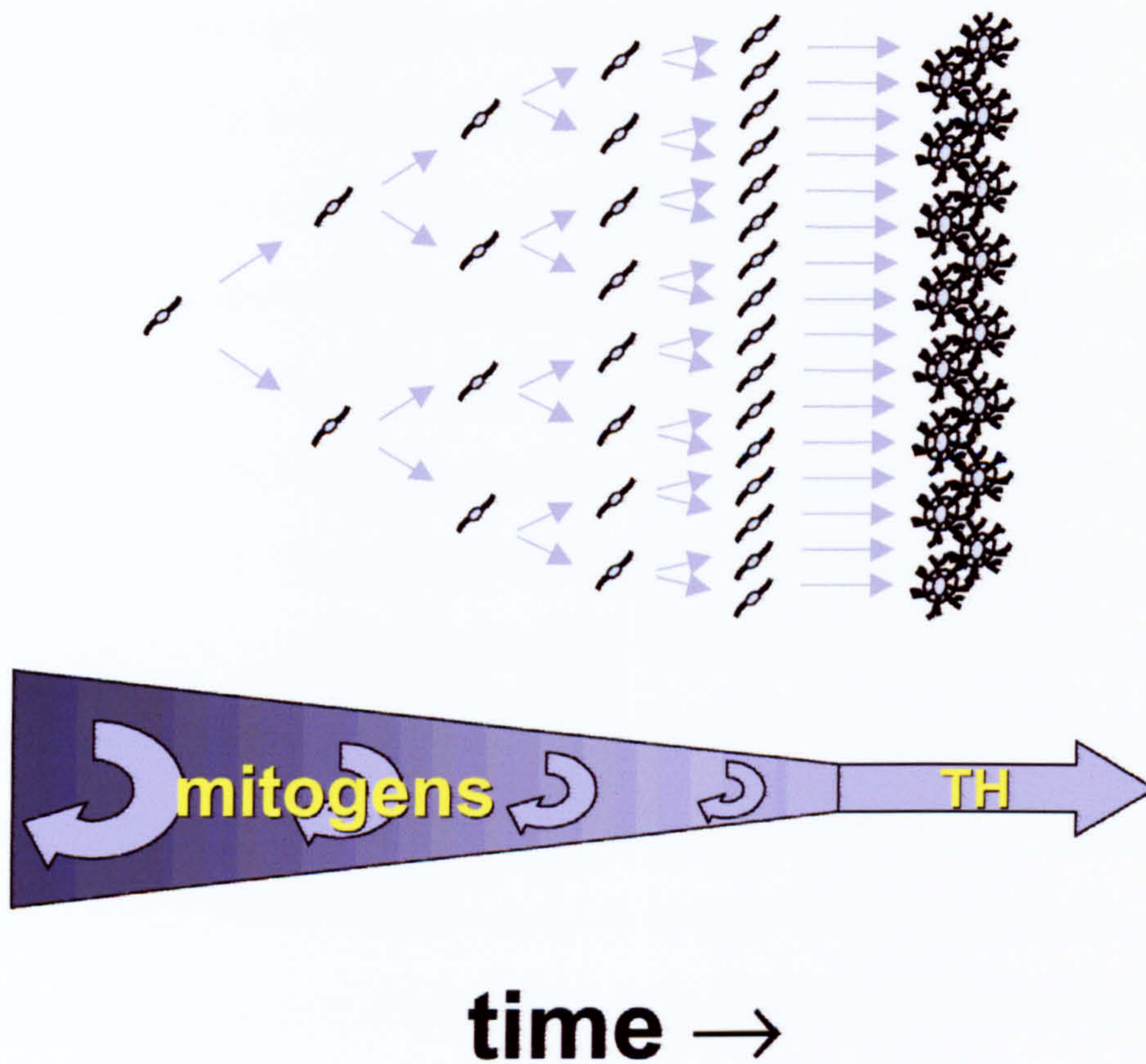


Figure 1-3: The oligodendrocyte precursor cell timer. The timer operates to limit the time during which precursor cells can divide, not the number of divisions, before they differentiate into post-mitotic oligodendrocytes. Mitogens drive a timing component that measures elapsed time, and TH triggers an effector component, which stops division and initiates terminal differentiation, once 'time is up'.



It seems likely that the intrinsic timer that operates in culture also governs the schedule of oligodendrocyte development *in vivo*. The timely development of oligodendrocytes in culture, which relies on the normal operation of the timer, matches the schedule observed *in vivo* (Raff, 1989; Raff *et al.*, 1985). Delivery of surplus PDGF to P8 rat optic nerves does not increase the proliferation of oligodendrocyte precursor cells in the nerve (Barres *et al.*, 1992b), suggesting that the precursors do not normally stop dividing and differentiate due to limiting concentration of mitogen, at least at this stage of development. Oligodendrocyte precursor cells purified from embryonic optic nerves tend to divide in culture for a longer time than precursor cells isolated from post-natal animals, suggesting that the timing component of the timer records the duration of the proliferative period *in vivo* (Gao and Raff, 1997). Lineage analysis of the developing CNS, using a retrovirus that indelibly labels cells and their progeny with  $\beta$ -galactosidase, suggested that clusters of labelled oligodendrocyte-lineage cells contained either oligodendrocytes or precursors, but never both. (Grove *et al.*, 1993). These results suggest that differentiation of oligodendrocyte precursor cells in the developing CNS occurs synchronously, as it does *in vitro* (Temple and Raff, 1986), providing the strongest evidence for the operation of the timer *in vivo*.

Although the timer may operate in precursor cells *in vivo*, there are probably many additional factors that control the timing of oligodendrocyte development in the optic nerve. For example, the Notch signalling pathway, whose components are expressed in the optic nerve during development, has been shown to influence the differentiation of oligodendrocyte precursor cells in culture (Wang *et al.*, 1998).

TH is required for the normal development of oligodendrocytes in the optic nerve: hypothyroid rats (Ahlgren *et al.*, 1997; Ibarrola and Rodriguez-Pena, 1997) and mice (Ahlgren *et al.*, 1997) have many fewer oligodendrocytes in their optic nerves at P7 than do wild-type animals. In addition to its effects on oligodendrocyte precursor cells, TH also has effects on the maturation of oligodendrocytes: for example, hyperthyroidism accelerates myelination whereas hypothyroidism delays it (Dussault and Ruel, 1987; Warringa *et al.*, 1987). TH influences the differentiation and maturation of precursor cells of many vertebrate lineages, including brain, lung, muscle, bone and erythroid progenitor cells (Oppenheimer, 1991; Legrand, 1986; Schwartz, 1983). Myoblasts and erythroid progenitor cells appear to use counting mechanisms similar to oligodendrocyte precursor cells (Quinn *et al.*, 1985; Allen and Dexter, 1982; Dainiak *et al.*, 1978). The rodent thyroid gland becomes active before birth and TH levels progressively increase during the first postnatal weeks in the rat (Puymirat, 1992; Dussault and Ruel, 1987; Samel, 1968). TH is well suited, therefore, to co-ordinate events, such as oligodendrocyte development and myelination, that occur during this period. The possibility exists that TH may help the systemic co-ordination of vertebrate development, especially at the stage of organogenesis, just as it orchestrates the events of amphibian metamorphosis (Gilbert and Frieden, 1981; Shi *et al.*, 1996; Tata, 1993).

The sensitivity of oligodendrocyte precursor cells to the effects of TH progressively increases with time as they proliferate in culture. Precursors isolated from the optic nerves of P14 rats are more sensitive to TH than P1 precursor cells, which correspondingly express fewer TH  $\beta$ -receptors (Barres *et*

*al.*, 1994). Part of the intrinsic timer appears to involve an accumulation of TH  $\beta$ -receptors: the expression increases in perinatal precursor cells, in the absence of TH, and reaches a plateau at a time when most cells would have differentiated (figure 1-4). Moreover, the rate of accumulation is faster when precursor cells are cultured at 33°C than at 37°C, conditions where the timing component of the timer runs faster (Gao *et al.*, 1998).

How might the intrinsic timer measure elapsed time, and how does it stop the cell cycle and initiate terminal differentiation once time is up? Whatever the mechanism, it is likely to interact eventually with the intracellular machinery that controls progression through the cell cycle. In principle, the timer could depend upon: (1) the decay of stimulatory proteins, such as cyclins or CDKs, that normally drive progress through the cell cycle; (2) the accumulation of inhibitory proteins, such as the CDK inhibitors (CKIs), that act to retard progress through the cell cycle; or (3) both of these mechanisms. It seems most likely that both of these mechanisms are involved as this would ensure that the stopping mechanism is more robust.

The eukaryotic cell cycle is controlled by a family of CDKs that are cyclically activated to trigger the different phases of the cell cycle at the right time and in the correct order (Sherr, 1994; Morgan, 1995; Lees, 1995). The decision to initiate cell division is made in the first gap phase of the cycle ( $G_1$ ): once cells pass through a restriction point in  $G_1$  they are committed to undergo another round of the division cycle (Pardee, 1989). In making this choice, cells integrate a wide variety of extracellular signals, such as growth factors, mitogen antagonists, differentiation inducers, and spatial cues. Without extracellular mitogens, most mammalian cells withdraw from the cell cycle in

G<sub>1</sub>, and become quiescent. Mitogens stimulate the synthesis of D-type cyclins, and the assembly of cyclin D/CDK4 and cyclin D/CDK6 holoenzymes stimulate the cell to re-enter the cycle and progress through G<sub>1</sub> into S phase.

Inhibition of the cell cycle in G<sub>1</sub> is, in part, mediated by CKIs, which bind to and inhibit CDKs. The CKIs have been divided into two families based upon their structures and CDK targets. The first class includes the INK4 proteins, so-named for their ability to inhibit the catalytic subunits of CDK4 and CDK6, specifically p16<sup>Ink4a</sup> (Serrano *et al.*, 1993), p15<sup>Ink4b</sup> (Hannon and Beach, 1994), p18<sup>Ink4c</sup> (Guan *et al.*, 1994), and p19<sup>Ink4d</sup> (Chan *et al.*, 1995). The second class comprises the more broadly acting Cip/Kip family of proteins that affect the activities of cyclin D-, E-, and A-dependent kinases. The latter class includes p21<sup>Cip1</sup> (Gu *et al.*, 1993; Harper *et al.*, 1993; el-Deiry *et al.*, 1993; Xiong *et al.*, 1993; Dulic *et al.*, 1994; Noda *et al.*, 1994), p27<sup>Kip1</sup> (Polyak *et al.*, 1994a; Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994), and p57<sup>Kip2</sup> (Lee *et al.*, 1995; Matsuoka *et al.*, 1995), which bind to both cyclin and CDK subunits. Over-expression of these proteins in various cell lines arrests the cell cycle in G<sub>1</sub> (Sherr and Roberts, 1995; Hunter and Pines, 1994). Moreover, several CKIs are up-regulated when developing cells exit the cell cycle, suggesting a possible role in stopping proliferation at the appropriate time (Harper and Elledge, 1996; Jiang *et al.*, 1994; Steinman *et al.*, 1994). In yeast, for example, the CKI Far1 mediates cell-cycle arrest mediated by mating pheromone (Peter and Herskowitz, 1994). An increase in p21 expression is associated with cell-cycle exit prior to terminal differentiation in several mammalian lineages, including keratinocytes (Harvat *et al.*, 1998) and myocytes (Walsh and Perlman, 1997; Halevy *et al.*, 1995; Parker *et al.*, 1995). Similarly, the

senescence of fibroblasts proliferating in culture, which can be viewed as a special kind of terminal differentiation, is associated with the accumulation of p16 (Palmero *et al.*, 1997; Hara *et al.*, 1996; Serrano *et al.*, 1996; Hayflick, 1965). Cell-cycle arrest has been associated with an increase in p27 expression in various circumstances, such as contact inhibition and treatment with TGF $\beta$  (Sherr and Roberts, 1995; Polyak *et al.*, 1994a). Levels of p27 are high in many tissues where cells are exiting the cell cycle and terminally differentiating (Kato *et al.*, 1994; Nourse *et al.*, 1994).

Several lines of evidence suggest that the accumulation of the p27 is part of the timer in oligodendrocyte precursor cells (Durand *et al.*, 1998; Durand *et al.*, 1997). First, p27 expression is high in all oligodendrocytes, whereas it is variable in their precursors. Second, when precursor cells are deprived of PDGF in culture, the rate at which p27 rises is indistinguishable from the rate at which the cells commit to cell-cycle withdrawal and differentiation, raising the possibility that p27 is part of the effector mechanism that stops the cell cycle. Third, p27 progressively accumulates as precursor cells proliferate in culture in the presence of PDGF but in the absence of TH, even though most cells do not stop dividing and differentiate in these conditions. It reaches a plateau of expression at around the same time that most precursor cells would have stopped dividing and differentiated into oligodendrocytes had TH been present, suggesting that the progressive rise in p27 expression is part of the timing component (figure 1-4). The level of p27 expressed by precursor cells also rises as they proliferate *in vivo* (Yasuhito Tokumoto, unpublished observations). Since the cells in culture continue to proliferate with this high level of p27, however, it is clear that the rise in p27 is not sufficient on its own

to stop the cell cycle and that p27 is only one component of the timer. Fourth, p27 levels rise much faster when oligodendrocyte precursor cells are cultured at 33°C than at 37°C, conditions in which the timer runs faster (Gao *et al.*, 1997). Fifth, precursor cells isolated from the optic nerves of p27-deficient mice undergo one or two extra divisions in clonal culture in the presence of PDGF and TH before they differentiate than do precursors from wild-type mice. Detailed analysis of the p27-deficient precursor cells suggests that they are defective in both the timing (Durand *et al.*, 1998) and effector (Casaccia-Bonofil *et al.*, 1997; Durand *et al.*, 1997) components of the timer.

p27-deficient mice grow more rapidly and are about one-third larger than normal mice, despite normal serum levels of growth hormone and insulin-like growth factor 1 (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996). Interestingly, mice with only one copy of the p27 gene inactivated are intermediate in size between wild-type and homozygous-null animals. All of the organs examined in the p27-deficient animals are increased in size and contain more cells than normal, apparently as a result of increased proliferation rather than a decrease in cell death. For example, the hearts of p27-deficient mice are larger and contain more cells than wild-type animals because cardiac myocytes fail to arrest proliferation at the normal time (Poolman *et al.*, 1999). The generalised hyperplasia observed in these animals generalises the findings regarding the role of p27 in timing oligodendrocyte differentiation. Could the increased number of cells be due a failure in multiple p27-dependent timing mechanisms operating in the precursor cells of many different lineages?

CKIs play a role in the timing of cell-cycle arrest and differentiation in developing invertebrates. In *dacapo* mutants of *Drosophila*, cells in several

tissues, including the central nervous system, fail to stop dividing at the appropriate time during embryogenesis (de Nooij *et al.*, 1996; Lane *et al.*, 1996). Most strikingly, epidermal cells, which normally undergo cell-cycle arrest in G<sub>1</sub> of cycle 17 (Foe, 1989; Edgar and O'Farrell, 1990), proceed through a complete additional division cycle, resulting in a 70% increase in epidermal cell number. Ectopic expression of *dacapo* in the epidermis prematurely stops cell division. The *dacapo* gene encodes a CKI that is homologous to mammalian Cip/Kip CKIs and inhibits *Drosophila* cyclin E/CDK2 complexes. Although in *dacapo* mutant embryos the normal timing of cell-cycle exit is delayed, after the additional cycle most cells do become quiescent, probably because of a fall in expression of cyclin E (Knoblich *et al.*, 1994). Thus, *dacapo* normally functions to achieve a precisely timed exit from the cell cycle, rather than being required for cell-cycle arrest *per se*.

The *dacapo* gene is controlled transcriptionally, and is only expressed transiently at high levels when cells exit the cell cycle (de Nooij *et al.*, 1996; Lane *et al.*, 1996). In contrast, p27 accumulates progressively in oligodendrocyte precursor cells as they proliferate (Durand *et al.*, 1997) and remains high in post-mitotic oligodendrocytes throughout life (Yasuhito Tokumoto, unpublished observations). Its expression is controlled by post-transcriptional mechanisms, largely by protein sequestration (Polyak *et al.*, 1994b) and ubiquitin-dependent proteolysis (Pagano *et al.*, 1995). Despite these differences, there is a clear similarity in the role that these CKIs play in determining the precise timing of cell-cycle arrest: in their absence, stopping is delayed but not prevented.

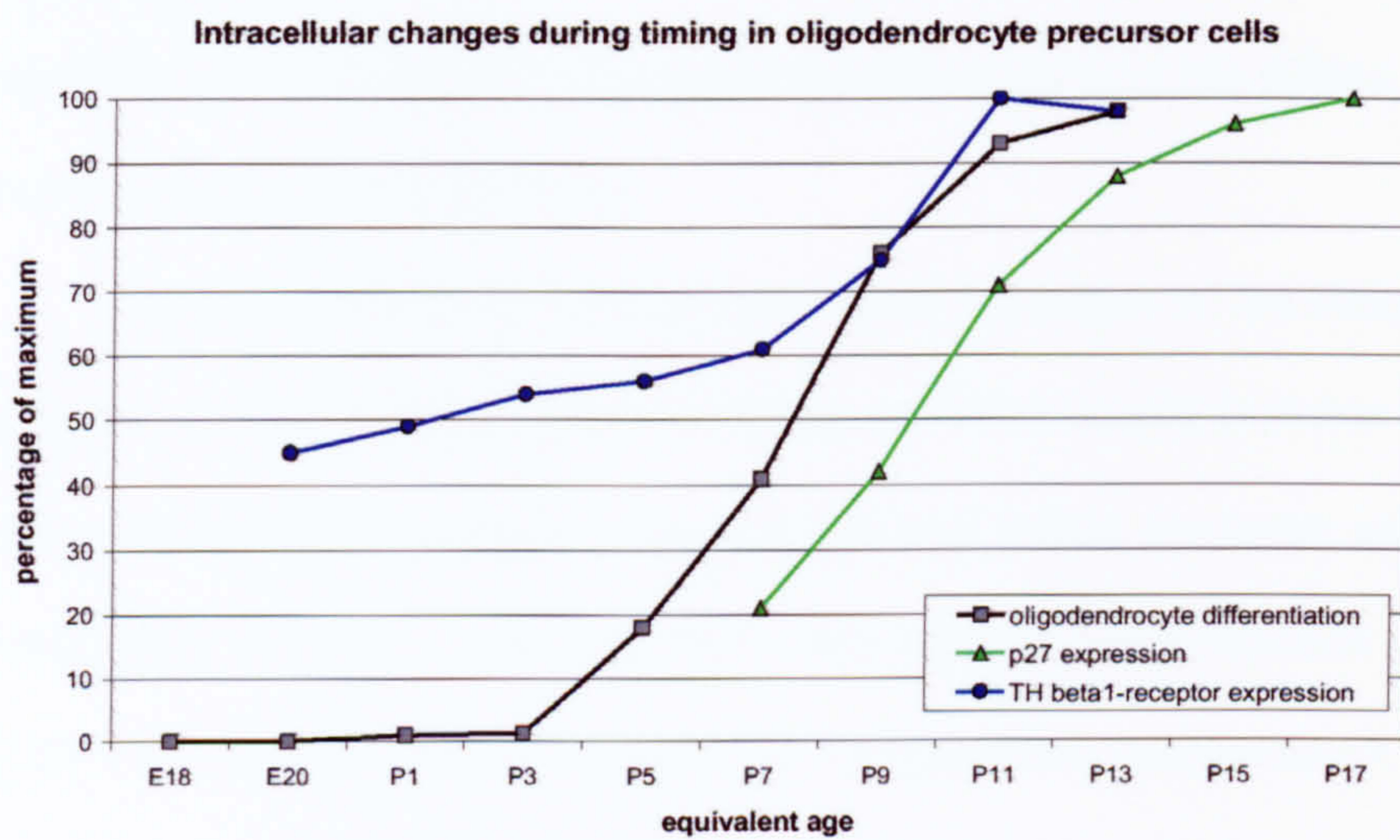


Figure 1-4: Intracellular events during timing in oligodendrocyte precursor cells proliferating in culture. Both p27 and TH  $\beta$ 1-receptor accumulate as the timing component measures elapsed time. All values are expressed as percentages of maximum values. Data compiled from Gao *et al.*, 1998; Durand *et al.*, 1997.



## ***Aims of this study***

It has been nearly twenty years since the discovery of developmental timers in glial cells of the CNS (Abney *et al.*, 1981). We now possess considerable knowledge about the cellular and molecular biology of the oligodendrocyte precursor cell timer, but we still do not understand how it works. How are the changes in the biochemistry of the precursor cells controlled, and how are these changes integrated to produce the effects that we observe as the output of the timer?

One especially perplexing question about the nature of the timer forms the basis for the studies presented in this thesis. What exactly is it that the cell timer controls? Does it measure the time allowed before precursor cells stop proliferating, or does it meter the time until terminal differentiation begins? Perhaps it controls both processes by independent mechanisms? These questions are important because their answers hold the key to the nature of the timer itself.

The predominant view has been that the timer primarily controls the time when oligodendrocyte precursor cells stop dividing. In this view, terminal differentiation is not itself timed, but follows passively as a consequence of cell-cycle arrest. (Gao *et al.*, 1998; Barres *et al.*, 1994). This viewpoint has arisen because in the absence of mitogenic stimulation cultured precursor cells stop dividing and differentiate within a few days (Temple and Raff, 1985; Raff *et al.*, 1983), however, there is no direct evidence that the timer primarily controls cell proliferation. The major barrier to answering this question has been the lack of a means to arrest precursor cell proliferation in the presence of mitogens, which are required to drive the timing component of the timer.

I aimed to inhibit the proliferation of precursor cells that were at an early stage of their timer, in the presence of PDGF. If the cells stopped dividing and differentiated immediately, it is likely that that major influence of the timer is to control when precursors normally exit the cell cycle. Alternatively, if precursors stopped dividing, but did not differentiate until the appropriate time, it is likely that the timer principally controls the timing of terminal differentiation.

## **2 Experiments using transient transfection**

### **methods**

## **2.1 Introduction**

It is not known how the oligodendrocyte precursor cell timer regulates oligodendrocyte development. Does it control the time to cell-cycle exit or the time to terminal differentiation?

Differentiation is the default fate for cultured oligodendrocyte precursor cells when mitogenic signals are withdrawn (Temple and Raff, 1985; Raff *et al.*, 1983). This has fostered the view that the timer primarily controls when oligodendrocyte precursor cells stop dividing, with terminal differentiation following as a direct consequence of exit from the cell cycle. (Gao *et al.*, 1998; Barres *et al.*, 1994). As PDGF is required for the normal running of the timer, a means of arresting proliferation without removing PDGF is necessary to dissociate the controls of proliferation and differentiation. However, in spite of many attempts, it has not been possible to stop the proliferation of oligodendrocyte precursor cells without removing mitogens. Classical chemical inhibitors of the cell cycle, such as hydroxyurea and aphidicholin, have been tried but induce death of PDGF-stimulated oligodendrocyte precursor cells (Martin Raff, personal communication).

I planned to over-express biochemical inhibitors of the cell cycle in oligodendrocyte precursor cells in order to stop proliferation in the presence of PDGF. It was first necessary to develop an effective system for transfecting oligodendrocyte precursor cells. Several conventional methods, such as electroporation and calcium phosphate precipitation, were found to be toxic to these cells (my unpublished observations; Béatrice Durand, personal communication).

I chose two methods for transfecting cultures of oligodendrocyte precursor cells. Lipofection has proven effective for the transfection of many cell types, including primary cells, at high efficiency and low cytotoxicity. It involves binding vector DNA to the surface of cationic liposomes, which are endocytosed by cells *in vitro*. Several commercial organisations have improved the method by including a proprietary reagent that enhances the binding DNA to the liposomes and subsequent uptake by cells. To establish optimal experimental conditions for lipofection, I used a GFP expression vector as a reporter to assay the efficiency of transfection.

The second method involved the use of the Penetratin membrane-translocating peptide. Penetratin is a sixteen amino acid peptide, derived from the third  $\alpha$ -helix of the Antennapedia homeodomain, and is the minimal sequence required for crossing biological membranes (Derossi *et al.*, 1994). The peptide is able to enter cells with high efficiency, in an energy-independent fashion and without requirement for a specific receptor (Derossi *et al.*, 1996). Penetratin consists of several positively charged amino acids and a crucial pair of tryptophans. It has been proposed that cationic Penetratin dimers bind to negatively charged sialic acid residues on the surface of target cells. The tryptophans destabilise the lipid bilayer to form inverted micelles and ultimately release the peptide at the cytosolic surface. The positively charged amino acids then direct Penetratin to become concentrated in the nucleus (Prochiantz, 1996).

Penetratin can be used as a vector to transport peptides or oligonucleotides into cells (Prochiantz, 1996; Derossi *et al.*, 1998). I obtained two peptides consisting of Penetratin fused to the active regions of the cyclin-

dependent kinase inhibitors p16<sup>Ink4a</sup> and p21<sup>Cip1</sup>, termed pen-p16 and pen-p21, respectively (Ball *et al.*, 1997; Fahraeus *et al.*, 1996). These two proteins are the canonical members of the two families of CKIs, which often act in concert to arrest the cell cycle in G<sub>1</sub> (Reynisdottir *et al.*, 1995). To establish optimal experimental conditions, biotinylated Penetratin was used in a reporter assay.

I lipofected mixed optic nerve cells with a GFP reporter plasmid and was able to detect oligodendrocyte precursor cells that were expressing GFP. However, the lipofection procedure seemed to be toxic to the cells: transfected cultures appeared to be unhealthy and did not grow in the same way as control cultures. I was able to show that Penetratin could enter oligodendrocyte precursor cells when added to the culture medium. Unfortunately, when Penetratin peptides were used at concentrations required for biochemical activity they appeared to be toxic to the cells. Consequently, I chose to develop an alternate means to introduce and express exogenous genes in oligodendrocyte precursor cells.

## **2.2 Results**

### **2.2.1 Lipofection of mixed cultures of optic nerve cells**

Mixed cultures of P7 optic nerve cells were transfected with a GFP expression plasmid. After two days, cultures were fixed and stained with antibodies to GFP (Sawin, 1999) and A2B5, to identify oligodendrocyte precursor cells.

Many cells were identified as being GFP<sup>+</sup>/A2B5<sup>+</sup>, indicating that oligodendrocyte precursor cells had been successfully transfected (figure 2-1). However, the efficiency of transfection was always low: generally, around 10% of A2B5<sup>+</sup> cells were also GFP<sup>+</sup> (data not shown).

Of greater concern was the quality of cultures following transfection. Following lipofection, although most cells appeared to remain viable, they looked unhealthy. Numerous oligodendrocyte precursor cells withdrew their processes and clumped together in small groups. Astrocytes, which normally grow into a dense monolayer, remained sparse. Many oligodendrocytes contained condensed and highly refractile nuclei, a sign indicative of apoptosis.

### **2.2.2 Penetratin enters oligodendrocyte precursor cells**

Cells were dissociated from P7 optic nerves and cultured for two days. Biotinylated Penetratin was added to the medium for one hour to allow uptake by the cells, before the medium was replaced and the cells cultured over-night. The cells were fixed, permeabilised, and incubated with streptavidin-conjugated horseradish peroxidase (streptavidin-HRP). The HRP was revealed

by immunocytochemistry using the substrate diaminobenzidine (DAB), which forms dark deposits when oxidised.

Cells incubated with biotinylated Penetratin took up the compound after only one hour, with an efficiency approaching 100% (figure 2-2). The concentrations of pen-p16 or pen-p21 required for optimal biochemical activities are 25 $\mu$ M and 20 $\mu$ M, respectively (Ball *et al.*, 1997; Fahraeus *et al.*, 1996). When either of these peptides, or the biotinylated control, were used at or above 1 $\mu$ M, all of the oligodendrocyte precursor cells died (data not shown). These tests were repeated several times with similar results.



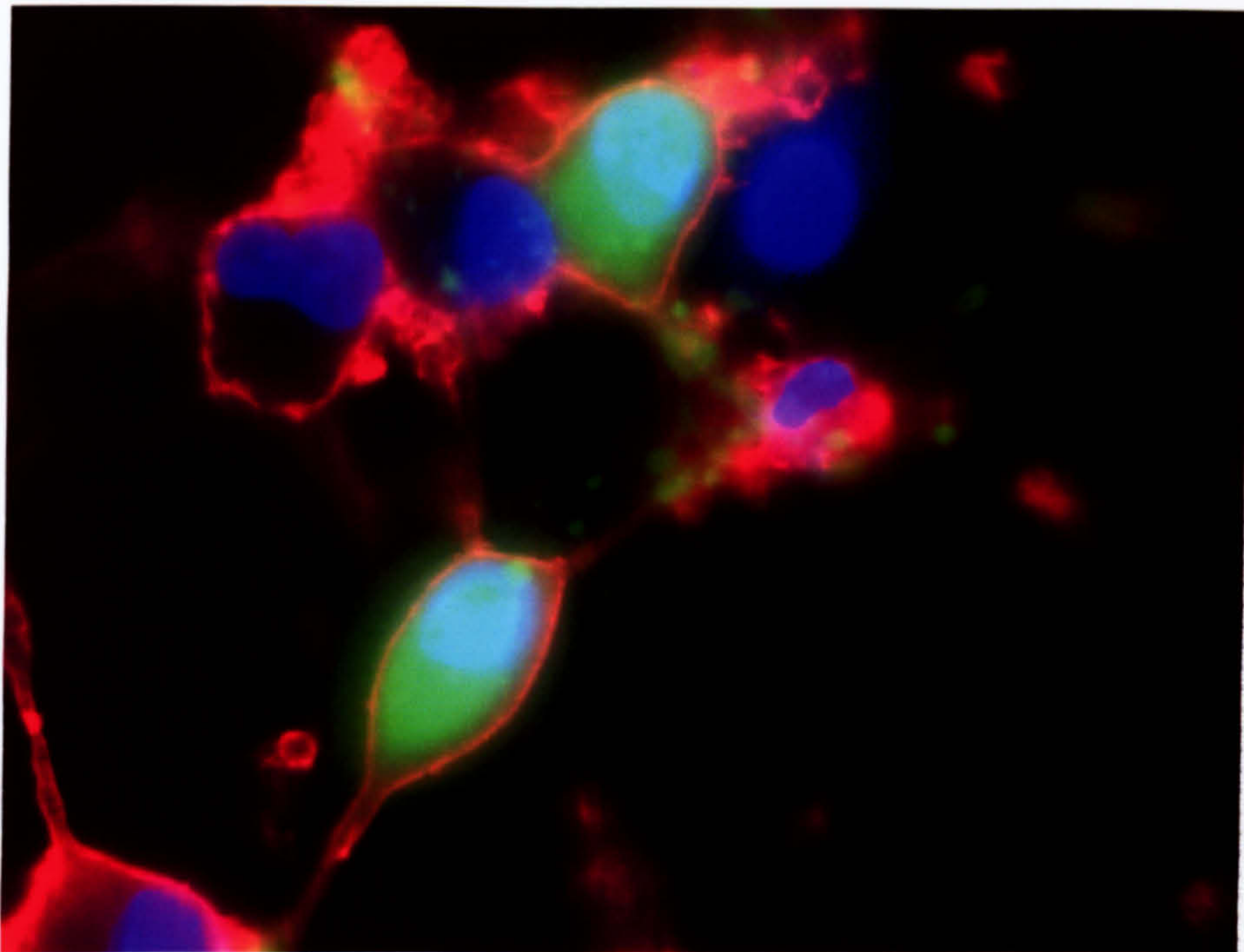


Figure 2-1: Micrograph of oligodendrocyte precursor cells in culture that have been transfected with a GFP expression plasmid. Mixed cultures of P7 optic nerve cells were lipofected with  $0.04\mu\text{g}$  of pEGFP-C1 and stained for GFP (green), A2B5 (red) and DNA (blue).

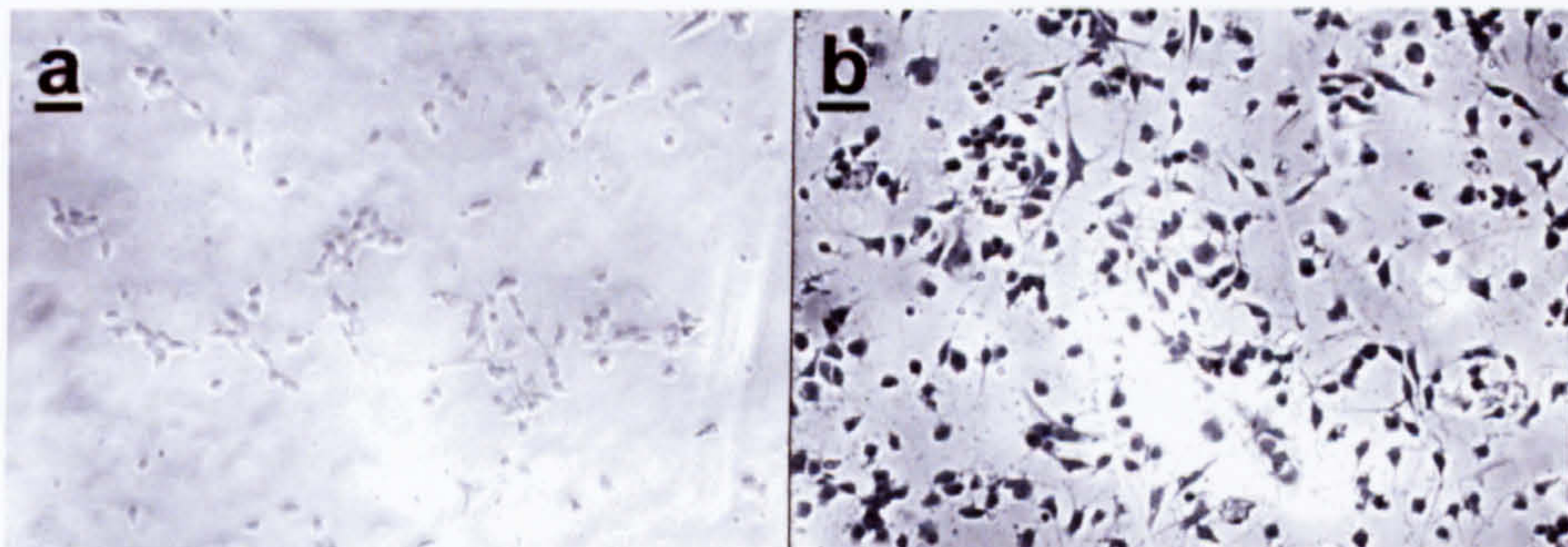


Figure 2-2: Penetratin enters oligodendrocyte precursor cells. Mixed cultures of P7 optic nerve cells were incubated with (a) or without (b)  $0.5\mu\text{M}$  biotinylated Penetratin for one hour. Biotin was detected by immunocytochemistry against streptavidin-HRP using DAB as a substrate.

## **2.3 Discussion**

I wanted to study the effect of prematurely inhibiting the cell cycle of oligodendrocyte precursor cells during the normal running of the intrinsic timer. To this end, I designed new protocols to transfect oligodendrocyte precursor cells in culture, with a view to over-expressing protein cell-cycle inhibitors.

The *in vitro* culture system used to study the development of optic nerve oligodendroglia has significant advantages. Oligodendrocyte precursor cells can be prepared with high purity (>99.5%) and grown in defined medium at clonal density (Barres *et al.*, 1994). Despite these merits, two shortcomings have limited the kinds of study that can be undertaken. Firstly, because of the restricted amount of material available (the best preparations provide only a few thousand cells per nerve) biochemical analysis has proven near impossible. Secondly, the apparent fragility of oligodendrocyte precursor cells has made them difficult to transfect. Electroporation and calcium phosphate precipitation are impracticable because they compromise cell viability for transfection efficiency, and microinjection is inappropriate because the cells are too small.

I have tried two approaches in an attempt to introduce exogenous molecules into oligodendrocyte precursor cells: lipofection and Penetratin-mediated peptide uptake.

Lipofection was relatively more successful, with many oligodendrocyte precursor cells expressing the GFP reporter gene. To my knowledge, this was the first successful expression of GFP in oligodendrocyte precursor cells. Although the efficiency of transfection was low, this should not prove too great an obstacle as only a few transfected cells are required for clonal analysis.

The main problem with the use of lipofection for transfecting oligodendrocyte precursor cells is its deleterious effect on cell viability. Cultures were affected adversely by the procedure, failing to grow normally after exposure to the lipofection reagent. Specifically, oligodendrocyte precursor cells withdrew their processes and bunched into dense clumps, often clustering on astrocytes. These are clear signs that their normal growth was upset. The very nature of this study is to measure processes that govern the normal growth and development of oligodendrocyte precursor cells in culture. As these processes are grossly perturbed by the experimental method, the results obtained would be uninterpretable.

Penetratin has been used successfully to introduce exogenous compounds into many cell types, including primary neurones (Hall *et al.*, 1996). The pen-p16 and pen-21 fusion peptides have been shown to inhibit the proliferation of cell lines (Ball *et al.*, 1997; Fahraeus *et al.*, 1996). Using a sensitive detection method, I showed that Penetratin was taken up rapidly by oligodendrocyte precursor cells. However, I was forced to terminate the experiments because the reagent was toxic at concentrations required for biochemical activity.

## **2.4 Methods**

All chemicals were purchased from Sigma (Poole, UK) unless otherwise stated.

### **2.4.1 Optic nerve dissection and dissociation**

Sprague-Dawley rats were obtained from the animal facility at University College London. Neo-natal (P0) or P5 rat pups were sacrificed by decapitation and the upper part of the skull removed to expose the brain. The olfactory bulb was lesioned and the anterior part of the brain raised to reveal the optic nerves and the optic chiasm. The chiasm was cut free from the cortex and each nerve was severed distally, as near to the back of the eye as possible. The chiasm with adjoining nerves was transferred to a petri dish containing HEPES-buffered minimal Eagle's medium (MEM-HEPES; GibcoBRL, UK). Observing the tissue under a dissecting microscope, the optic nerves were cut from the chiasm and any adhering blood vessels were removed.

The individual nerves were transferred to 4ml MEM-HEPES in a sterile plastic bijou (Sterilin) and chopped into small pieces. Nerves were enzymatically dissociated by addition of 160 units of papain solution (Lorne Laboratories) and 2mg cysteine, and incubation at 37°C for 30 minutes. Proteolysis was halted by addition of a low concentration of ovomucoid (Boehringer-Mannheim) and bovine serum albumin (BSA; 1.5mg.ml<sup>-1</sup> of each) in MEM-HEPES. After brief centrifugation, the nerves were resuspended in a high concentration of ovomucoid and BSA (6mg.ml<sup>-1</sup> of each) in MEM-HEPES. Cells were mechanically dissociated from the nerves by repeated trituration

with a Gilson Pipetteman set at 500 $\mu$ l and subsequent filtration through 20 $\mu$ m nylon mesh. The average yield of cells was approximately  $20 \times 10^3$  per nerve for a P0 animal and  $40 \times 10^3$  per nerve for a P5 animal.

#### **2.4.2 In vitro culture of mixed optic nerve cells and purified oligodendrocyte precursor cells**

Mixed optic nerve cells were cultured in serum-free medium, termed B-S medium, with added growth factors, mitogens and other signalling molecules (modified from Bottenstein and Sato, 1979). Basic B-S medium consists of Dulbecco's modified Eagle's medium (DMEM) with high glucose and sodium pyruvate and containing bovine insulin ( $10\mu\text{g.ml}^{-1}$ ), human transferrin ( $100\mu\text{g.ml}^{-1}$ ), BSA ( $100\mu\text{g.ml}^{-1}$ ), progesterone ( $60\text{ng.ml}^{-1}$ ), putrescine ( $16\mu\text{g.ml}^{-1}$ ), sodium selenite ( $40\text{ng.ml}^{-1}$ ), N-acetylcysteine ( $60\mu\text{g.ml}^{-1}$ ), forskolin ( $5\mu\text{M}$ ), penicillin ( $100\text{U.ml}^{-1}$ ) and streptomycin ( $100\mu\text{g.ml}^{-1}$ ). In every experiment we added platelet-derived growth factor (PDGF; Preprotech) ( $10\text{ng.ml}^{-1}$ ) and neurotrophin-3 (NT-3; Preprotech) ( $5\text{ng.ml}^{-1}$ ).

Cells were cultured in either 25cm<sup>2</sup> flasks (Falcon) with 3ml of medium, 10cm<sup>2</sup> slide-flasks (Nunc) with 2ml of medium, or on 11mm glass coverslips (Chance Proper) in 24 well tissue-culture dishes (Falcon) with 1ml medium. All culture surfaces were coated with poly-D-lysine (PDL),  $10\mu\text{g.ml}^{-1}$  in distilled water overnight, prior to use. The cultures were maintained in an 8% CO<sub>2</sub> incubator at 37°C and were fed by replacing half of the medium with fresh medium (containing enough new PDGF, NT-3, and TH, if required, for the entire volume) every 2-4 days.

### **2.4.3 Lipofection of mixed optic nerve cultures**

Optic nerve cells were prepared as described and cultured overnight on PDL-coated coverslips in B-S medium containing PDGF (10ng.ml<sup>-1</sup>) and NT-3 (5ng.ml<sup>-1</sup>). The following morning the cells were transfected with a GFP reporter plasmid, pEGFP-C1 (figure 3-5; Clontech #6084-1; Genbank #U55763), using Lipofectamine PLUS (GibcoBRL) as follows.

PLUS reagent, 3µl, was mixed with 0.04µg of plasmid DNA and 25µl of B-S medium without mitogens, and left to stand at room temperature for 15 minutes. After addition of 0.75µl Lipofectamine and 25µl medium the mixture was incubated for a further 15 minutes at room temperature. Growth medium was aspirated from cultures and replaced with 200µl B-S medium containing PDGF and NT-3. The DNA/Lipofectamine/PLUS mixture was added, dropwise, to the cultures which were then returned to the incubator for three hours. After this time, the lipofection medium was removed and replaced with 1ml of fresh B-S medium containing PDGF and NT-3. After two days culture at 37°C, coverslips were fixed and stained with antibodies against GFP and A2B5.

### **2.4.4 Immunofluorescence staining**

For staining cells on their surface for A2B5, cultures were washed in PBS and fixed in 4% paraformaldehyde for five minutes at room temperature. After washing, they were incubated for 30 minutes at room temperature in A2B5 monoclonal antibody (Eisenbarth *et al.*, 1979; ascites fluid, diluted 1:100 in 20% goat serum in TBLS {50mM Tris-HCl, pH 7.4, 1% BSA, 10mM L-

lysine, 85mM sodium chloride}), followed by Texas Red-conjugated goat anti-mouse IgM (Amersham; diluted 1:100 in TBLs).

For intracellular staining of GFP, the cells were permeabilised with ethanol/acetic acid (20:1) for five minutes. After thorough washing, the coverslips were stained with rabbit anti-GFP (a kind gift from Dr Ken Sawin, Imperial Cancer Research Fund, London, UK; diluted 1:500 in TBLs; Sawin, 1999) for 30 minutes at room temperature, followed by FITC-conjugated anti-rabbit Ig (Amersham; diluted 1:100 in TBLs) and  $1\mu\text{g.ml}^{-1}$  bisbenzimidazole #33342 to stain DNA. The coverslips were mounted in Citifluor mounting medium (Citifluor UKC, UK) on glass slides, sealed with nail varnish, and viewed with a Zeiss Axioplan fluorescence microscope (Thornton, NY).

#### **2.4.5 Penetratin-mediated transfection of optic nerve cells cultures**

Mixed cell cultures were prepared from P7 optic nerves and cells were plated at  $25 \times 10^3$  per PDL-coated coverslip. Cultures were grown in B-S medium including PDGF and NT-3 for two days at  $37^\circ\text{C}$ . Stock solutions of biotinylated Penetratin, 2.5mM in dimethylsulphoxide (DMSO), were stored at  $-20^\circ\text{C}$  and, if necessary, working dilutions made in DMSO immediately prior to use. The volume of medium was reduced to 250 $\mu\text{l}$  and biotinylated Penetratin was added to a final concentration of 0.5 $\mu\text{M}$ . For the controls, an equal amount of DMSO alone was added. The concentration of DMSO in cultures never exceeded 0.5% (v/v). Cultures were incubated at  $37^\circ\text{C}$  for one hour before the medium was replaced with fresh B-S medium containing

mitogens. The following days, cultures were fixed with ethanol/acetic acid (20:1) for five minutes. After thorough washing in PBS, streptavidin-conjugated horseradish peroxidase (Amersham; diluted 1:200 in 20% goat serum, 0.1% Tween in PBS) was added for 30 minutes at room temperature. Horseradish peroxidase was detected by reaction with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.03% cobalt (II) chloride and 0.02% hydrogen peroxide for seven minutes at room temperature. The coverslips were washed in PBS, post-fixed in 4% paraformaldehyde and mounted on glass slides in Citifluor mounting medium. Samples were viewed with a Zeiss Axioplan microscope using phase contrast optics to detect the black deposits of DAB.



### **3 Construction and testing of two new retroviral vectors**

### **3.1 Introduction**

I aimed to develop a method to over-express exogenous transgenes in oligodendrocyte precursor cells. Previous attempts, using conventional transfection methods, failed because the procedures employed were too harsh and proved toxic to the cells.

I chose to use a retroviral method for gene transduction. Retroviruses have a proven efficacy in this lineage, both as vectors for gene over-expression and as lineage tracers (Groves *et al.*, 1993; Raff *et al.*, 1993; Cepko, 1988; Price *et al.*, 1987). For these studies, they have several advantages over conventional transfection procedures. Firstly, under most circumstances, retroviruses infect dividing cells but not post-mitotic cells (Ito and Kedes, 1997). This ensures that in a mixed population only precursor cells, and not oligodendrocytes, are transduced. Secondly, once the retroviral genome has integrated into the genome of the host cell, it is replicated with host DNA during cell division. Consequently, the transduced genes are stably inherited by every daughter cell produced after the initial infection event. This contrasts with transient transfection in which vector DNA is randomly distributed between successive generations and is eventually titrated out of the population. Thirdly, all daughter cells that carry the integrated provirus receive the same dose of the expressed gene(s). When cells are transiently transfected, however, one effect of vector titration is that each generation receives half the gene dose of the one before.

I wanted to study the effect of gene over-expression on oligodendrocyte precursor cells over several days in culture at clonal density. It is extremely difficult to obtain retroviral titres that are high enough to infect all cells in a

culture (Morling and Russell, 1995). In developing this system, it was necessary to provide a means of reliably identifying cells that were carrying the transduced genes, without having to fix and stain infected cultures. Consequently, I designed a new retrovirus that would co-express green fluorescent protein (GFP; reviewed in Tsien, 1998) as a marker. With this facility, it should be possible to observe infected and uninfected clones of cells as they expand over several days.

I constructed two retroviruses that were based upon the Moloney murine leukaemia retrovirus backbone of the pBabe series of vectors (Morgenstern and Land, 1990). Each retrovirus was designed to use a different strategy to co-express GFP and a transgene of interest from its genome. The first used an internal ribosome entry site (IRES) to allow the translation of both proteins from a single mRNA transcript (Jang *et al.*, 1990; Jang and Wimmer, 1990; Jang *et al.*, 1989). The second contained two promoters to express the gene of interest and GFP from independent transcripts.

Each method of co-expression has particular merits. Using an IRES to express both genes from a single transcript ensures that expression of GFP reports expression of the upstream transgene. However, the efficiency of translation from an IRES is generally of the order of 1-3% of that from a 5'-capped end of a mRNA, and at best around 30% (Borman *et al.*, 1994; Kaminski *et al.*, 1994; Kuhn *et al.*, 1990). Such reduced expression may be sufficient for detection of an enzymatic marker, as the marker can produce an amplified signal through histochemical catalysis, but may be problematic for detection of stoichiometric markers such as GFP. Conversely, expression from a separate promoter will produce a sufficient amount of GFP for direct

detection but additional validation will be necessary to prove that GFP expression faithfully reports co-expression of the upstream transgene.

## **3.2 Results**

I constructed two retroviruses to co-express a transgene of interest and GFP in oligodendrocyte precursor cells. The first, pFoxIV, was designed to express both genes from a single transcript using an IRES sequence to permit re-initiation and translation of the second protein. The second, pBird, was designed using separate promoters to express each protein.

I experienced serious technical difficulties with the construction of pFoxIV that rendered it unusable. Although it may be possible to rescue this vector, the construction of pBird was successful and, consequently, I used this latter vector in the remainder of my experiments.

Here, I summarise the important steps of the design and subsequent construction of the retroviral vectors. For a detailed description the reader should refer to the Methods section of this chapter (see §3.4.1.6). Full sequences of the constructs described are to be found in the appendix.

### **3.2.1 The single-transcript vector: pFoxIV**

#### **3.2.1.1 Design and construction**

I aimed to construct a retroviral vector containing an IRES sequence to drive the expression of GFP. I purchased a commercially available vector, pIRES-EGFP, that contained the IRES derived from the encephalomyocarditis virus (EMCV) fused to enhanced GFP (figure 3-1; Cormack *et al.*, 1996). To enhance the quantum efficiency of fluorescence, the EGFP coding sequence was selectively mutagenised to change three amino acids, producing a brighter variant known as KGFP (Jay Morgenstern, personal communication). In

addition, the IRES was augmented (IRES<sup>+</sup>) to increase the efficiency of translation initiation (Derek Knight, personal communication).

The modified vector, pIRES<sup>+</sup>-KGFP, was tested for functional expression of GFP by transfection into NIH3T3 cells and was found to be significantly brighter than the parent vector, pIRES-EGFP (data not shown).

To create a more useful multiple cloning site (MCS) in the final vector, the IRES<sup>+</sup>-KGFP sequence was subcloned into pGimscript, a form of pBluescript whose MCS had been extended by insertion of a synthetic oligonucleotide. Finally, the MCS-IRES<sup>+</sup>-KGFP fragment was excised from pGimscript and subcloned into the retroviral vector pBabe.PURO (figure 3-2) to form pFoxIV (figure 3-3).

### **3.2.1.2 Sequencing and expression**

To verify the construction of pFoxIV, the vector was subjected to DNA sequence analysis and expression screening. To test for expression of GFP, this construct was electroporated into NIH3T3 cells, however, no green fluorescence was observed. When the construct was transfected into an ecotropic packaging line and supernatant collected, no infectious viral particles, as determined by titration for puromycin resistance, were detected. The sequence is detailed in full in the appendix and is summarised in figure 3-4. There was a large deletion of approximately 150 base pairs at the beginning of the IRES. In addition, there were several single base insertions or substitutions. Many of these clustered in the untranslated part of the retroviral *gag* gene that is present in the vector to enhance the expression of the viral genome. Another substitution (G→T) occurred within the KGFP open reading frame resulting in

a codon change from Asp102→Phe. This suggests that there had been a serious technical failure during the construction of this vector, which rendered it dysfunctional.

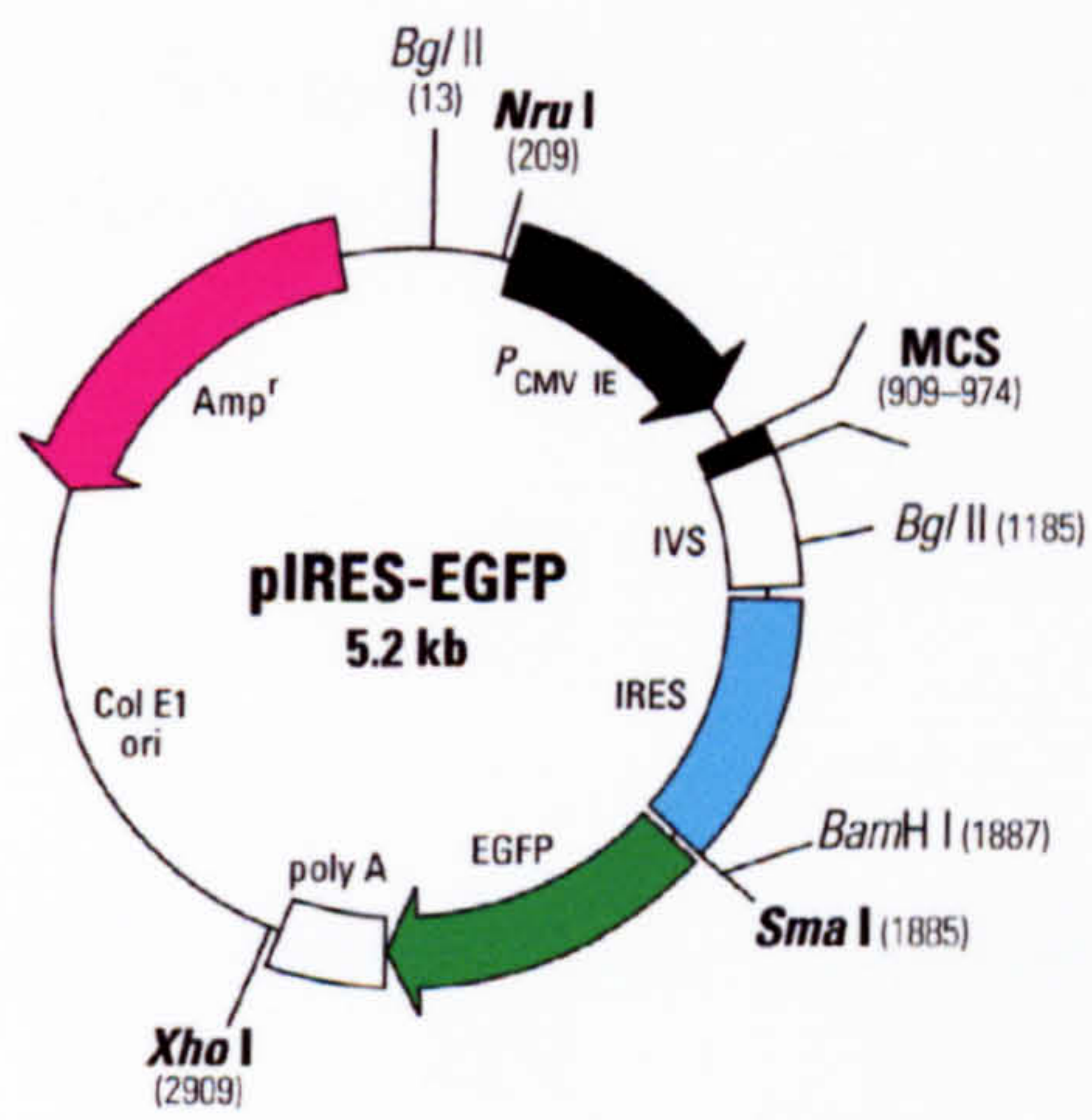


Figure 3-1: Map of pIRES-EGFP highlighting important regions of sequence



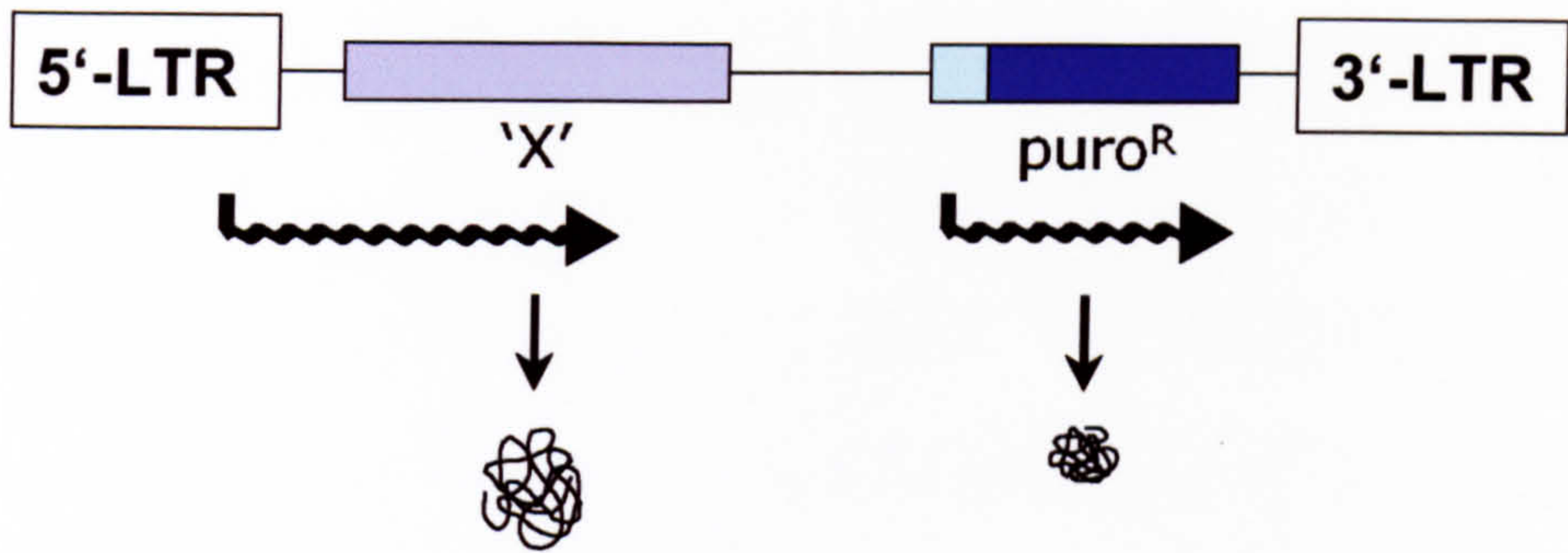


Figure 3-2: Schematic map of pBabe.PURO. The gene of interest ('X') is expressed from the retroviral 5'-long terminal repeat (LTR) promoter and the puromycin resistance gene ( $puro^R$ ), used to select for stable integration of the provirus, is expressed from a separate promoter.

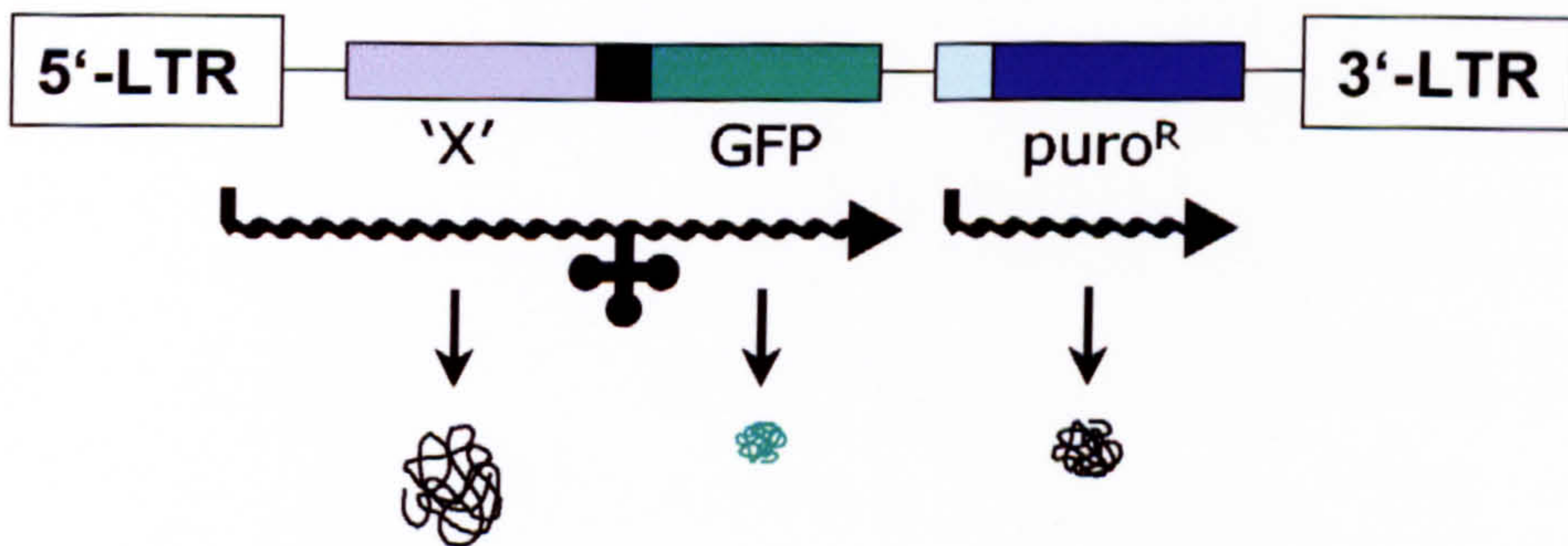


Figure 3-3: Schematic map of pFoxIV. The gene of interest ('X') is expressed from the retroviral 5'-long terminal repeat (LTR) promoter. The IRES, represented by a cloverleaf structure in the transcript, allows reinitiation of translation permitting expression of the GFP coding sequence. The puromycin resistance gene ( $puro^R$ ) is expressed from a separate promoter.

```

pFoxIV design.seq  ACGTTGGGTTACCTTCTGCTCTGCAGAAATGGCCAACTTTAAAGTGGGATGGCCGCGAGACGGCACCTTTAACCGAGACC 1108
pFoxIV sequence.seq ACGTTGGGTTACCTTCTGCTCTGCAGAAATGGCCAACTTTAAAGTNGGATGGCCGCGAGACGGCACCTTTAACCGAGACC 80

pFoxIV design.seq  TCATCACCAGGTTAAGATCAAGGCTTTTTCACCTGGCCCGCATGGACACCCAGACCAGGT-CCCTACATCGTGACCTG 1187
pFoxIV sequence.seq TCATNANCCAGGTTAAGATNAAGGNTTTTTCACCTGGCCNGCATGGANANCCAGACCAGGTNCCCTACATCGTGACCTG 160

pFoxIV design.seq  GGAAGCCTTGGCTTTTGACCCCC-TCCTGGGTCAAGCCCTTTGTACACCCTAAGCCTCCGCTCCTCTTCTCCATCC 1266
pFoxIV sequence.seq GGAAGCCTTGGCTTTTGACCCCCTTCCCTGGGTCAAGCCCTTTGTACACCCTAAGCCTCCGCTCCTCTTCTCCATCC 240

pFoxIV design.seq  GCGCGTCTCTCCCCCTTGAACCTCCTCTTTGACCCCGCCTCAATCCTCCCTTTATCCAGCCCTCACTCCTTCTCTAGG 1346
pFoxIV sequence.seq GCCCGTCTNTCCCCCTTGAACCTCCTCGTTGACCCCGCCTCGATCCTCCCTTTATCCAGCCCTCACTCCTTCTCTAGG 320

pFoxIV design.seq  CGCCGG-CCGGATCCTTCGAAGCGCTGTGACTACGTATCCGGAGAATTCCGTACGCCGGGCCGCGGTGCGACTGCAG 1425
pFoxIV sequence.seq CGCCGGCCGGATCCTTCGA-GNGCTGTGACTACGTATCCGGAGAATTCCGTACGCCGGGCCGCGGTGCGACTGCAG 399

pFoxIV design.seq  GTCGAGCATGCATCTAGGGC-GGCCAATTCGCCCCCTCTCCCTCCCCCCCCCTAAAGTTACTGGCCGAAGCCGCTTGA 1504
pFoxIV sequence.seq GTCGAGCATGCATCTAGGGCCGGCCAATTCGCCCC-T-----CTCCCCCCCCC-----C-----CT--C----- 451

pFoxIV design.seq  ATAAGGCCGGTGTGCGTTTGTCTATATGTGATTTCCACCATATTGCGTCTTTTGGCAATGTGAGGGCCCGAAACCTG 1584
pFoxIV sequence.seq -----TC----- 453

pFoxIV design.seq  GCCCTGTCTTCTTACGAGCATTCTAGGGGTCTTTCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAG 1664
pFoxIV sequence.seq -----CGCCAAAGGAATG-AAGGTNTGTTGAATGTCGTGAAG 489

pFoxIV design.seq  GAAGCAGTTCCCTCTGGAAGCTTCTTGAAGACAAACAACGCTGTAGCGACCCCTTTCAGGCAGCGGAACCCCCACCTGG 1744
pFoxIV sequence.seq GAAGCAGTTCCCTCTGGAAGCTTCTTGAAGACAAACAACGCTGTAGCGACCCCTTTCAGGCAGCGGAACCCCCACCTGG 569

pFoxIV design.seq  CGACAGGTGCCTCTGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAAGCGGCACAACCCAGTGCCACGTTGTGA 1824
pFoxIV sequence.seq CGACAGGTGCNTHT---GCCAAAAGCCACGTGTATAAGATACACCTGCAAAAGCGGCACAACCCAGTGCCACGTTGTGA 646

pFoxIV design.seq  GTTGGATAGTTGTGGAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCAGAGGTACCC 1904
pFoxIV sequence.seq GTTGGATAGTTGTGGAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCAGAGGTACCC 726

pFoxIV design.seq  CATTGTATG-GGATCTGATCTGG-----GGCCTCG-GTGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAACGTC 1977
pFoxIV sequence.seq CATTGTA-GTGG---GNTCTNNNNNNNGGCCT-GCGTGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAACGTC 800

pFoxIV design.seq  TAGGCCCCCGAACCCAGGGGACGTGGTTTT-CCTTTGAAAAACACGATGATAATATGGCCACAACCATGGGACCGGTCG 2056
pFoxIV sequence.seq TAGGCCCCCGAACCCAGGGGACGTGGTTTTNCCTTTGAAAAACACGATGATAATATGGCCACAACCATGGGACCGGTCG 880

pFoxIV design.seq  CCACCATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGTGGTGCCCATCCTGGTGCAGCTGGACGGCGACGTAAACGGC 2136
pFoxIV sequence.seq CCACCATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGTGGTGCCCATCCTGGTGCAGCTGGACGGCGACGTAAACGGC 960

pFoxIV design.seq  CACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCCTGAAGTTCATCTGC-ACCACCG 2215
pFoxIV sequence.seq CACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCCTGAAGTTCATCT-CGACCACCG 1039

pFoxIV design.seq  GCAA 2219
pFoxIV sequence.seq GCAA 1043

```

Figure 3-4: Sequence alignment between the designed pFoxIV ('design') and the real pFoxIV ('sequence'). Apart from several single nucleotide insertions and substitutions, the main feature is the large deletion that corresponds to the first 150bp of the IRES. Matched bases are shown in black and joined by a vertical line. Bases in red are mismatched. N = base not determined during sequencing. - = no corresponding base.

### **3.2.2 The dual-promoter vector: pBird**

The aim was to construct a retrovirus to express a gene of interest and GFP from separate promoters. There were two possible ways to accomplish this: the gene of interest could be expressed from the retroviral LTR promoter and GFP from a sub-genomic promoter, or *vice versa*. Retroviral LTR promoters are estimated to express genes at lower levels than other constitutive promoters that are used in modern expression vectors, such as SV40 or CMV, may express genes at orders of magnitude above endogenous levels (Guo *et al.*, 1996; Ray and Gage, 1992). I chose to express the gene of interest from the LTR promoter so that the level of gene expression, relative to the endogenous amount, would not be too great.

Starting with pBabe.PURO, I replaced the puromycin resistance gene cassette with the EGFP expression cassette from pEGFP-C1 (figure 3-5). As I did not intend to create stable packaging cell lines containing this virus, the antibiotic selection gene was redundant. The CMV promoter and EGFP coding sequence were amplified from pEGFP-C1 using the polymerase chain reaction (PCR) incorporating oligonucleotide primers that contained unique restriction sites at either end. The PCR product was directionally cloned into the pBabe backbone, from which the puromycin cassette had been excised using the same two restriction enzymes. The resultant vector, pBird (figure 3-6), was identified by restriction mapping.

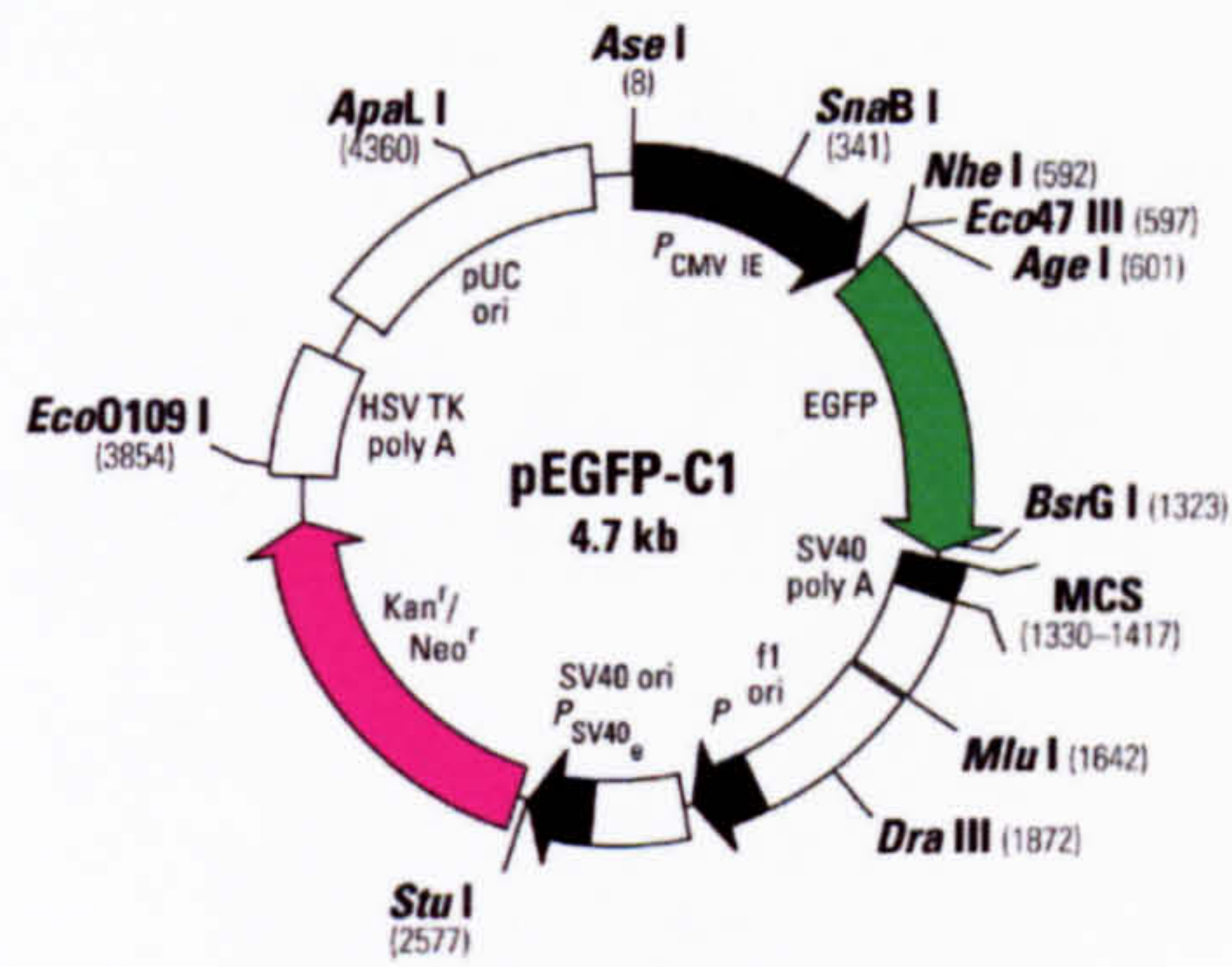


Figure 3-5: Map of pEGFP-C1 highlighting regions of important sequence.

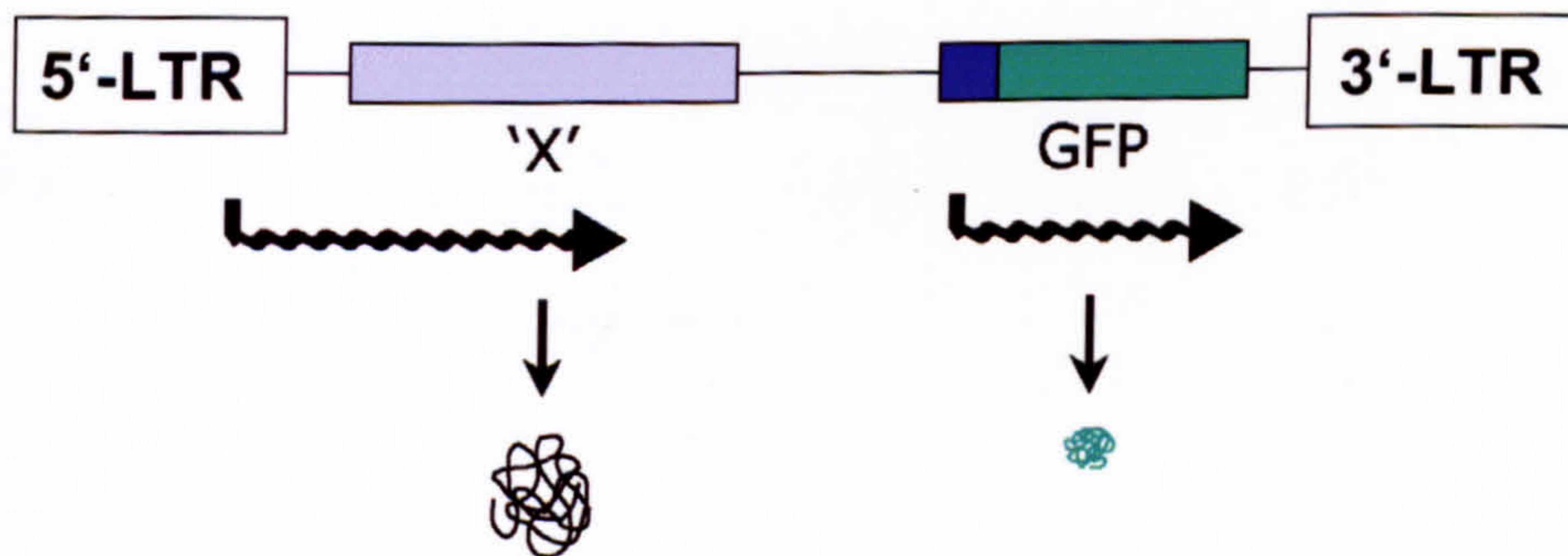


Figure 3-6: Schematic map of pBird. The gene of interest ('X') is expressed from the 5'-retroviral long terminal repeat (LTR) promoter and GFP from a separate promoter.

### **3.2.3 Oligodendrocyte precursor cells infected with pBird express detectable levels of GFP**

Restriction mapping identified that the subcloning steps had produced a vector with a structure that was consistent with pBird. However, before continuing with the construction of recombinant retroviruses, I wanted to ensure that pBird could express detectable levels of GFP from its internal promoter in oligodendrocyte precursor cells.

Oligodendrocyte precursor cells were purified from P7 optic nerves by immunopanning and cultured overnight. Supernatant containing pBird retrovirus was prepared by transient transfection of an ecotropic packaging cell line with the pBird vector. Cultures were infected by exposure to pBird supernatant for three hours. After five days, cultures were observed using a Zeiss Axioplan fluorescence microscope.

The GFP signal from infected cells was clearly observed. Infected cells fluoresced strongly and did not photobleach quickly. Most GFP<sup>+</sup> cells appeared morphologically as oligodendrocyte precursor cells (figure 3-7a) but there were a few GFP<sup>+</sup> oligodendrocytes (figure 3-7b).

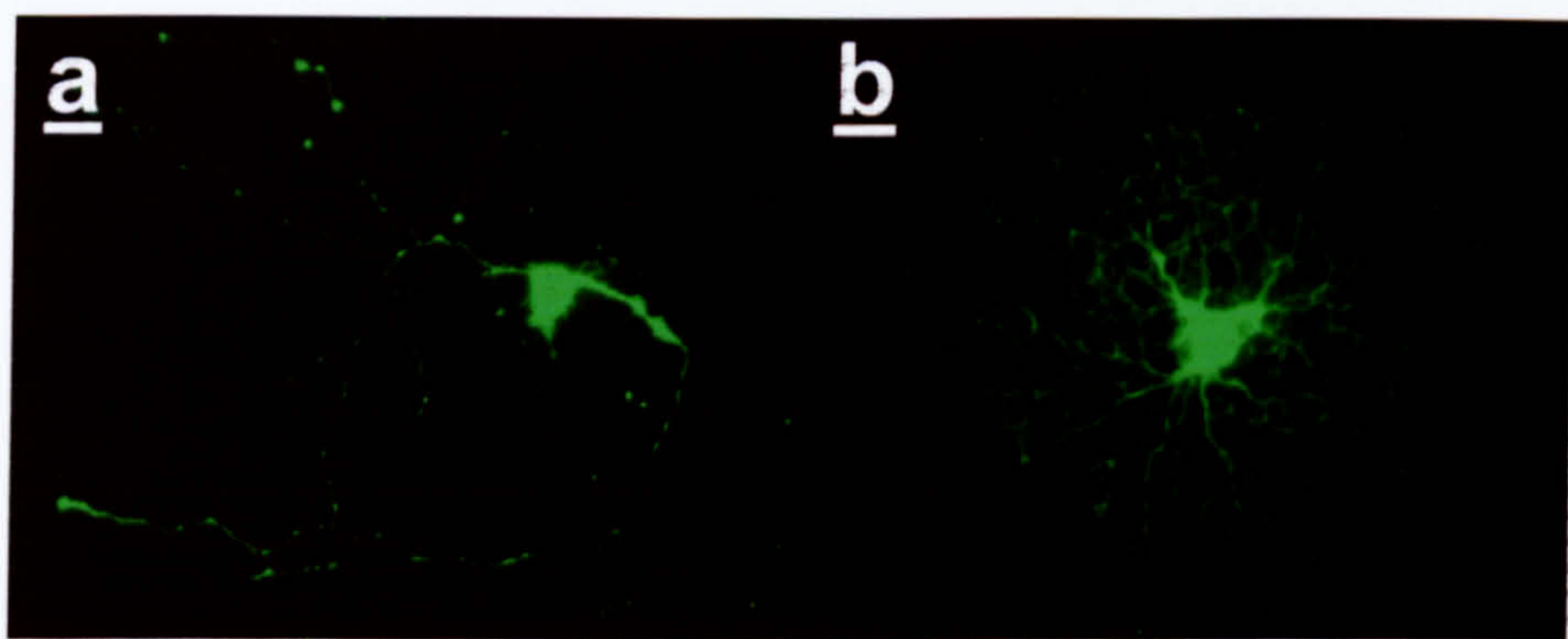


Figure 3-7: Fluorescence micrographs of (a) an oligodendrocyte precursor cell, and (b) an oligodendrocyte expressing GFP following infection with pBird.

### **3.2.4 GFP expression faithfully reports co-expression of the upstream transgene**

The design of pBird results in each gene being expressed from separate, independently regulated promoters. It was necessary, therefore, to show that expression of GFP from the internal CMV promoter faithfully reported co-expression of the upstream gene from the viral LTR promoter. To interpret results obtained with this vector, I had to be confident that all GFP<sup>+</sup> cells co-express the transgene of interest, and that all cells that express the transgene are GFP<sup>+</sup>.

I attempted to prove the efficacy of this technique using three independent approaches. Three different retroviruses were constructed to co-express the following genes together with GFP (table 3-1). Expression of  $\beta$ -galactosidase can be detected using a histochemical reaction with the X-gal substrate (Maniatis *et al.*, 1982). Constitutively-active Harvey Ras (Ha-Ras<sup>V12</sup>) can be detected functionally because it induces neuronal differentiation of the rat pheochromocytoma cell line, PC12 (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985). Finally, p27 expression can be detected by indirect immunofluorescence in cells derived from mice that have had both copies of the p27 gene ablated by homologous recombination (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996).

Table 3-1 – A list of retroviruses constructed and the transgenes that they contained.

Transgene	Retrovirus
$\beta$ -galactosidase	pBird. $\beta$ gal
Ha-Ras <sup>V12</sup>	pBird.Ras
human p27 <sup>Kip1</sup>	pBird.p27

### **3.2.5 Co-expression of GFP and $\beta$ -galactosidase**

Purified oligodendrocyte precursor cells were infected with pBird. $\beta$ gal, which was produced by concentration of supernatant derived from a transiently transfected packaging cell line. After five days, the cells were observed using an MRC-600 inverted confocal fluorescence microscope. Patches of cells that expressed GFP were marked and micrographs of GFP fluorescence were recorded. The cells were then fixed, permeabilised and, for detection of  $\beta$ -galactosidase, incubated with the X-gal substrate overnight at room temperature. The following day, transmission micrographs of the marked patches of cells were taken and compared with the images of GFP fluorescence recorded previously.

In all cases, cells that expressed GFP were also stained with the blue product of the X-gal histochemical reaction (figure 3-8). This was the first piece of evidence to establish that expression of GFP faithfully reported expression of the transgene driven by the retroviral LTR promoter.



### **3.2.6 Co-expression of GFP and constitutively-active Ras**

The day prior to infection, stock cultures of PC12 cells were replated into six-well tissue culture dishes, at a density of  $10^5$  cells per well, and were returned to the incubator over-night. The culture medium was replaced with 2.0ml per well of supernatant containing the pBird.Ras retrovirus, which encodes a constitutively-active Ras transgene, collected from transiently transfected packaging cells, containing  $8\mu\text{g}\cdot\text{ml}^{-1}$  polybrene to enhance infection (Themis *et al.*, 1998). After three hours incubation at  $37^\circ\text{C}$ , the medium was replaced with fresh growth medium, and the cells were returned to the incubator for a further three days.

Cultures were lightly fixed and bisbenzimidazole was added to co-stain DNA. The cells were observed using a Zeiss Axioskop fluorescence microscope, and micrographs were recorded using a Hamamatsu CCD camera. Montages of the GFP and bisbenzimidazole fluorescence images were created using Adobe Photoshop.

Expression of constitutively-active Ras induced differentiation of PC12 cells into a neuronal phenotype (figure 3-9). Characteristically, transduced cells stop dividing and extend neurites that can be visualised easily by transmission microscopy. In the image presented, which is a typical of many fields observed in two independent experiments, cells expressing GFP can be seen to have extended neurites. Cells that were not expressing GFP did not extend neurites.

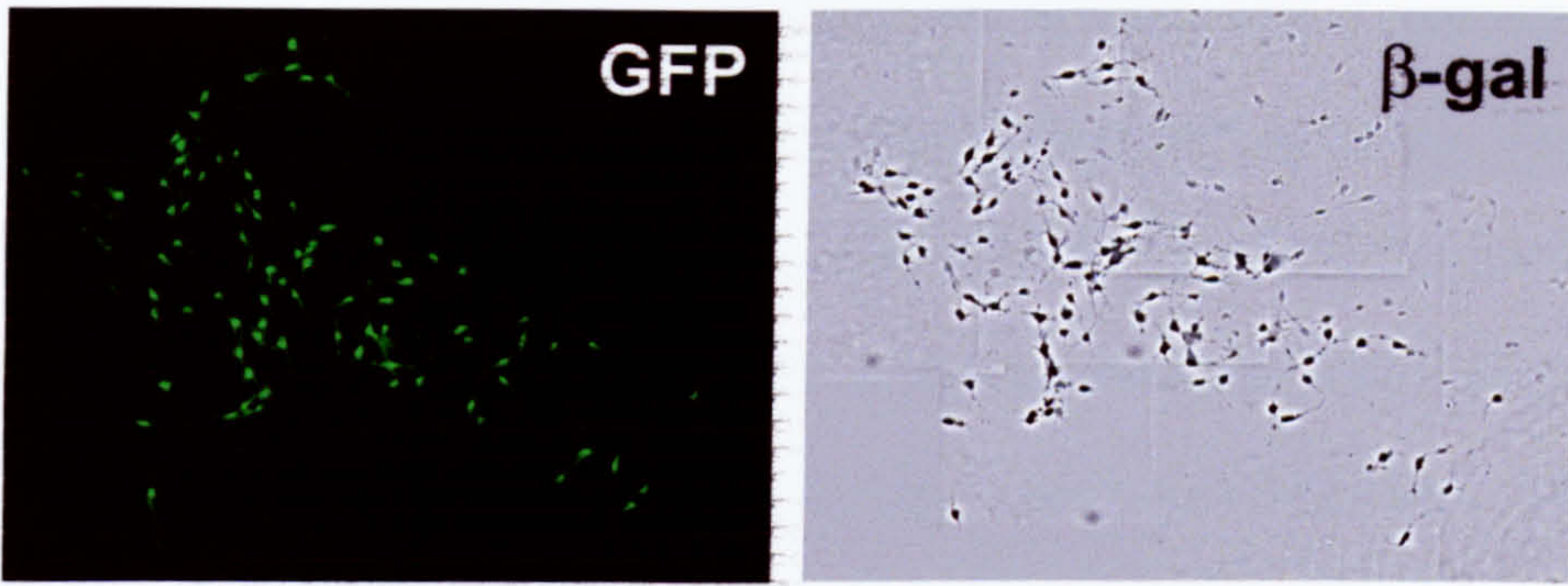


Figure 3-8: Micrographs of GFP fluorescence and  $\beta$ -galactosidase histochemistry. The images show the same field and indicate that every cell that expresses GFP co-expresses  $\beta$ -galactosidase, and *vice versa*.

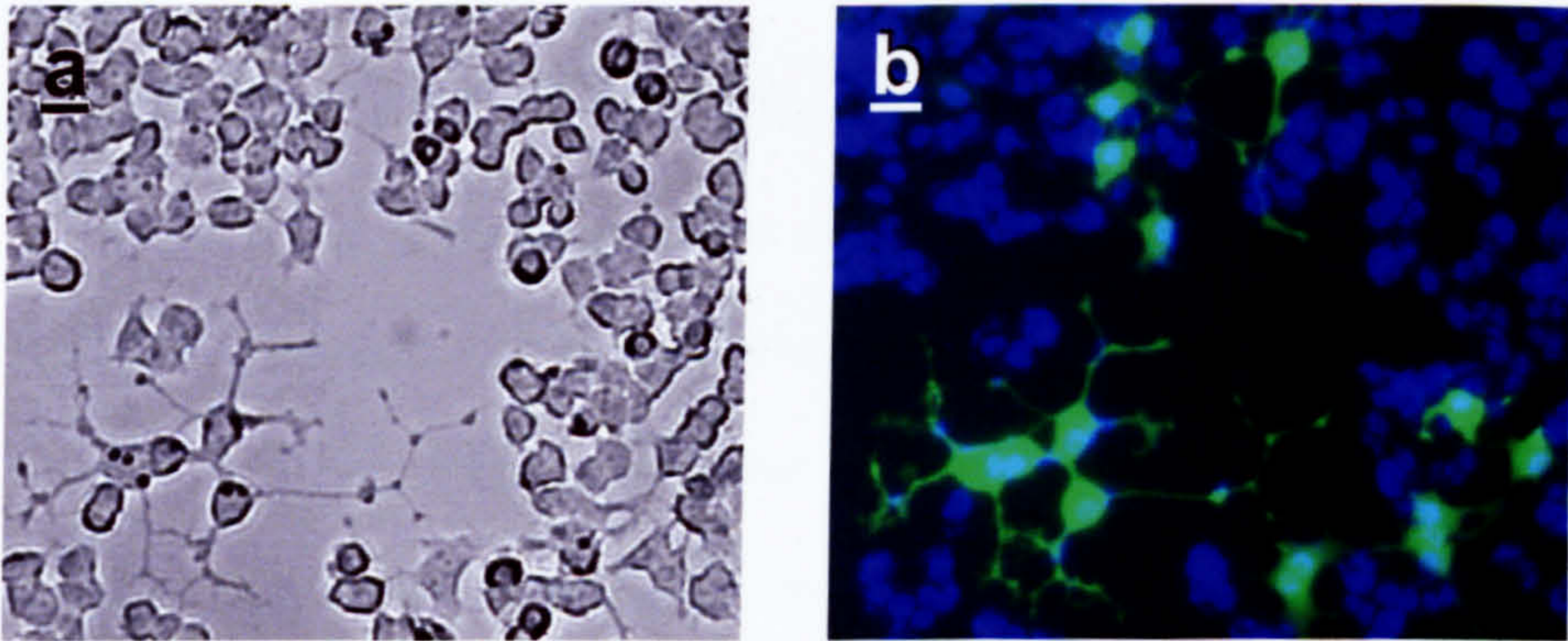


Figure 3-9: Micrographs of PC12 cells infected with pBird.Ras. (a) Transmission image, and (b) fluorescence image of the same field showing nuclei of all cells (blue) and GFP in infected cells (green). Cells that express GFP exhibit a neuronal phenotype, a consequence of expressing constitutively-active Harvey Ras.

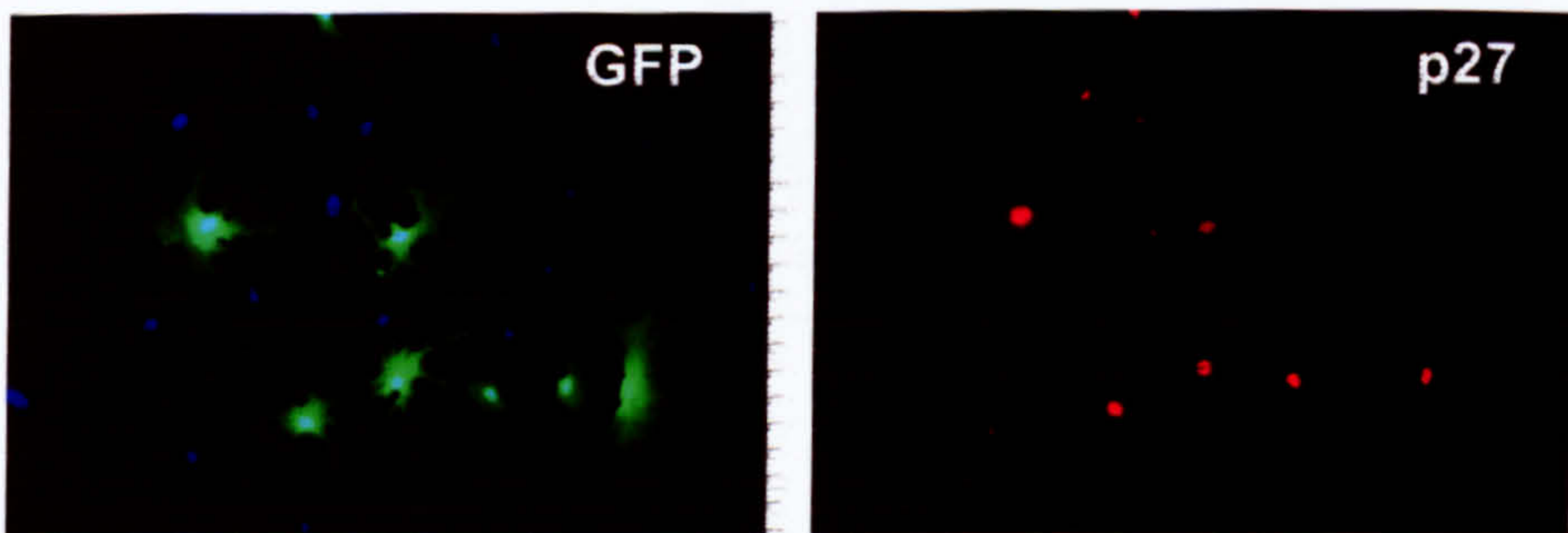


Figure 3-10: Fluorescence micrograph of murine optic nerve cells infected with pBird.p27. All cells that express GFP also co-express p27; the reverse is also true.

The level of the GFP signal from infected cells varied markedly in intensity. Interestingly, cells that emitted the strongest GFP signal also showed the most profound neuronal phenotype. This indicates that the GFP signal not only reports qualitatively whether or not a cell is infected but may also report semi-quantitatively to what degree the viral genome is expressed. These experiments establish that the virus can be used to functionally express an exogenous transgene and that GFP faithfully reports co-expression of that gene.

### **3.2.7 Co-expression of GFP and p27**

Cultures of mixed optic nerve cells were prepared from neonatal mice that were homozygous null for p27. These cells were grown for two days in B-S medium including PDGF, NT-3 and 0.5% FCS. Cultures were infected by addition of concentrated pBird.p27 supernatant, in the absence of polybrene, for three hours at 37°C. Subsequently, the virus was removed and fresh medium added before the cultures were returned to the incubator for a further five days.

Infected cultures were fixed, permeabilised and stained by indirect immunofluorescence for both p27 and GFP. Bisbenzimidazole was added to co-stain the DNA of all cells, and the slides were visualised using a Zeiss Axioskop fluorescence microscope. Micrographs were recorded using a Hamamatsu CCD camera and montages of the three fluorescence images of each field were compiled using Adobe Photoshop.

There is no endogenous p27 in these cells, as the mice from which they were derived had both copies of the endogenous p27 gene ablated by homologous recombination. In the uninfected cultures, no cells were stained by the p27 antibody, indicating that the antibody did not react non-specifically with these cells (data not shown). Infected cells could be detected by immunostaining for GFP. These cells, and only these cells, co-stained for p27. In every case, a GFP<sup>+</sup> cell was p27<sup>+</sup> and, conversely, a p27<sup>+</sup> cell was GFP<sup>+</sup> (figure 3-10). This experiment established that the pBird.p27 retrovirus co-expresses both p27 and GFP and that detection of GFP can be used as a reliable indicator of p27 expression. The intensity of the GFP signal appeared to correlate with the intensity of p27 staining, suggesting that with this retrovirus, as with pBird.Ras, the reporting is semi-quantitative.

### **3.3 Discussion**

The aim of the work described in this chapter was to develop a retroviral method to introduce exogenous transgenes in oligodendrocyte precursor cells. In addition to expressing the transgene under study, it was necessary for the retrovirus to co-express GFP to permit identification of infected cells in a mixed population of living cells. These goals required the design and construction of a novel retroviral vector, and I chose two alternate designs to meet the criteria described.

#### **3.3.1 The single-transcript vector: pFoxIV**

The first design employed an IRES sequence within the retrovirus to co-express two genes. Unfortunately, this attempt failed because of technical problems with the molecular biology used to construct the vector. The vector failed to express GFP when transfected into NIH3T3 cells, and infectious virus was not produced when transfected into a suitable packaging cell line.

After DNA sequence analysis it was clear that crucial parts of the vector were missing, in particular a deletion of 150bp was discovered at the start of the IRES sequence. This deletion may have arisen as a result of recombination events during propagation in bacterial hosts. It is probably not a result of errors during mutagenesis because GFP was expressed from the IRES when an intermediate vector in the subcloning process, pIRES-KGFP, was transfected into NIH3T3 cells (see §3.4.1.6). Steps were taken to use bacterial strains that are deficient in the most significant recombinase enzymes.

However, the presence of large regions of repetitive sequence in the vector, especially within the IRES, may have promoted recombination.

DNA sequencing also revealed several point mutations and substitutions in the vector that may offer an explanation of why the retroviral genome was not expressed. Firstly, there were several mutations affecting a region of sequence immediately distal to the 5'-LTR. This region of the vector consists of part of the sequence of the wild-type *gag* gene, with the initiating ATG deleted to prevent translation. This sequence has been shown empirically to improve expression from the retroviral LTR, thereby enhancing viral titres (Morgenstern and Land, 1990). Secondly, there were several mutations affecting the GFP open reading frame. None of these were truncation mutations but one is predicted to cause a codon change of Asp102→Phe. Although I do not know the effect that this would have on the expression of GFP, it is a significant change in the character of the amino acid and I would not be surprised if it affected the three-dimensional structure, and hence the fluorescence, of the protein.

### **3.3.2 The dual-promoter vector: pBird**

The second design relied on separate promoters to drive expression of the two genes. The transgene of interest is transcribed from the retroviral LTR promoter and GFP from a sub-genomic promoter. Although the design is more straightforward than the IRES-containing vector, the approach requires that the fidelity of reporting by GFP is verified before the virus can be used efficiently. Without direct measurement, one cannot be certain that the expression of GFP

correlates with expression of the upstream transgene, because their expression is controlled independently.

Initially, the vector was tested for production of infectious viral particles and subsequent GFP expression. Cultures of oligodendrocyte precursor cells infected with pBird expressed detectable amounts of GFP approximately 24 hours after infection. Most of the cells that expressed GFP showed the distinctive morphology of oligodendrocyte precursor cells, although some oligodendrocytes also appeared to be GFP<sup>+</sup>. As this retrovirus will only infect dividing cells, it is probable that the oligodendrocytes that were expressing GFP had been infected prior to differentiation. Therefore, infection with pBird did not block terminal differentiation. Clones of oligodendrocyte precursor cells were observed in which all of the constituent cells were expressing GFP, implying that the retroviral genome is inherited by the daughter cells following cell division (data not shown).

Three methods were used to validate the dual promoter retrovirus. In each case it was established that GFP is expressed in the same cells, and only in the same cells, as the upstream transgene. The first cells to visibly express GFP appeared around 24 hours after infection. The number of cells expressing GFP, and the overall intensity of fluorescence, increased for another 24 hours afterwards. After this time, there was a variation in the intensity of green fluorescence with some cells clearly expressing more GFP than others. Interestingly, the intensity of green fluorescence often appeared to report semi-quantitatively the level of the upstream gene. PC12 cells infected with pBird.Ras differentiated with a neuronal phenotype, arresting proliferation and extending neurites. The cells that appeared to have the strongest neuronal

character also exhibited the most intense green fluorescence. Similarly, the amount of p27 detected in cells infected with pBird.p27 correlated with the amount of GFP that they expressed.

The dual-promoter retrovirus, pBird, can be used to introduce and over-express transgenes in oligodendrocyte precursor cells. The use of GFP to identify cells that are expressing the transgene allows the observer to follow unique clones of cells as they expand in culture over several days. This method will prove an invaluable tool to extend studies on oligodendrocyte development because it allows the successful transfection of oligodendrocyte precursor cells and the identification of living transfected cells, which can be subsequently followed over several days in culture.



## 3.4 Methods

### 3.4.1 Molecular Biology

For a general reference to the molecular biology used in this chapter please see the excellent volume Harwood, 1996. All standard chemicals were from Sigma (Poole, UK) and equipment and reagents for molecular biology were purchased from Promega (Madison, WI) unless otherwise stated.

#### 3.4.1.1 Common media and solutions

Table 3-2: Compositions of common media and solutions

Luria Broth (L-broth)	1% bactotryptone, 0.5% yeast extract, 200mM sodium chloride; sterilised by autoclaving.
L-B agar	L-broth containing 15g.L <sup>-1</sup> agar.
TE	10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0; sterilised by autoclaving.
TBE gel running buffer	545g Tris base, 278g boric acid, 46.5g EDTA dissolved in 5L water for 10x stock.
TAE gel running buffer	242g Tris base, 57.1ml glacial acetic acid, 100ml of 0.5M EDTA pH 8.0; made up to 1L with water for 50x stock solution.
ethidium bromide (EtBr)	stock solution 10mg.ml <sup>-1</sup> , aqueous.
ampicillin	stock solution is 100mg.ml <sup>-1</sup> in 50% aqueous ethanol, stored at -20°C.
kanamycin	stock solution is 10mg.ml <sup>-1</sup> , aqueous, stored at 4°C.

### **3.4.1.2 Transformation of bacteria by electroporation**

Transformation describes the process of introducing exogenous DNA into bacterial cells, and can be achieved by several different methods. All transformations performed during the course of this work were effected by electroporation, using a strong electric field to reversibly permeabilise bacterial cells that were suspended in plasmid DNA. Electroporation has many advantages over alternative transformation procedures, such as chemical transformation, as it reliably results in a very high efficiency of plasmid uptake.

#### **3.4.1.2.1 Preparation of electrocompetent cells**

*E. coli* JS4 bacteria (a kind gift from Dr Parmjit Jat, Ludwig Institute for Cancer Research, London, UK), from a glycerol stock kept at -80°C, were streaked onto an L-B agar plate that was then incubated overnight at 37°C. A single colony was picked to inoculate a 5ml culture of L-broth, which was grown overnight at 37°C with vigorous shaking to ensure good aeration. The following day, this was used to inoculate 1 litre of L-broth that was subsequently grown at 37°C, with vigorous shaking, until the OD<sub>600</sub> reached 0.6. The culture was placed immediately on ice and left to chill for 20 minutes. The bacteria were sedimented and washed successively in ice-cold solutions of 0.1M Hepes (repeated twice) and 20ml of 20% (v/v) aqueous glycerol. The cells were resuspended in 3.0ml of 20% (v/v) aqueous glycerol to achieve a bacterial concentration of approximately  $3 \times 10^{10}$  cells per ml. The cells were then aliquotted into pre-chilled Eppendorfs, snap-frozen on dry-ice and stored at -80°C until required.

#### **3.4.1.2 Electrotransformation**

DNA (1-5 $\mu$ l in water or TE) was transferred to 0.1cm electroporation cuvettes (BioRad), which were then pre-chilled on ice. Aliquots of bacterial cells (45 $\mu$ l per transformation) were thawed on ice and added to the cuvettes. Directly prior to electroporation, the cuvettes were tapped firmly on the bench-top to fully mix the contents. The cuvettes were exposed to a single pulse of 2.5kV, 25 $\mu$ F and 200 $\Omega$  using a BioRad GenePulser II electroporator. Immediately, 200 $\mu$ l L-broth, containing 10mM glucose and 20mM magnesium sulphate, was added and the samples were left at room temperature for five minutes. Samples were plated onto L-B agar, containing appropriate antibiotics, and incubated overnight at 37°C to allow growth of transformed colonies.

#### **3.4.1.3 Small-scale preparation of plasmid DNA**

The rapid-boiling miniprep method is a classic, alternative method for plasmid DNA preparation, based on exactly the same principles as the alkaline lysis method, with the advantage of speed when processing large numbers of samples (Holmes and Quigley, 1981).

Liquid cultures of 2.0ml L-broth, containing either 100 $\mu$ g.ml<sup>-1</sup> ampicillin or 50 $\mu$ g.ml<sup>-1</sup> kanamycin, were inoculated with single bacterial colonies picked from transformation plates using a sterile pipette tip. The cultures were grown over-night at 37°C with vigorous shaking. The following day, 1.5ml of the cultures was transferred to sterile Eppendorf tubes and spun at 13,000rpm in a microfuge for three minutes. The supernatants were aspirated

and pellets resuspended in 200µl STET (5% Triton X-100, 50mM Tris-HCl pH 8.0, 50mM EDTA pH 8.0, 8% sucrose) containing 1.0mg.ml<sup>-1</sup> lysozyme. The tubes were plunged immediately into a boiling water bath for exactly 45 seconds, to denature bacterial proteins and DNA, and then spun at 13,000rpm for 10 minutes. The gelatinous pellets were removed using sterile pipette tips and 200µl isopropanol added to precipitate plasmid DNA. The tubes were centrifuged at 13,000rpm for five minutes and the pellets resuspended in 200µl TE. The residual bacterial protein was precipitated by addition of 100µl of 7M ammonium acetate followed by incubation on ice for 10 minutes. The tubes were spun at 13,000rpm and the supernatants were transferred to fresh Eppendorfs. The plasmid DNA was re-precipitated by addition of 600µl of 70% ethanol and the tubes were centrifuged at 13,000rpm for five minutes. The supernatants were aspirated and when the pellets had dried the plasmid DNA was resuspended in 100µl TE.

#### ***3.4.1.4 Large-scale preparation of plasmid DNA***

Large volume (usually 250ml) liquid cultures of L-broth, containing appropriate selective antibiotic (see 'Small-scale' method, §3.4.1.3), were inoculated with 1ml of bacterial cultures. These were grown overnight at 37°C with vigorous shaking. The cultures were transferred to large centrifuge bottles and the bacteria sedimented by centrifugation at 4000rpm for 15 minutes. The pellets were resuspended in 20ml of 50mM glucose, 50mM Tris-HCl pH 8.0 and 10mM EDTA pH 8.0, by trituration using sterile 20ml pipettes. Bacteria were lysed by addition of 10ml of 1% SDS in 0.2M sodium hydroxide solution for 10 minutes at room temperature. This was neutralised by addition of 30ml

of 3M potassium/5M acetate and bacterial debris was removed by centrifugation at 4000rpm for 10 minutes. The supernatant was transferred to fresh centrifuge tubes and crude plasmid DNA was precipitated by addition of 55ml isopropanol and sedimented by centrifugation at 4000rpm for 10 minutes. The DNA was resuspended in a total volume of 3.6ml of TE containing exactly 4.0g of caesium chloride and 400 $\mu$ l of EtBr, to give a final density approaching 1.55g.ml<sup>-1</sup>. This solution was centrifuged at 10,000rpm for 10 minutes in a JA-20 rotor at 16°C and the supernatant was transferred to 3.9ml Quick-seal ultra-centrifuge tubes (Beckmann). After careful balancing, the tubes were sealed and spun overnight in a TLN-100 rotor in a bench-top Optima TLX ultra-centrifuge (Beckmann) at 100,000rpm and 16°C. At equilibrium, the plasmid DNA came to lie as a red band around the middle of the ultra-centrifuge tube. Contaminating genomic DNA, which is less dense than the supercoiled plasmid, floated above and RNA pelleted on the bottom and sides of the tube. Under long wavelength ultraviolet light, to aid visualisation, the plasmid DNA was drawn out of the tube using a syringe and 21 gauge needle. The volume was made up to 2ml with TE and EtBr was extracted by repeated washing with water-saturated butanol. The volume was made up to 4ml with TE and DNA was precipitated by addition of 8ml 70% ethanol. The pellet of pure plasmid DNA was resuspended, reprecipitated and finally dissolved in 500 $\mu$ l of TE. Typically, this method yielded DNA concentrations of around 1-5mg.ml<sup>-1</sup> when starting from 250ml bacterial cultures. This method is based upon Maniatis *et al.*, 1982; Birnboim and Doly, 1979.

### **3.4.1.5 Common subcloning procedures**

Subcloning procedures are used to transfer DNA fragments from one sequence context to another. During the course of this work they were routinely employed to construct expression systems, usually for splicing either promoters or open reading frames from one vector into the retroviral vectors that were tested.

Basic subcloning involves four steps. Firstly, vector and insert DNA fragments are prepared by digestion with appropriate restriction enzymes and subsequent purification. Secondly, the fragments are mixed with T4 DNA ligase and then transformed into an *E. coli* host. Finally, screening procedures are used to identify transformant containing recombinant plasmids with the desired structure.

#### **3.4.1.5.1 Digestion of DNA using restriction endonucleases**

The starting material for all subcloning strategies is vector and insert DNA. The vectors employed here were plasmids, obtained in quantity by either the small-scale or large-scale methods described above. The insert DNA was either contained in a separate plasmid or prepared using the polymerase chain reaction.

DNA, usually 1-10 $\mu$ g, was digested in a 30-100 $\mu$ l reaction containing not more than one-tenth of the volume of restriction enzyme<sup>2</sup> mixed with one-tenth of the volume of 10x restriction buffer, 2.5mM  $\beta$ -mercaptoethanol and

100 $\mu\text{g.ml}^{-1}$  BSA. Reactions were incubated at the appropriate temperature, generally 37°C but sometimes room temperature, for not less than one hour. A fraction of the sample, usually one-tenth of the volume, was then run out on an agarose gel to check for complete digestion.

#### *3.4.1.5.2 Agarose gel electrophoresis*

The electrophoretic separation of DNA fragments on an agarose gel is a rapid, convenient and cheap method for assessing the success of many manipulations in molecular biology. It can also be used in a preparative capacity to purify DNA fragments for use in subcloning procedures (see below).

For most analytical purposes during the course of this work, 0.8% (w/v) gels were cast using 0.8% agarose set in 0.5x TBE containing 5 $\mu\text{g.ml}^{-1}$  EtBr. The DNA samples were mixed with one-tenth of the volume of loading buffer {50% (v/v) glycerol, 50mM EDTA pH 8.0, 0.125% bromophenol blue (w/v), 0.125% (w/v) xylene cyanol} and electrophoresed at 120V until the bromophenol blue dye front reached the distal end of the gel. Samples were run in parallel with DNA size markers, either 1kB or 100bp ladder, depending on the sizes of fragments concerned. The gel was visualised with a high intensity short-wavelength ultraviolet trans-illuminator and photographed using a

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<sup>2</sup> Stock solutions of restriction enzymes contain 50% glycerol for cryopreservation during storage at -20°C. The final glycerol concentration in the digestion reaction should not exceed 10% glycerol to reduce the chance of the enzyme exhibiting star activity. Star activity refers to the recognition of secondary sites by the enzyme with such altered specificity leading to unwanted cleavage products.

Syngene CCD camera system and Sony UP-890CE thermal printer. For references please see Sealey and Southern, 1982; Aaij and Borst, 1972.

#### ***3.4.1.5.3 Blunting the termini of DNA fragments***

It is sometimes necessary to create DNA fragments with blunt termini, for example, to allow ligation of two fragments if compatible cohesive ends cannot be derived. Although some restriction enzymes naturally cleave DNA to produce blunt termini most do not and, depending on the type of enzyme used, one must either fill in 5'-extensions or chew back 3'-overhangs. Many methods, using alternative DNA polymerases and exonucleases, have been described. I chose to use a method employing the thermostable *Pfu* polymerase (Stratagene, CA), because it possesses both polymerase activity and 3'→5' exonuclease activity, and lacks terminal transferase activity.

DNA to be blunted was resuspended in 8µl of water. To this, 1µl 10x *Pfu* buffer, 1µl 10mM dNTP mix, and 1µl cloned *Pfu* polymerase (2.5U) was added. Samples were incubated at 72°C for 30 minutes, and were removed to ice. DNA was either purified or added directly to ligation reactions.

#### ***3.4.1.5.4 Purification of DNA fragments by gel electrophoresis and differential binding to glass beads***

Many methods have been described for purifying DNA by size selection on agarose gels and subsequent extraction. The method employed here is based on the capacity for sodium iodide to dissolve agarose gels and the



binding of DNA to the surface of glass beads at a high salt concentration (Vogelstein and Gillespie, 1979).

The DNA sample was run out on a 0.8% agarose gel cast in 1x TAE. The fragment to be purified was cut out of the gel in the smallest volume possible under long-wavelength ultraviolet trans-illumination, to minimise irradiation damage to the DNA, using a sterile razor blade (Wilkinson). The gel block was transferred to an Eppendorf tube containing 500µl sodium iodide solution and incubated at 55°C until the agarose had dissolved. Subsequently, 5µl of a 50% silica slurry in TE was added and the solution was allowed to cool to room temperature with occasional mixing. The silica was sedimented at 13,000rpm for two minutes in a bench-top microfuge and the pellet was washed three times in 50% ethanol, 100mM sodium chloride, 10mM Tris-HCl pH 7.5, 1mM EDTA. Finally, the DNA was eluted from the silica beads by two washes with 10µl TE.

#### ***3.4.1.5.5 Ligation of vector and insert fragments***

The ultimate goal of any subcloning strategy is to form novel recombinant DNA molecules from specific vector and insert combinations. Once the DNA fragments have been prepared, to be reasonably pure and possess compatible termini, they are mixed in combination with the DNA ligase from the T4 bacteriophage of *E. coli*. Ligation is an inherently inefficient process, largely because of entropic considerations, as bimolecular reactions occur at rates significantly lower than the competing intramolecular reactions. Whenever possible, the molar ratios of insert and vector are adjusted such that

the insert is present in excess over the vector, usually 3:1 or higher. This consideration becomes more important as the relative size of the vector increases. Occasionally, extra steps were employed to reduce background contamination caused by intramolecular re-ligation. For example, if a restriction site is present in the parent vector but absent in the desired recombinant, ligations can be digested to linearise the unwanted plasmid.

### **3.4.1.6 Construction of retroviral plasmids**

#### **3.4.1.6.1 The single-transcript vector: pFoxIV**

To construct a dicistronic retroviral vector I inserted an IRES sequence, fused to GFP, into pBabe.PURO. I purchased a plasmid that contained the IRES from encephalomyocarditis virus fused to an enhanced variant of GFP, pIRES-EGFP (figure 3-1; Clontech #6064-1). The coding sequence of EGFP was mutagenised to enhance the quantum yield of fluorescence. Using a mutagenesis kit (QuikChange Site-Directed Mutagenesis kit, Stratagene), I introduced three codon changes (six nucleotide changes) into the enhanced GFP variant to produce KGFP (Jay Morgenstern, personal communication). The relevant codon changes were F99S, M153T and V163A. The synthetic oligonucleotides used, with mismatched bases underlined, were:

F99S     5'-G GAG CGC ACC ATC AGC TTC AAG GAC GAC GG-3'

M153T    5'-GC CAC AAC GTC TAT ATC ACC GCC GAC AAG CAG AAG-3'

V163A    5'-G AAG AAC GGC ATC AAG GCC AAC TTC AAG ATC CGC C-3'

Mutagenesis was performed in three separate rounds, each time using the pooled products of the previous round as a template. Following all three rounds of mutagenesis, ten clones were picked and analysed by expression in NIH3T3 cells.

NIH3T3 cells were maintained in DMEM containing 10% FCS at 37°C. The day prior to transfection, cells were replated at approximately 40% confluency on 10cm tissue culture dishes to promote an exponential growth rate. Cells were trypsinised, resuspended in 500µl of OptiMEM-1 serum-free medium, and added to 0.4cm electroporation cuvettes. DNA was purified from the mutagenised clones and 10µg of each was added to the cells. The cuvettes were shocked with a single pulse of 260V, 950µF and  $\infty\Omega$ , and the cells were replated on glass coverslips in DMEM + 10% FCS. After two days, the coverslips were mounted on glass slides in Citifluor mounting medium and observed using a Zeiss Axioplan fluorescence microscope. Each clone was scored for fluorescence intensity, using a subjective scale, relative to the parent vector. The brightest clone was selected, termed pIRES-KGFP, and used in the subsequent subcloning steps.

The next stage was to augment the translation efficiency of the IRES. The vector used has an IRES that is attenuated with respect to the wild-type encephalomyocarditis virus sequence. In particular, a pair of ATG codons near the end of the IRES are missing from the pIRES-KGFP vector. These codons have been shown to be important for maintaining a high efficiency of translation from the IRES (Derek Knight, personal communication). I aimed to replace the region of missing sequence by using the polymerase chain reaction with oligonucleotide primers that contained the wild-type sequence.

The forward primer was homologous to a region of the IRES sequence located 5' to a unique restriction site, PmlI. The reverse primer contained some homologous sequence, to allow specific annealing to the template, fused to the wild-type sequence and another unique restriction site, AgeI (figure 3-11).

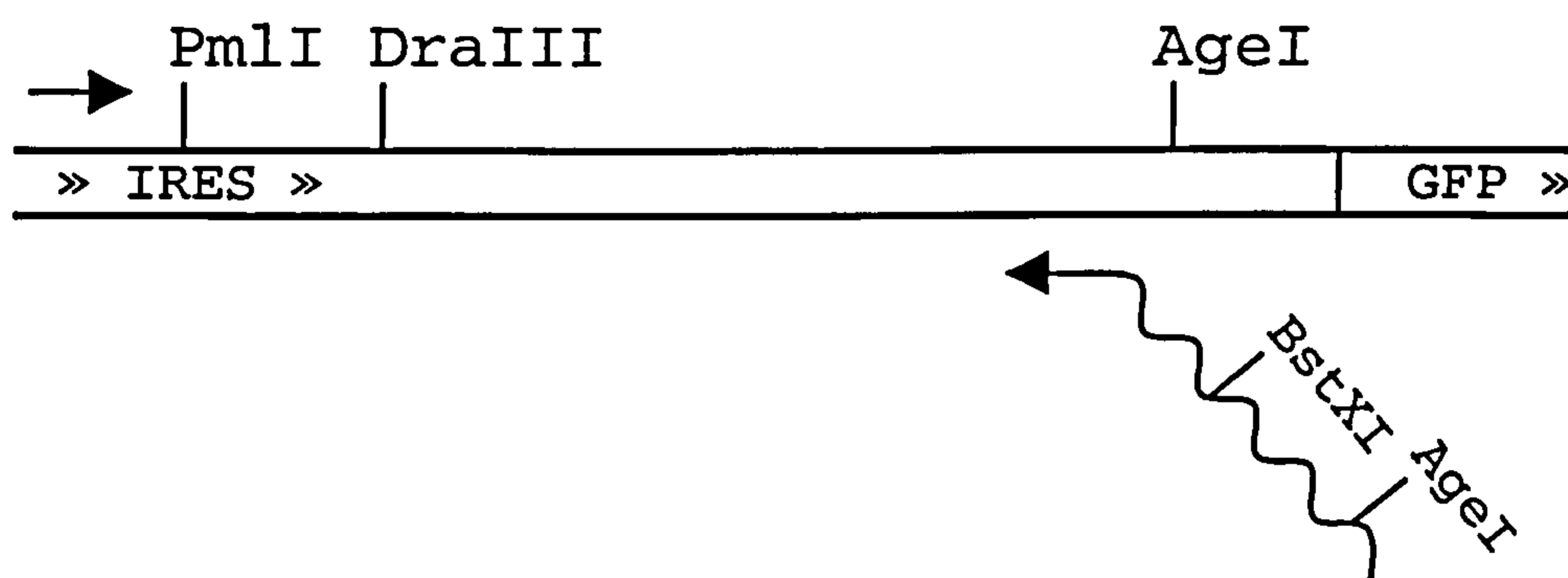


Figure 3-11: Map of pIRES-KGFP showing the border between the IRES and GFP coding sequences. The schematic details the strategy used to augment the IRES using the polymerase chain reaction and oligonucleotide primers (arrows). The wavy arrow depicts the region of wild-type sequence to be inserted into the vector.

The PCR product and pIRES-KGFP were digested sequentially with PmlI and AgeI. The fragments were purified and ligated. Recombinant clones, termed pIRES<sup>+</sup>-KGFP were identified by the presence of a new BstXI restriction site that was introduced during PCR (figure 3-11).

The original multiple cloning site (MCS) in pBabe.PURO is short and lacks many common restriction enzyme sites. To facilitate future subcloning of transgenes into pFoxIV, I wanted to extend the MCS. Therefore, I modified the MCS of the common cloning vector, pBluescript (Stratagene), with a synthetic oligonucleotide containing many restriction enzyme sites that would be unique in pFoxIV. Two 65mer oligonucleotides, containing 21 complementary bases,

were synthesised (figure 3-12). They were annealed by mixing 2µg of each, in restriction enzyme buffer, to 94°C followed by slow cooling to room temperature. To fill in double-stranded sequence, ten units of Klenow DNA polymerase and 0.2µl 25mM dNTP mix were added and the mixture was incubated at room temperature for 20 minutes. The enzyme was inactivated by heating to 75°C for ten minutes.

5' -TTG...GGAGAATTCCGTACGCCCGGGCCGCGGTCGC-3'  
 3' -CATGCGGGCCCGGCCAGCGCTGACGTCAA...AGC-5'

anneal and fill in  
with Klenow

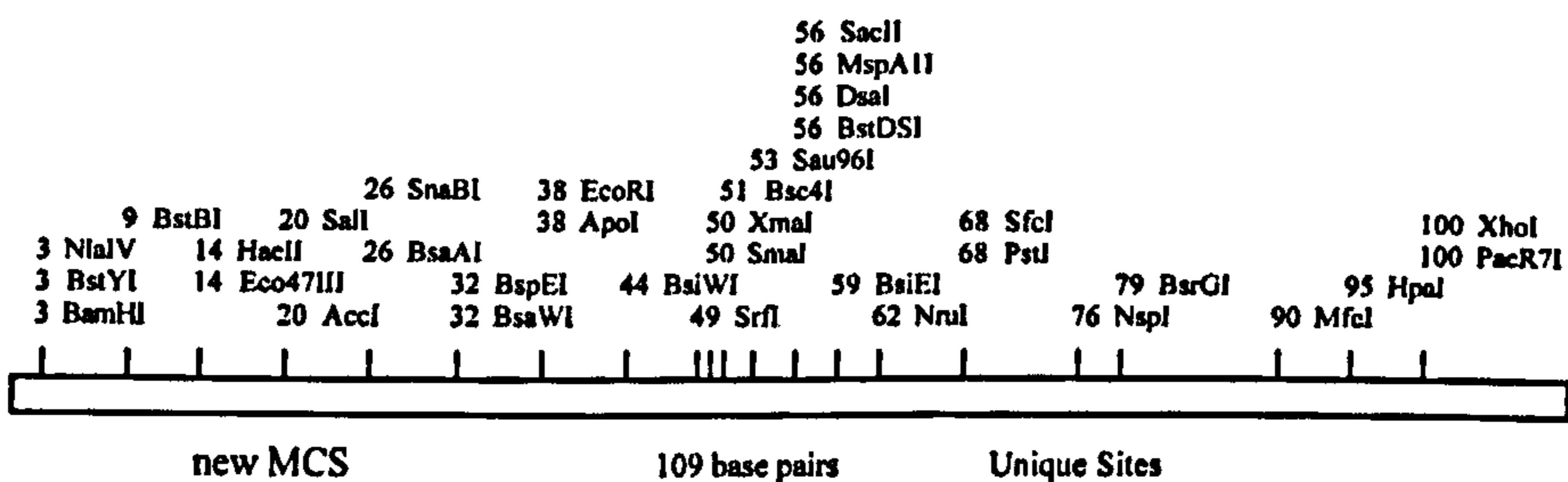


Figure 3-12: Constructing a new multiple cloning site. Two, partially complementary oligonucleotides were annealed, and double-stranded sequence was filled in with Klenow DNA polymerase and dNTPs.

The new MCS was digested with BamHI and XhoI, and ligated into pBluescript, which had been cut previously with BamHI and XhoI, and purified, to create pGimscript. The new cloning vector was identified by

several unique sites, such as SnaBI and BsrGI, that were present only in the oligonucleotide.

The IRES<sup>+</sup>-KGFP cassette was excised from pIRES<sup>+</sup>-KGFP by sequential digestion with PstI and BsrGI, and purified. pGimscript was cut with the same restriction enzymes and, after purification, ligated with the IRES<sup>+</sup>-KGFP.

The new construct, pGimscript.IRES<sup>+</sup>-KGFP, was digested with BamHI and XhoI to excise the MCS.IRES<sup>+</sup>-KGFP. This fragment was purified and ligated into pBabe.PURO that had been cut with BamHI and SalI. XhoI and SalI are different restriction enzymes that create compatible cohesive ends. However, once an XhoI-cut fragment is ligated with a SalI-cut fragment both restriction sites are destroyed. Initially, the background level of bacterial transformation was high, presumably because of contamination of the ligations with the parent vectors. Consequently, the ligation reactions were digested with XhoI and SalI before transformation into bacteria. This extra step significantly reduced the background level of transformants because it selected for plasmids without XhoI and SalI sites, as only circular vectors can replicate in the bacterial host. Using this method, the task of identifying true recombinants was simplified considerably.

Clones that matched the design of pFoxIV were identified by restriction mapping. Specifically, I determined maps for the following enzymes, each of which should produce a unique pattern of DNA fragments relative to the parent vectors: BsrGI, XbaI+HindIII, EcoRI+MfeI, EcoRI-NotI, EcoRI-ClaI, PstI, and XbaI.

To verify the construction of pFoxIV, clones with restriction maps that matched the designed vector were subjected to expression screening and DNA sequence analysis. Expression screening aimed to test two features of pFoxIV: that GFP could be expressed from the IRES, and that infectious viral particles could be produced. To test for expression of GFP, vector DNA was electroporated in NIH3T3 cells (as detailed above) and cells were observed using a Zeiss Axioskop fluorescence microscope. None of eight clones chosen showed green fluorescence above the background level observed in mock transfected cells. To assay viral titre, clones were electroporated into Phoenix cells, an ecotropic packaging line that produces a high titre of pBabe.PURO following transient transfection. Supernatant was collected from transfected Phoenix cells and, in the presence of  $8\mu\text{g}\cdot\text{ml}^{-1}$  polybrene, used to infect exponentially growing cultures of NIH3T3 cells. The infected cells were replated at one-tenth density and selected with  $2.5\mu\text{g}\cdot\text{ml}^{-1}$  puromycin for seven days. Whereas pBabe.PURO supernatant contains around  $10^6$  infectious particles per ml, none of the pFoxIV clones produced a titre that was above the background level.

To identify the cause of the problems with pFoxIV, DNA sequence analysis was performed on a single clone whose restriction map was identical to the others tested. Initially, the IRES and KGFP regions of vector were sequenced.

DNA sequencing was performed manually using a commercially available sequencing kit (AmpliCycle, Perkin Elmer). Briefly, reactions were performed in 30 $\mu\text{l}$  volume containing 2.0 $\mu\text{l}$  oligonucleotide primer (10 $\mu\text{M}$ ), 1.0ml [ $\alpha$ - $^{33}\text{P}$ ]-dATP (10mCi. $\text{ml}^{-1}$ ; Amersham), 4.0 $\mu\text{l}$  10x sequencing mix and

approximately 1nmol template DNA. Reactions were incubated in a programmable thermal cycler (PTC-100, MJ Research) as follows: 120 seconds at 95°C, and 35 cycles of 60 seconds at 95°C, 60 seconds at 68°C, and 60 seconds at 72°C. After cycling, 4.0µl stop solution was added to each tube, mixed, and 3.5µl was loaded on to a 6% polyacrylamide gel. Gels were run at between 50 and 70 watts, to maintain a constant temperature of 55°C. The gels were dried and used to expose Kodak X-omat film at -80°C.

Whilst analysing the sequence data, I discovered that there were several differences between the actual and designed sequences of pFoxIV (figure 3-4). There were several single nucleotide insertions and substitutions in the untranslated *gag* region of the virus. This sequence is part of the retroviral *gag* gene and, although it is not translated in the recombinant vector, it is required in the genomic RNA transcript to obtain high retroviral titres (Morgenstern and Land, 1990). The directed mutations are present in the KGFP coding sequence, providing a direct explanation for the enhanced fluorescence observed following expression of pIRES-KGFP. However, there are several additional mutations, including a G→T substitution that results in a codon change from Asp102→Phe. This is a significant change of amino acids and although I cannot predict the exact effect of this mutation, I would not be surprised if it had a adverse effect on the three-dimensional structure of GFP, if it is translated at all.

The most serious discrepancy that I found was a deletion of 150 bp at the beginning of the IRES sequence. The full IRES is around 600 bp in length and this represents a significant loss of the sequence. The IRES functions as a secondary structure in mRNA by recruiting the small ribosomal subunit, in



combination with an accessory factor called polypyrimidine-binding protein (Borovjagin *et al.*, 1994; Witherell *et al.*, 1993). This deletion is likely to ruinously disrupt the IRES sequence, which would explain why I failed to detect expression of GFP.

#### ***3.4.1.6.2 The dual-promoter vector: pBird***

An alternative method to co-express a gene of interest and GFP, using a retroviral vector, is to use separate promoters for each gene. Most current retroviral expression systems use two promoters to express the gene under study and a gene for antibiotic resistance. The resistance gene is a selectable marker of cells that are expressing the viral genome, and permits the establishment of stable producer cell lines. Many of the genes whose effect on oligodendrocyte precursor cells I intended to study are negative regulators of cell growth and division. It is not possible to select for stably transformed lines containing such genes because proliferation is necessary for antibiotic selection. Consequently, I planned to harvest retrovirus using transient transfection, thus obviating the requirement for the antibiotic resistance gene.

I decided to remove the antibiotic resistance gene from pBabe.PURO, and replace it with a GFP expression cassette. Of the many GFP cassettes available, I chose one derived from a commercially available plasmid named pEGFP-C1 (figure 3-5; Clontech #6084-1; GenBank accession #U55763). It consists of the open reading frame of the enhanced GFP gene driven by the strong cytomegalovirus (CMV) immediate early promoter, called CMVegfp.

We used the polymerase chain reaction (PCR) to amplify a 1360 bp fragment that encompassed the entire CMVegfp sequence. The oligonucleotide

primers employed added unique restriction sites to the 5' and 3' ends, to facilitate directional subcloning. The primers sequences, with the restriction sites underlined, were:

SalCMVegfp      5'-GAT GCG TAA GTC GAC TAG TTA TTA ATA GTA ATC AAT TAC GGG G-3'  
CMVegfpCla      5'-TCA ATG ATC GAT TAC TTG TAC AGC TOG TOC ATG CCG-3'

The PCR reactions contained: 5µl 10x buffer, 5µl 25mM MgCl<sub>2</sub>, 1µl 10mM dNTP mix, 5µl 10µM each primer, 5 units Taq polymerase, 200ng pEGFP-C1, made up to a final volume of 50µl. The reaction was incubated in a programmable thermal cycler (PTC-100, MJ Research) as follows: five cycles of 30 seconds at 94°C, 30 seconds at 42°C, and 30 seconds at 72°C; followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C, and 30 seconds at 72°C; followed by two minutes at 72°C. The PCR reaction was analysed by agarose gel electrophoresis and a single product of approximately 1360 bp was observed. This matched the size of fragment expected if the CMVegfp sequence was amplified correctly.

The parent vector, pBabe.PURO, was digested with ClaI and SalI to excise the puromycin resistance gene and the SV40 promoter that drives its expression. The CMVegfp PCR fragment was digested using the same enzymes to generate complementary sticky ends. Both the vector and the CMVegfp insert were purified, and the relative concentrations of each were assessed by gel electrophoresis. Ligations were performed and transformed into competent *E. coli* DH5α (GibcoBRL) by electroporation. Antibiotic resistant

colonies were picked and restriction mapping, with ClaI and Sall, identified several recombinants matching the expected fragment pattern of pBird.

#### ***3.4.1.6.3 Recombinant vectors based upon pBird***

##### ***3.4.1.6.3.1 pBird.βgal***

pBird.βgal was constructed by subcloning the *lacZ* gene from pcDNA3.1.lacZ (Invitrogen) into pBird. The pcDNA3.1.lacZ plasmid was linearised with HindIII and termini were blunted using *Pfu* DNA polymerase. The vector was digested with XbaI and the excised *lacZ* fragment was purified. pBird was digested with EcoRI, blunted, digested with XbaI, and purified. After ligation, recombinants were identified by PCR screening 160 colonies with oligonucleotide primers: the forward primer was homologous to a region of *lacZ* and the reverse primer to a region of GFP. Positive clones were verified by restriction mapping.

##### ***3.4.1.6.3.2 pBird.Ras***

The Ha-Ras<sup>V12</sup> open reading frame was excised from pBabe.Ras.PURO (a kind gift from Dr Alison Lloyd, University College London, London, UK) by digestion with BamHI and Sall. After purification, it was directionally cloned into pBird that had been digested previously with the same enzymes and purified. Recombinants were identified by restriction mapping.

#### **3.4.1.6.3.3 *pBird.p27***

The open reading frame of human p27 was excised from pCMV.NEO (a generous gift from Dr Parmjit Jat, Ludwig Institute for Cancer Research, London, UK) by digestion with BamHI. After blunting with *Pfu* DNA polymerase, the fragment was purified and ligated into pBird that had been digested with SnaBI (an enzyme that naturally creates blunt termini). Recombinants were identified and the orientation of the insert was verified by restriction mapping with XmaI+BamHI.

#### **3.4.2 Preparation of retrovirus by transient transfection of Phoenix cells**

Phoenix cells, an ecotropic packaging line (Grignani *et al.*, 1998), were maintained in DMEM containing 10% FCS, penicillin (100U.ml<sup>-1</sup>), streptomycin (100µg.ml<sup>-1</sup>) and glutamine (PSG; GibcoBRL) (DMEM-10) at 37°C in 8% CO<sub>2</sub>. The day prior to transfection, cells were replated in fresh medium on 100mm cell culture dish to ensure optimal growth and density.

Cells were transfected using an enhanced lipofection reagent, Lipofectamine PLUS, previously described (see chapter two, §2.4.3). Recombinant retroviral plasmid DNA (8µg per transfection) was mixed with 20µl PLUS reagent in 750µl serum-free medium (OptiMEM-1, GibcoBRL) and incubated at room temperature for 15 minutes. In a separate tube, 30µl Lipofectamine reagent was mixed with 750µl OptiMEM-1 which was subsequently added to the DNA/PLUS and incubated for a further 15 minutes at room temperature. Phoenix cells were washed with OptiMEM-1 and the

DNA/PLUS/Lipofectamine was added in a further 5.0ml OptiMEM-1. The cells were incubated for three hours at 37°C in 8% CO<sub>2</sub> and the medium was replaced with 5.0ml DMEM-10 and left overnight. Viral supernatant was harvested by twice daily replacement of 5.0ml DMEM-10 for seven days thereafter, snap-frozen on dry ice and stored at -80°C.

### **3.4.3 Purification of oligodendrocyte precursor cells by immunopanning**

Oligodendrocyte precursor cells were purified by sequential immunopanning as described previously (Barres *et al.*, 1992a, modified from Huettner and Baughman, 1986). A suspension of optic nerve cells, prepared either freshly from rats or by trypsinisation of *in vitro* cultures, is passed consecutively over two antibody-coated dishes. The first is a negative selection to deplete the major unwanted cell population, usually astrocytes, meningeal cells and microglia, and the second positively selects for oligodendrocyte precursor cells. Using this two-step procedure, a purity of about 99.5% can be achieved (Barres *et al.*, 1992a).

Two 100mm bacteriological petri dishes (Falcon) were incubated overnight at 4°C with 10ml of 50mM Tris-HCl pH 9.5 containing either affinity-purified goat anti-mouse IgG (H+L) or IgM ( $\mu$ -chain specific) at 10 $\mu$ g.ml<sup>-1</sup> (Jackson Immunochemicals). The plates were washed three times with PBS and then coated with either: (1) RAN-2 IgG<sub>2a</sub> supernatant (Bartlett *et al.*, 1980) diluted 1:1 with PBS containing 0.2% BSA, or (2) A2B5 monoclonal

antibody (Eisenbarth *et al.*, 1979) ascites fluid diluted 1:2000 with PBS containing 0.2% BSA.

Dissociated optic nerve cells were resuspended in 10ml L15-Air medium (GibcoBRL) containing 0.2mg.ml<sup>-1</sup> BSA and 10ug.ml<sup>-1</sup> bovine insulin. These were transferred to the RAN-2 dish (negative selection) and incubated at room temperature for 30 minutes. Non-adherent cells were transferred to the A2B5 dish (positive selection) and incubated similarly.

The dish was washed eight to ten times with 6ml MEM-Hepes to remove all non-adherent cells and then twice with 6ml Earle's balanced salt solution (EBSS; GibcoBRL). The remaining adherent oligodendrocyte precursor cells were dislodged by incubation with 0.012% trypsin (Boehringer-Mannheim) in EBSS for three minutes at 37°C and gentle washing with a high concentration of ovomucoid and BSA (6mg.ml<sup>-1</sup> of each) in MEM-Hepes. Cells were then washed, resuspended in serum-free growth medium and plated out on poly-D-lysine-coated culture dishes.

#### **3.4.4 Histochemistry for detection of $\beta$ -galactosidase**

Cells were fixed in 4% paraformaldehyde for five minutes at room temperature and then permeabilised with 0.1% Tween. After washing in PBS, the cultures were incubated in PBS containing 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM magnesium chloride, and 1mg.ml<sup>-1</sup> X-gal (5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside) at room temperature until the blue reaction product was visible, usually overnight. Cells were visualised using a Zeiss Axioplan microscope and phase contrast optics.

### **3.4.5 In vitro culture of purified oligodendrocyte precursor cells**

Purified oligodendrocyte precursor cells were cultured in the same conditions as mixed optic nerve cells (see §2.4.2).

### **3.4.6 Immunostaining cultures for p27**

Immunofluorescence was carried out as described in the methods section of chapter two (§2.4.4). p27 was detected with a murine monoclonal IgG1 anti-p27 (1:100; Santa Cruz sc-1641, clone F-8), followed by biotin-conjugated anti-murine IgG1 (1:100; Amersham) and Texas Red-conjugated streptavidin (1:100; Amersham).

**4 Does p27 over-expression, in the absence of thyroid hormone, induce differentiation?**



## **4.1 Introduction**

Oligodendrocytes are post-mitotic cells that are produced from highly proliferative precursor cells. The normal schedule of oligodendrocyte development depends upon a cell-intrinsic timer that limits the proliferation of precursor cells. The timer consists of two separable components. Whilst the precursor cells are dividing, a timing component measures the elapsed proliferative time. It seems not to operate by counting the number of cell divisions, but rather it measures time in some other way (Gao *et al.*, 1997). Once the timing component signals that 'time is up', an effector mechanism triggers cell-cycle arrest and initiates terminal differentiation. The intrinsic programme is modulated by extracellular signals, particularly the mitogen, PDGF, which drives the timing component, and TH, which is required for the effector component to operate in the presence of mitogens. TH is not absolutely required for differentiation as precursor cells differentiate upon withdrawal of PDGF in the absence of TH (Barres *et al.*, 1994; Temple and Raff, 1985).

It is not understood how the timer controls oligodendrocyte development. Does it act, primarily, by arresting the cell cycle once the permitted proliferative period has ended, with terminal differentiation following consequentially? Alternatively, does it direct the onset of terminal differentiation, with cell-cycle arrest being merely a part of this co-ordinated change in lineage-specific gene expression?

These questions have never been answered despite their obvious importance to understanding the regulation of oligodendrocyte development. The major obstacle to solving this problem has been an experimental one: it has

not been possible to inhibit the proliferation of oligodendrocyte precursor cells without removing mitogens. As mitogens are necessary for the timing component of the timer to run normally, independently of their role in promoting the cell cycle, another means of arresting cell division is required.

I aimed to over-express the cyclin-dependent kinase inhibitor, p27, in oligodendrocyte precursor cells. This protein acts to slow progress through the cell cycle by inhibiting the cyclin-dependent kinases that are active in G<sub>1</sub>. During the G<sub>1</sub> phase of the cell cycle, cells integrate many different signals, before deciding whether or not to commit to another autonomous round of cell division. The cyclin-dependent kinases, and the cyclins that stimulate them, are positive signals: when they are active, cells pass quickly through G<sub>1</sub> into S phase and divide rapidly. p27 antagonises their effect by blocking their activity and reducing the likelihood that cells will traverse G<sub>1</sub> and enter S phase (reviewed in Sherr and Roberts, 1999). Over-expression of p27 in various cell lines arrests the cell cycle in G<sub>1</sub> (Sherr and Roberts, 1995).

p27 has been shown to play a role in the oligodendrocyte precursor cell timer. In the presence of PDGF and the absence of TH, which are conditions where the timing component operates but the effector mechanism does not, the level of p27 expression progressively increases in oligodendrocyte precursor cells as they proliferate in culture (Durand *et al.*, 1997). The timing of the increase is consistent with the role of p27 as part of the timing component. When mitogens are withdrawn and oligodendrocyte precursor cells stop dividing and differentiate, p27 expression rises further still, raising the possibility that p27 may also be part of the effector mechanism. Moreover, the timing component in oligodendrocyte precursor cells isolated from the optic

nerves of p27-deficient mice is aberrant: many precursor cells undergo one or two more divisions, in the presence of PDGF and TH, before they differentiate than do precursor cells from wild-type mice (Durand *et al.*, 1998).

A significant part of this body of work has been concerned with developing a method to accomplish the goal of over-expressing p27 in oligodendrocyte precursor cells. In chapter two, I used transient transfection protocols to introduce transgenes into oligodendrocyte precursor cells, but, despite moderate success, they proved impracticable for my ultimate aims. In chapter three, I described a new method, employing a retroviral vector, pBird, for expressing exogenous genes into oligodendrocyte precursor cells.

I wanted to assess the effect of p27 over-expression on the precursor cell cycle in the absence of TH. This enables one to study the proliferation of oligodendrocyte precursor cells, independently of the TH-dependent effector mechanism. Will raising the level of p27 above normal levels arrest the precursor cell-cycle and, if so, will this induce terminal differentiation?

I found that over-expression of p27 is not, by itself, sufficient to cause cell-cycle arrest and terminal differentiation of oligodendrocyte precursor cells in the absence of TH. Rather, p27 acts to slow progress through the cell cycle: in the case of one clone studied, the cell-cycle time was extended to more than four days.

## **4.2 Results**

### **4.2.1 Clonal analysis of the effects of over-expression of p27 in oligodendrocyte precursor cells**

Oligodendrocyte differentiation is associated with withdrawal from the cell-cycle (Raff *et al.*, 1983), but the relationship between the two processes remains uncertain (Temple and Raff, 1986). The timing mechanism might control the onset of oligodendrocyte differentiation, with the cessation of proliferation following as a consequence. Alternatively, it might control the cessation of proliferation, with oligodendrocyte differentiation following as a consequence. Although the latter hypothesis has been favoured (Barres *et al.*, 1994; Gao *et al.*, 1998), all attempts to arrest proliferation of oligodendrocyte precursor cells in the presence of PDGF have failed (Martin Raff, personal communication) precluding the separation of the two processes.

I aimed to determine whether over-expression of p27 in oligodendrocyte precursor cells would elicit cell-cycle arrest and concomitant differentiation. Cultures of mixed optic nerve cells, which had been growing *in vitro* for two days, were infected with retroviruses that express either GFP alone (pBird), or in combination with human p27 (pBird.p27) or  $\beta$ -galactosidase (pBird. $\beta$ gal). Oligodendrocyte precursor cells were purified after 24 hours by immunopanning and replated at clonal density in the presence of PDGF and the absence of TH. In these conditions, the timer runs normally but the TH-dependent effector mechanism is inactive and oligodendrocyte precursor cells continue to proliferate. After several days in culture, the size and composition of the clones was scored to determine what effect p27 over-

expression might have on oligodendrocyte development. Oligodendrocyte and their precursors were identified by their distinctive morphologies (figures 1-2b and 3-7).

#### **4.2.2 Over-expression of p27 did not induce differentiation into oligodendrocytes**

Mixed cell cultures were prepared from P5 rat optic nerves and grown *in vitro* for two days. The cells were infected with concentrated pBird.p27 supernatant for three hours and were returned to the incubator over-night. Subsequently, oligodendrocyte precursor cells were purified from the infected cultures by immunopanning and were cultured in the presence of PDGF and absence of TH for four days.

Over-expression of p27 in oligodendrocyte precursor cells did not appear to induce differentiation into oligodendrocytes. Some GFP<sup>+</sup> oligodendrocytes were observed in the cultures, but the proportion of these cells did not differ between p27 and control experiments and was not different from the proportion of uninfected oligodendrocytes (figure 4-1).

#### **4.2.3 Effect of p27 on the cell-cycle time of oligodendrocyte precursor cells**

Preliminary experiments revealed that clones over-expressing p27 were smaller than non-infected clones growing in the same flask. After four days in culture, uninfected clones contained an average of two more cells than clones over-expressing p27. Whereas uninfected clones always comprised six or more

cells, 80% of clones infected with pBird.p27 contained five cells or less (figure 4-2). The difference in clone sizes were not a result of an increased proportion of dead cells in the p27 over-expressing clones. Rather, the division of infected cells appeared to be slowed.

To maximise the effect of changes in division rate on clone size, the experiment was repeated, allowing a longer period of culture after replating before scoring clones. After six days in culture at clonal density, p27 over-expressing clones tended to contain fewer cells than uninfected clones growing in the same flask (figure 4-3a). The clone sizes in control infected cultures, which expressed GFP alone, were not different from uninfected clones growing in the same flask (figure 4-3b).

Clones that over-expressed p27 expanded more slowly than clones expressing GFP alone. After six days in culture, control clones contained an average of 49 cells whereas the p27-over-expressing clones contained an average of 32 cells. The proportion of dead cells in the cultures was low and did not differ between p27 over-expressing and control cells (data not shown). On average, p27-over-expressing cells divided with a doubling time of 29 hours compared to the control cells, which doubled every 26 hours (figures 4-3 and 4-4).

**Over-expression of p27 does not induce oligodendrocyte differentiation**

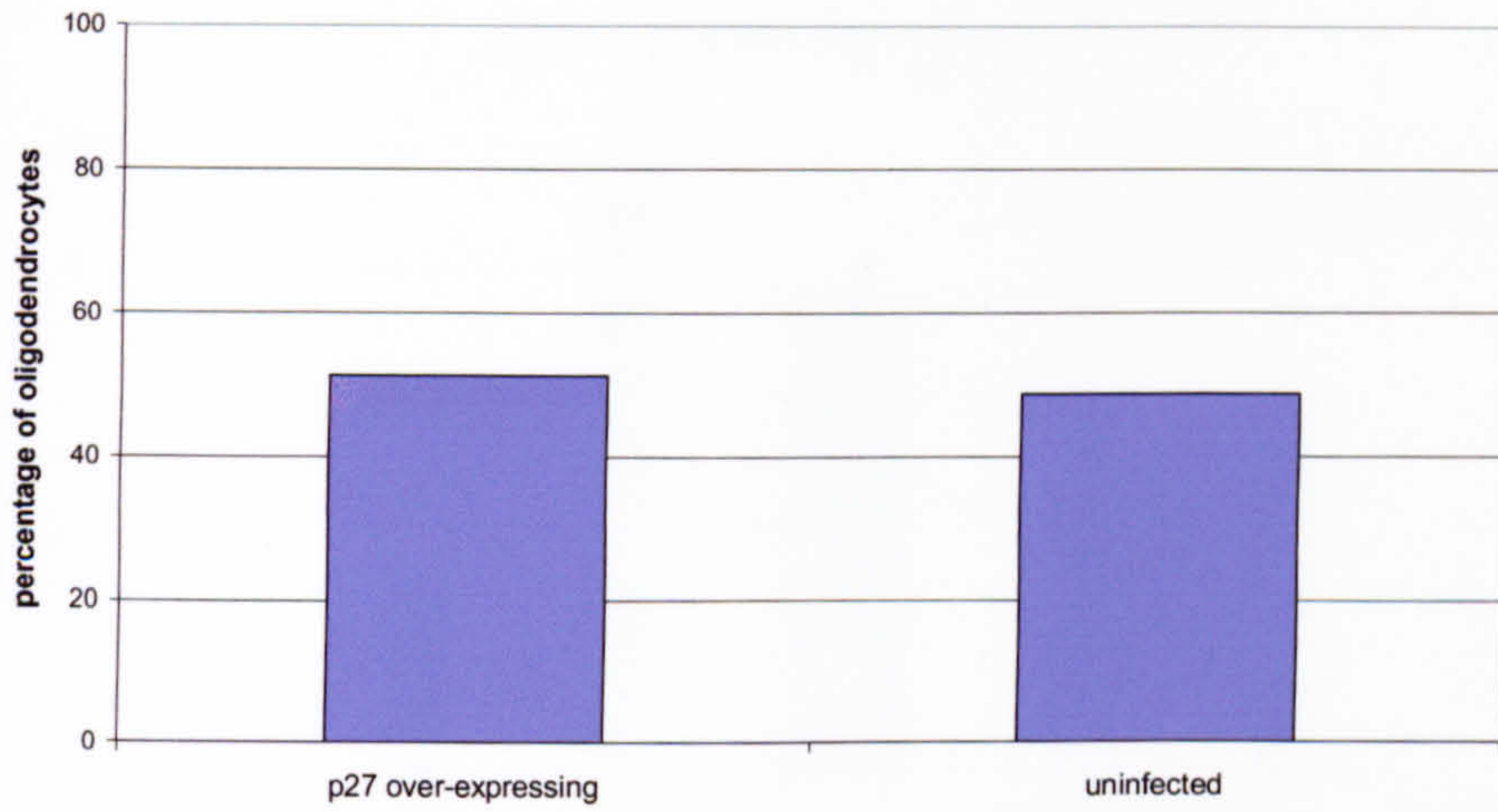


Figure 4-1: Over-expression of p27 in oligodendrocyte precursor cells does not elicit oligodendrocyte differentiation in the absence of TH.

**OPCs over-expressing p27 divide slowly**

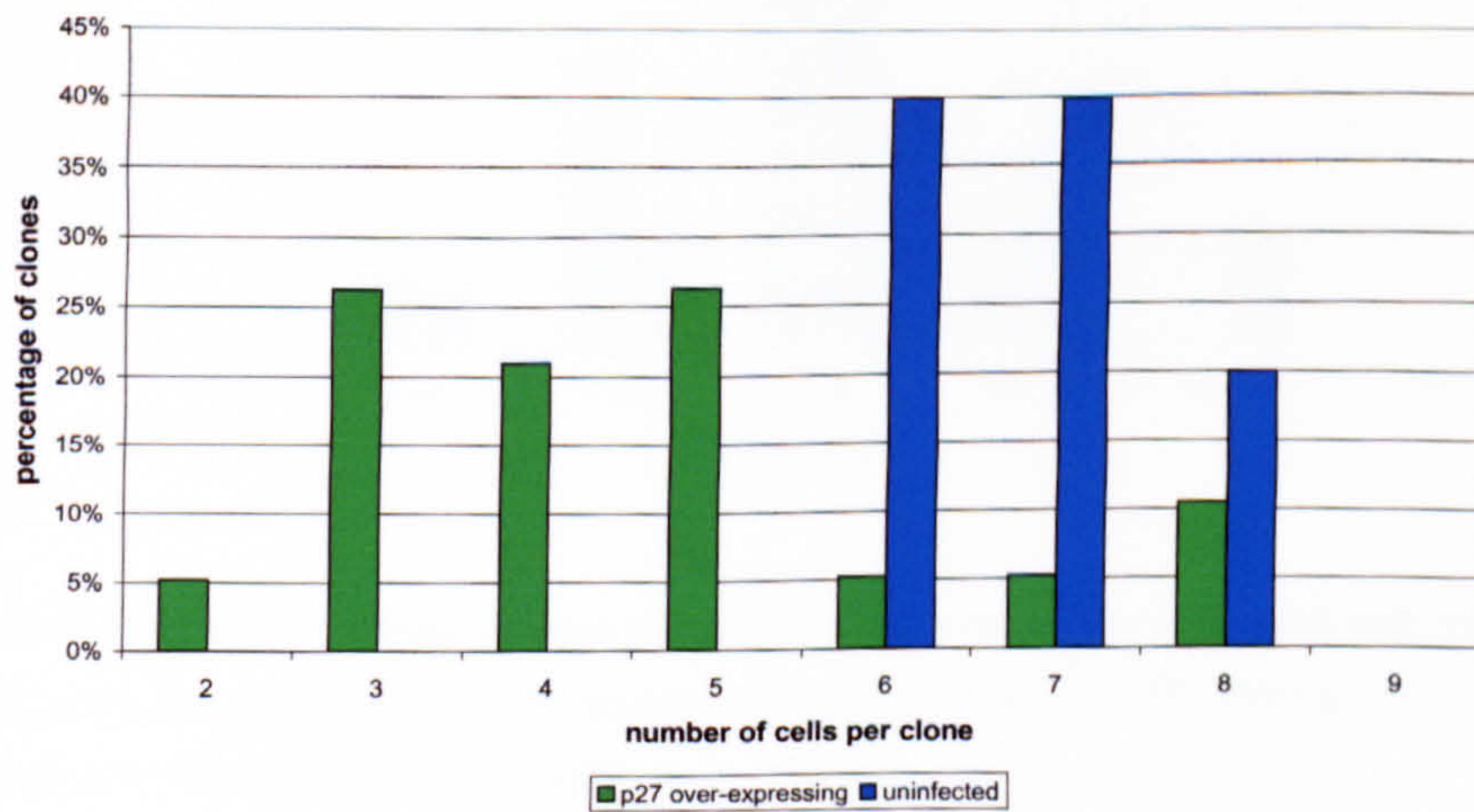
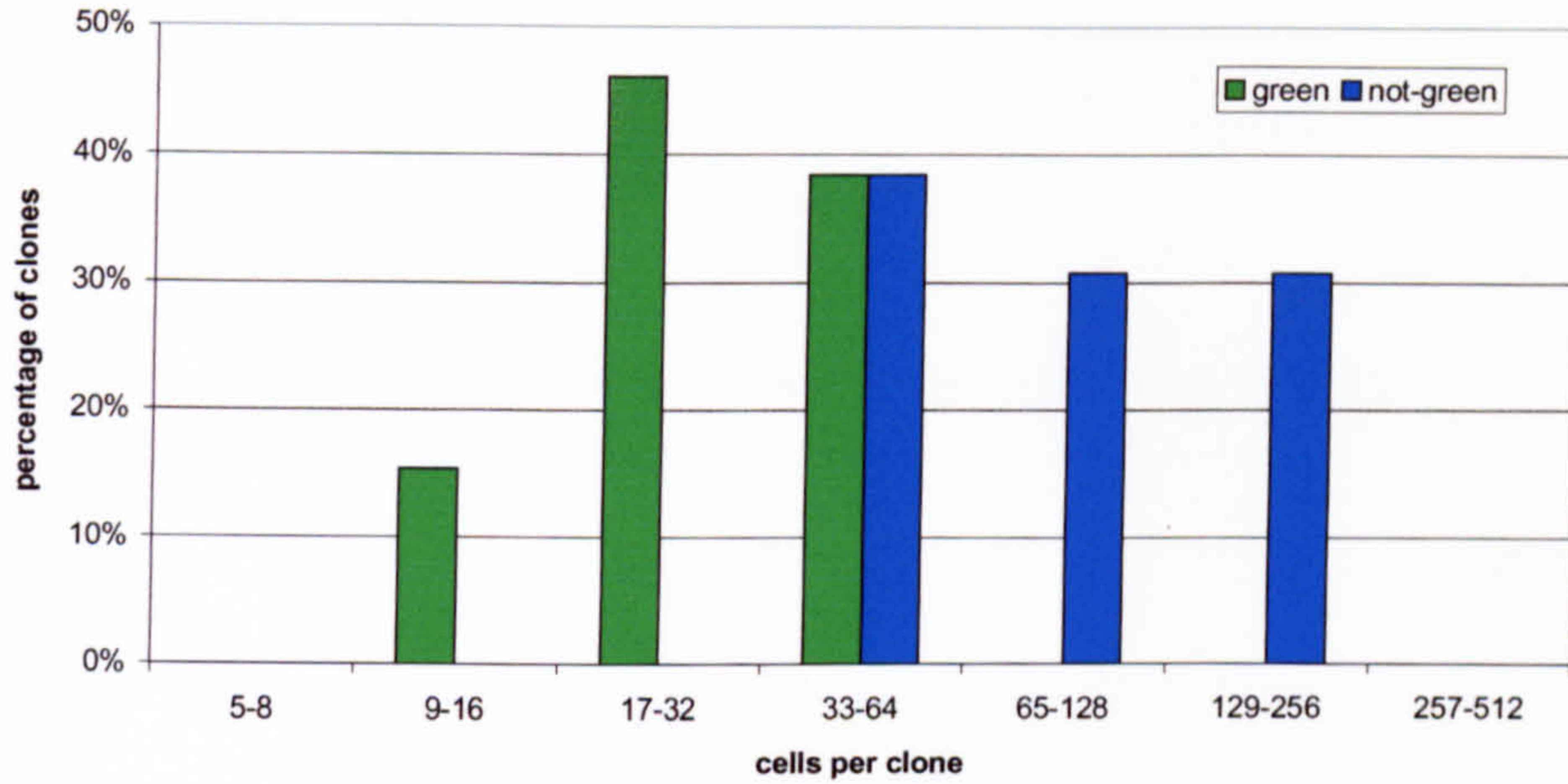


Figure 4-2: Clones that over-expressed p27 contained, on average, two fewer cells than uninfected clones growing in the same flask.

(a) Cultures infected with pBird.p27



(b) Cultures infected with a virus that expresses GFP alone

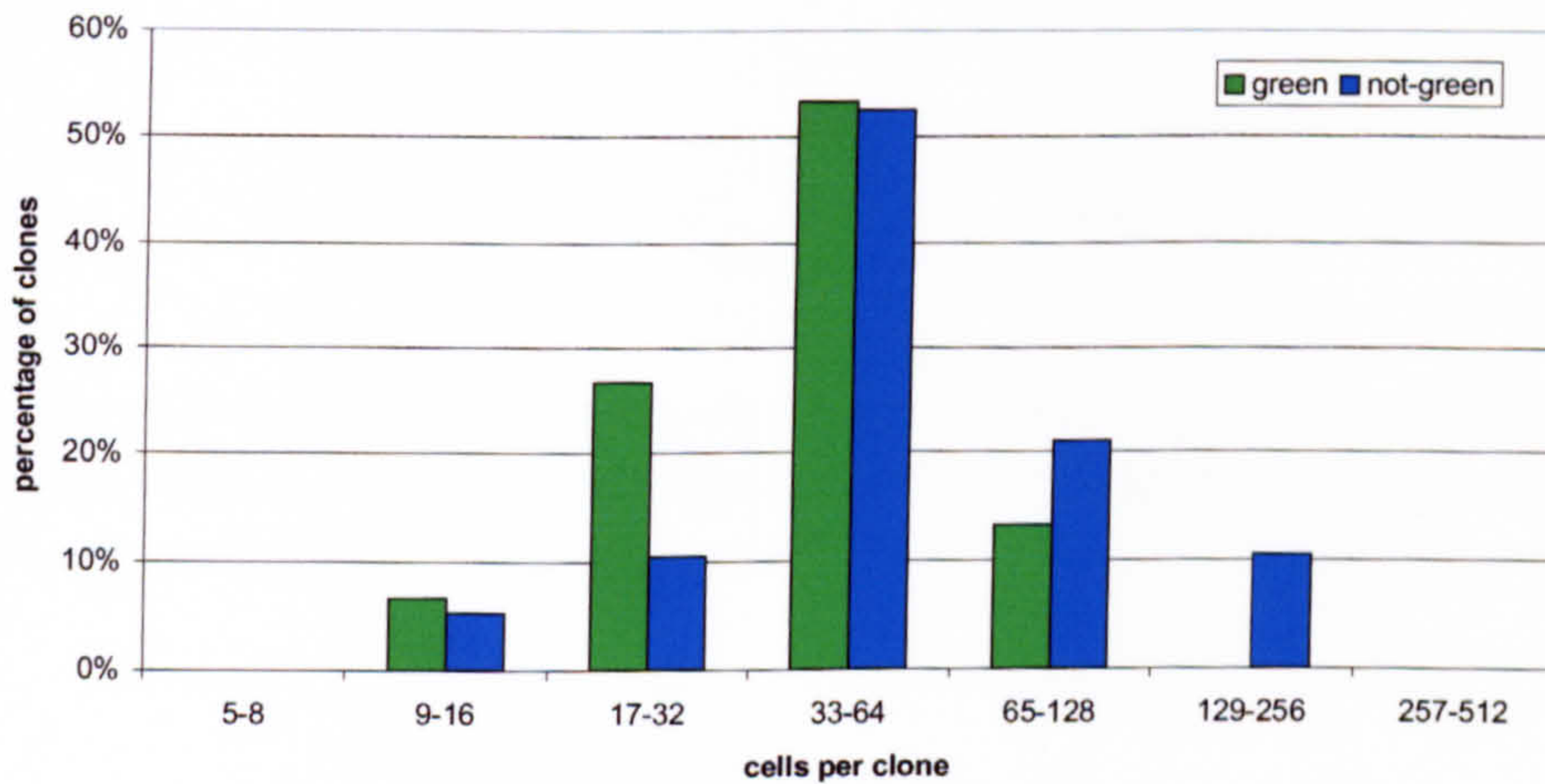


Figure 4-3: Graphs showing number of cells per clone for cultures infected with either (a) a retrovirus designed to over-express p27 and co-express GFP (pBird.p27), or (b) a retrovirus designed to express GFP alone (pBird).



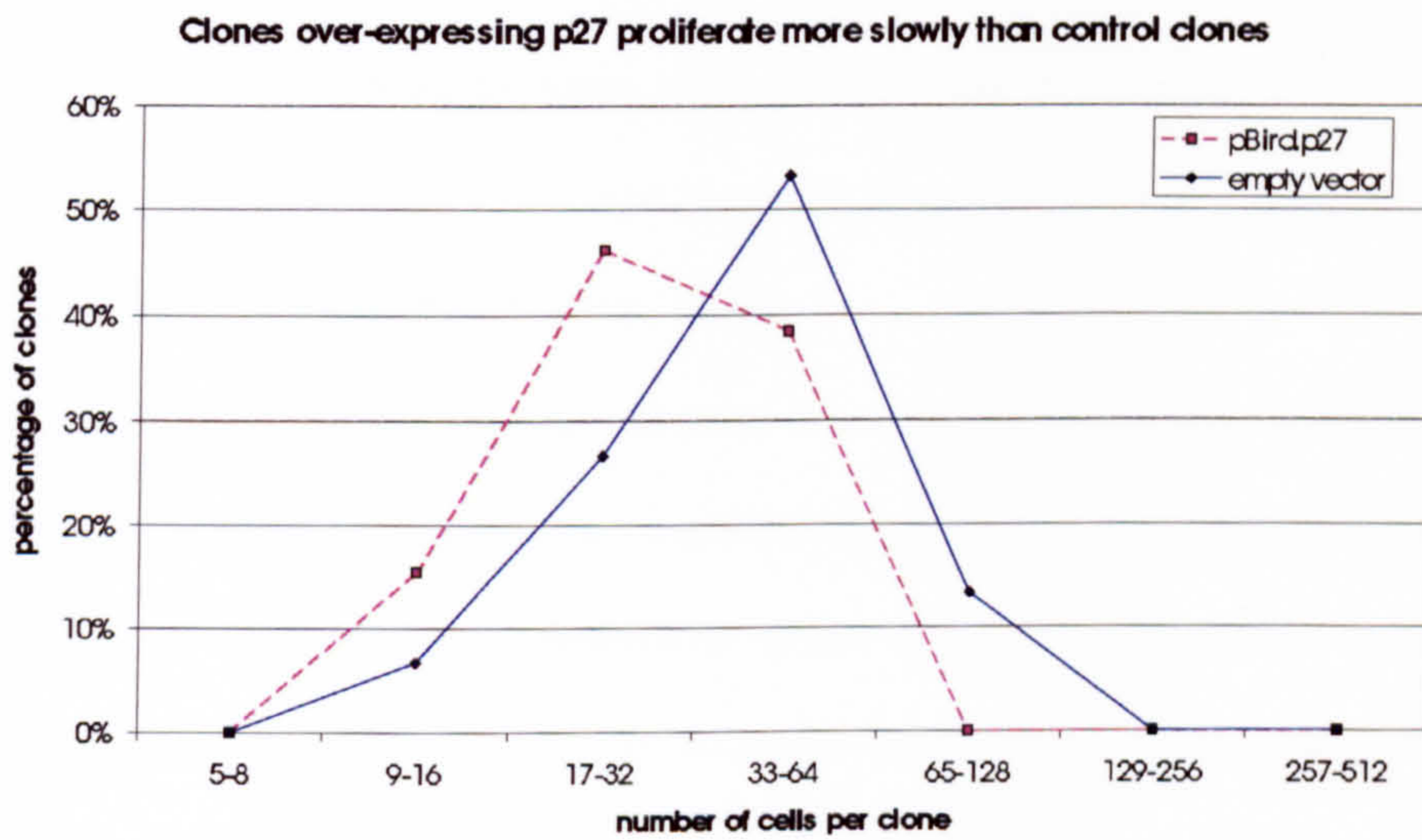


Figure 4-4: Clones that over-express p27 expand more slowly than control clones. This graph shows a comparison of the data from p27 over-expressing clones and clones infected with an empty vector, shown in figure 4-4.

#### **4.2.4 Analysis of the effect of p27 over-expression within oligodendrocyte precursor cell clones**

An important feature of the mechanism by which retroviruses infect host cells allowed us to take the clonal analysis one step further. By a small modification of the experimental procedure, we were able to compare cells over-expressing p27 with control cells within the same clone. This approach has significant advantages over comparing separate clones as individual precursor cells purified from optic nerves can vary considerably in their proliferative potential (Barres *et al.*, 1994).

Before a retrovirus can express its constituent genes, it must first reverse transcribe its genome and successfully integrate it into the genome of the host cell. This process is dependent on the host cell undergoing a round of DNA replication, which is why retroviruses only infect dividing cells. Thus, following adsorption and endocytosis of the retroviral particle by the host cell, there is a lag before expression of retroviral genes begins. We exploited this delay to purify and replate infected oligodendrocyte precursor cells at clonal density before they had time to divide. Subsequently, the first division of the clonal founder gives rise to two daughter cells, only one of which contains the integrated provirus. As the clone expands, the behaviour of the cells derived from the founder cell expressing the viral genome can be compared to the clonally related cells that do not, as the infected cells co-express GFP.

The expansion of a single clone was followed over the course of one week in culture, beginning three days after plating (figure 4-5). Initially, the clone contained twelve GFP<sup>-</sup> cells and two GFP<sup>+</sup> cells. The GFP<sup>+</sup> cells were grouped together and easily distinguished with an inverted fluorescence

microscope. Over the following four days, the uninfected cells proliferated to produce 85 cells. The GFP<sup>+</sup> cells did not divide until the seventh day whereupon one of the pair divided. In this case, the cell-cycle time of the cells over-expressing p27 was 4.3 times slower than the cycle time of the uninfected cells.

I wanted to study further the effect of p27 over-expression on the division rate of oligodendrocyte precursor cells, looking within single clones. The experimental procedure was modified to optimise the number of intra-clonal infection events: rather than waiting 24 hours after infection before purifying oligodendrocyte precursor cells, cells were infected for three hours and then purified immediately. Using this protocol, more than half of the clones that contained GFP<sup>+</sup> cells also contained GFP<sup>-</sup> cells. The remaining clones were entirely GFP<sup>-</sup> or entirely GFP<sup>+</sup>. Seven days after replating, mixed GFP<sup>+</sup>/GFP<sup>-</sup> clones were scored for total cell number and the proportion of GFP<sup>+</sup> cells that they contained.

The data show that in cultures infected with a control virus, expressing GFP in combination with  $\beta$ -galactosidase, the proportion of green cells within clones varied in a range from 33% ↔ 70%, with a mean of  $48.6 \pm 9.9\%$  (figure 4-6). In cultures infected with pBird.p27, the intra-clonal subset of cells over-expressing p27 comprised 3% ↔ 32%, with a mean of  $14.2 \pm 7.9\%$  (figure 4-7). In both the control- and pBird.p27-infected cultures, the proportion of dead cells that were GFP<sup>+</sup> was not different from those that were GFP<sup>-</sup> (data not shown).

Over-expression of p27 in a subset of cells within a single clone

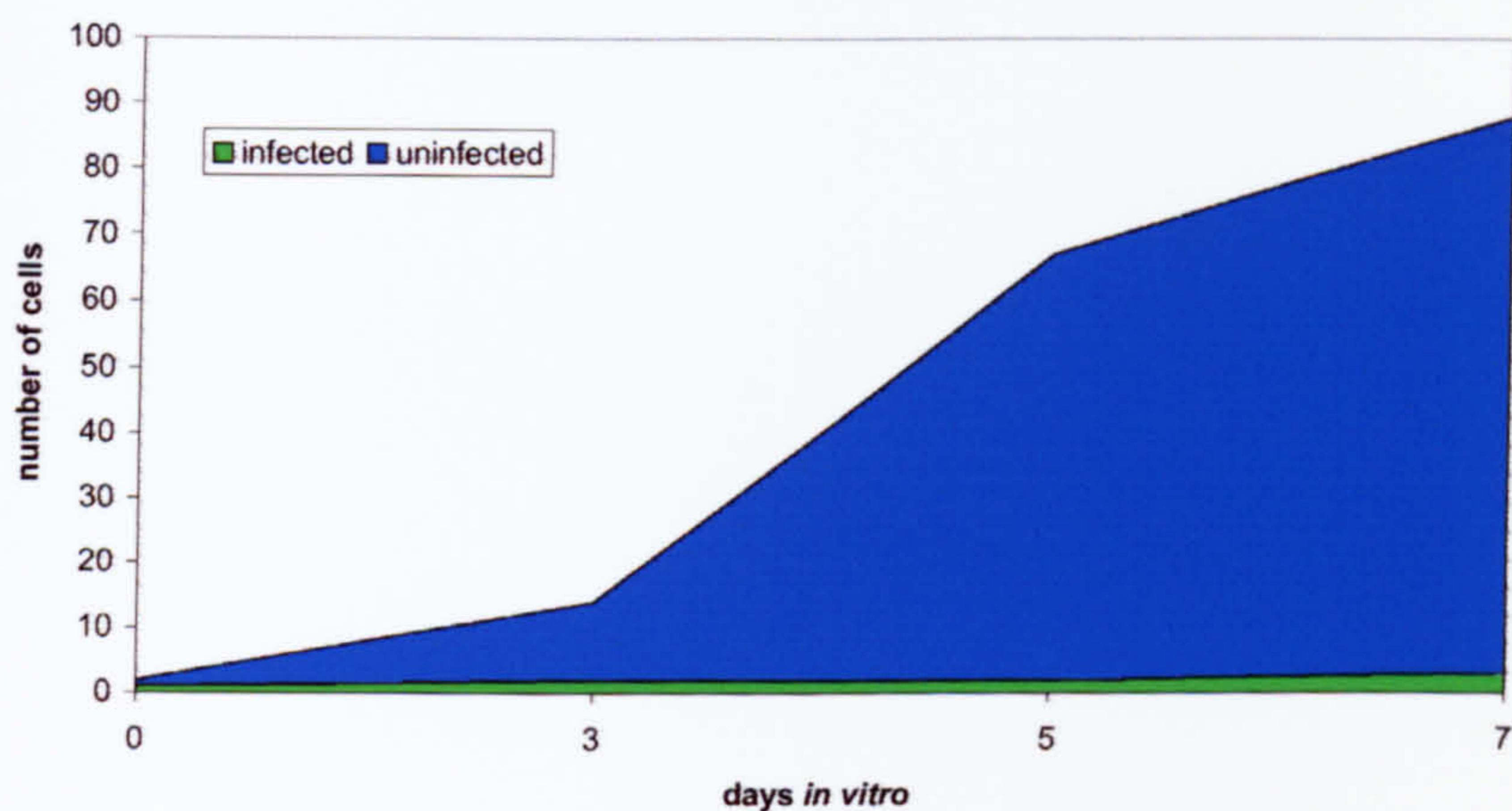


Figure 4-5: Graph showing expansion of an oligodendrocyte precursor cell clone containing a mixture of infected (GFP<sup>+</sup>) and uninfected (GFP<sup>-</sup>) cells. The oligodendrocyte precursor cells that over-expressed p27 proliferated four times more slowly than uninfected cells and the increase in cell number over time is largely due to expansion of the uninfected cells.

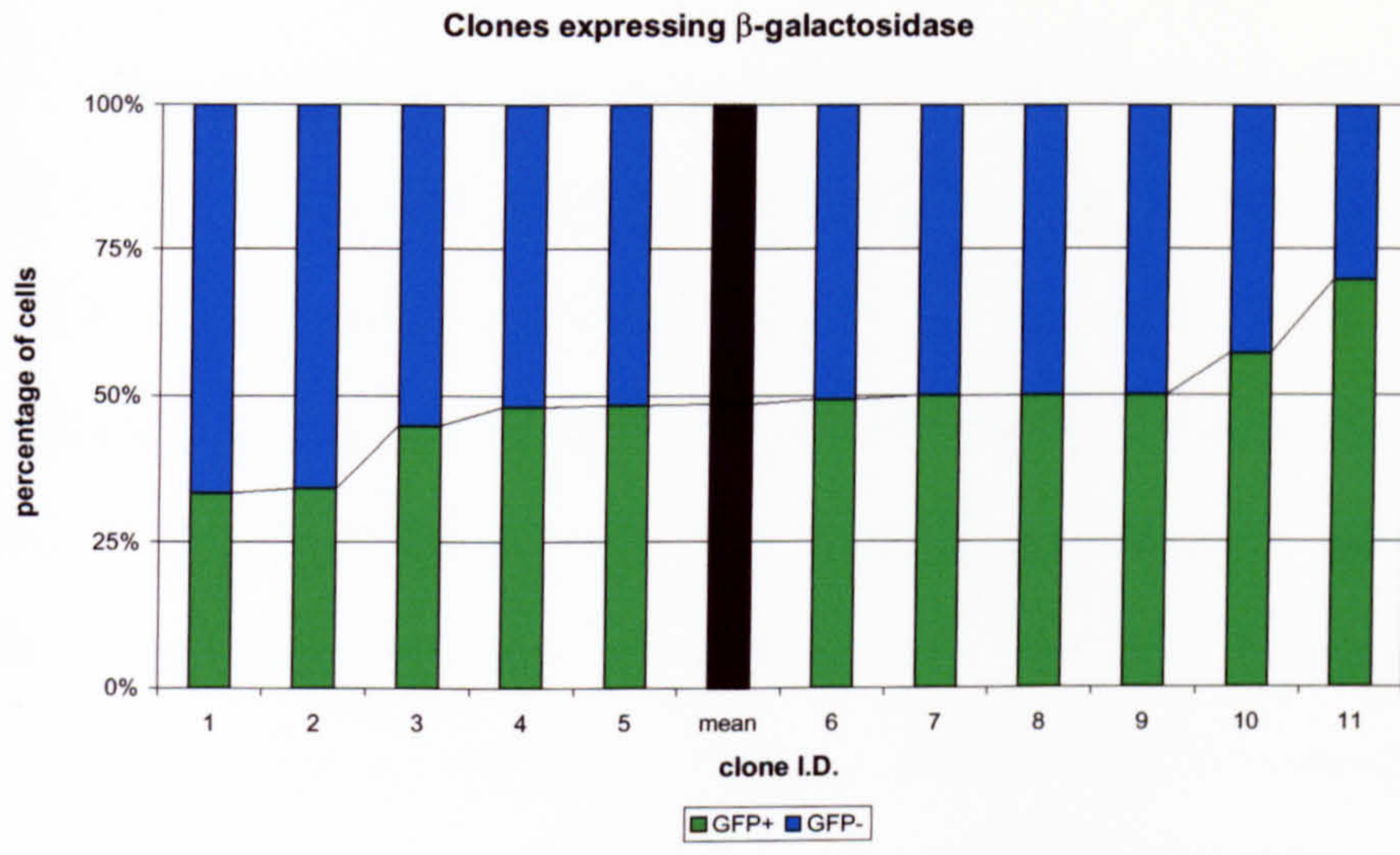


Figure 4-6: Histogram showing the proportion of GFP<sup>+</sup>/GFP<sup>-</sup> cells in eleven clones counted in this experiment. The stippled bar shows the mean of the values. These data are typical of those obtained in two independent experiments using a retrovirus that expresses GFP alone (pBird).

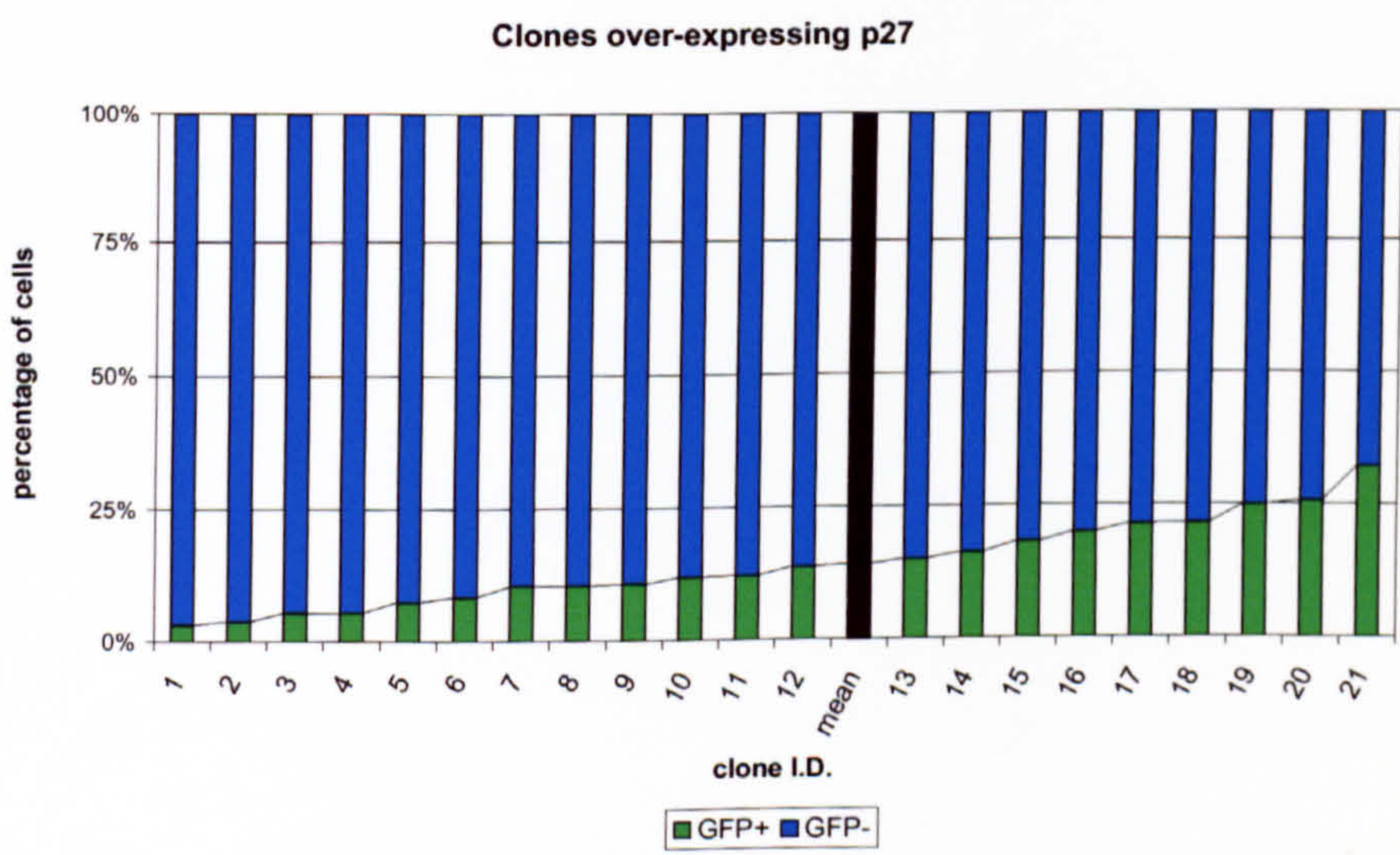


Figure 4-7: Histogram showing proportions of GFP<sup>+</sup>/GFP<sup>-</sup> cells in twenty-one clones counted in this experiment. The stippled bar shows the mean of the values. These data are typical of those obtained in three independent experiments using the pBird.p27 retrovirus in which 77 clones were scored.

These experiments were repeated using an empty vector control (pBird) instead of pBird.βgal, and similar data were obtained. The average proportion of GFP<sup>+</sup> cells in clones over-expressing p27 was  $16.4 \pm 2.1\%$ . In control experiments, the proportion of GFP<sup>+</sup> cells was  $47.6 \pm 2.3\%$ . The amount of cell death was not affected by over-expression of p27. These data are the means of three experiments and encompass data from 160 individual clones (figure 4-8).

In the control clones, infected cells are equal in number to uninfected cells, implying that they divide at a similar rate. In mixed GFP<sup>+</sup>/GFP<sup>-</sup> clones, the cells over-expressing p27 represented around one sixth of the total clone. As the proportion of dead cells within clones was not affected by infection with either pBird.p27 or the control virus, increasing the amount of p27 in precursor cells reduces their rate of division, on average by 1.5 times.

Over-expression of p27 reduces precursor cell proliferation

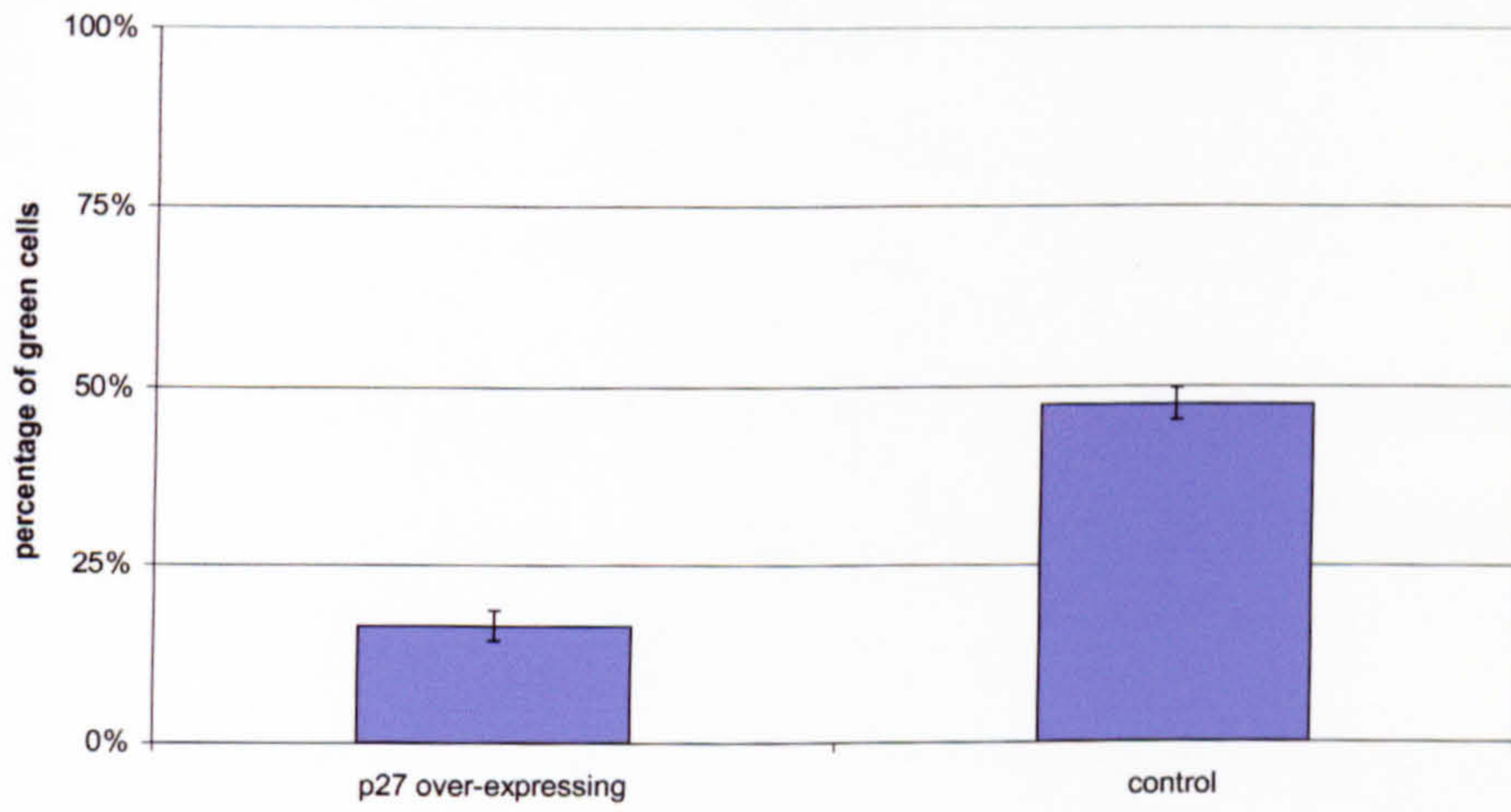


Figure 4-8: Over-expression of p27 reduces the rate of oligodendrocyte precursor cell proliferation. The histogram shows the mean percentage of green cells with standard deviations of data compiled from three independent experiments (encompassing 77 p27 clones and 83 control clones).

### **4.3 Discussion**

In this chapter, the effect of p27 over-expression was studied in the presence of mitogens but in the absence of TH. Under these conditions, the timing component of the timer still operates but the TH-dependent effector component does not. I aimed to inhibit the proliferation of oligodendrocyte precursor cells and determine if cell-cycle arrest is sufficient to induce differentiation.

#### **4.3.1 Over-expression of p27 is insufficient to cause cell-cycle arrest of oligodendrocyte precursor cells and differentiation into oligodendrocytes in the absence of TH**

I found that over-expression of p27 did not cause cell-cycle arrest of oligodendrocyte precursor cells in the presence of PDGF but in the absence of TH. Neither did it affect the proportion of oligodendrocytes that differentiated in these conditions. In the controls, one would not expect to see many oligodendrocyte precursor cells differentiating into oligodendrocytes, as mitogens were frequently replenished to keep them at or near saturating concentrations and TH was not present.

At the end of the experiment, very few cells had differentiated into oligodendrocytes. The small number of oligodendrocytes that were observed could be separated into two classes. The first class comprised single oligodendrocytes that were not associated with clones of cells, indicating that they had not divided prior to differentiation. These cells may have newly differentiated or committed to differentiation prior to replating, but remained



A2B5<sup>+</sup> and so were purified with oligodendrocyte precursor cells during panning; alternatively, they may have become insensitive to mitogens after replating, perhaps due to loss of mitogen receptors as a result of the trypsinisation. The second class comprised break-through oligodendrocytes, which were produced asynchronously and at a low frequency within clones. Such break-through differentiation has been studied previously (Ibarrola *et al.*, 1996; Gao *et al.*, 1998) and is proposed to arise due to stochastic events that operate independently of the TH-dependent effector mechanism.

Thus, the level of p27 expression achieved by transfection in my experiments is insufficient by itself to cause oligodendrocyte precursor cells to stop dividing and differentiate into oligodendrocytes. The possibility remains, however, that if the level of p27 was increased further it would be sufficient, even in the absence of TH. Yet, in the light of previous work showing that high levels of p27 are insufficient to promote differentiation (Durand *et al.*, 1997), it seems most likely that other intracellular events are required in order for the effector mechanism to be triggered. Perhaps the expression of other CKIs is necessary to trigger cell-cycle arrest in these cells, as has been demonstrated in other systems (Reynisdottir *et al.*, 1995).

#### **4.3.2 Over-expression of p27 in oligodendrocyte precursor cells retards clonal expansion**

I found that precursor clones over-expressing p27 contained fewer cells than control clones. The proportion of dead cells was not different in p27 over-expressing and control clones. Therefore, the size of a clone is a direct read-out of the cell-cycle time, and it appears that p27 acts by lengthening the cell-cycle

time. This observation is consistent with the known function of p27, which is mainly to inhibit cyclin-dependent kinases and thereby inhibit progression through the cell cycle.

In the absence of cell death, the number of cells in a clone increases exponentially over time according to equation 4-1, where  $N$  is the number of cells,  $t$  is the time in culture and  $d$  is the doubling time.

$$N = 2^{(t/d)}$$

Equation 4-1

Rearranging equation 4-1 gives an expression for the doubling time, thus:

$$d = \frac{t}{\log_2 N}$$

Equation 4-2

The p27 over-expressing clones were present in the same flask as the control clones and so were exposed to the same culture medium. Yet the mean clone sizes were 32 and 49 cells for control and infected clones, respectively, which corresponds to doubling times of 26 and 29 hours. This shows that the p27 over-expressing cells are proliferating at just less than 90% of the rate of the control cells.

### **4.3.3 Oligodendrocyte precursor cells that over-express p27 divide more slowly than wild-type cells in the same clone**

One concern when assessing the properties of clones within a population is that one may not be comparing like with like. Oligodendrocyte

precursor cells purified from optic nerves vary considerably in both their proliferative capacity (Barres *et al.*, 1994) and the amount of endogenous p27 that they express (Durand *et al.*, 1997). It has been assumed that this variation reflects heterogeneity in the maturation of oligodendrocyte precursor cells within the nerve (Gao and Raff, 1997), although it has never been shown directly that the level of p27 expressed by an oligodendrocyte precursor cell correlates with its maturation *in vivo*.

I exploited a peculiar feature of the retroviral life cycle to surmount many of these problems. Following infection and the requisite host cell division, only one of the daughter cells inherits and expresses the retroviral genome. When combined with clonal analysis, this characteristic of retroviral vectors allows one to study the effect of a transduced gene in a subpopulation of cells within a single clone.

Infection of precursor cells, with either the control viruses or pBird.p27, did not appear to affect their viability during the course of these experiments. The proportion of dead cells was very low and the amount of cell death was not different between GFP<sup>+</sup> and GFP<sup>-</sup> cells. Consequently, I will concentrate my analysis on the effects of p27 over-expression on the rate of cell division.

If a gene has no effect on the cell-cycle time, the infected and control cells will comprise equal proportions of the clone. Conversely, if a gene slows or accelerates the cell cycle, the infected and control cells will be unequal in number.

I found that p27 over-expressing cells comprised  $16.4 \pm 2.1\%$  (n=77) of the clones in which they were found. Cells that expressed either GFP alone, or GFP in combination with  $\beta$ -galactosidase, comprised  $47.6 \pm 2.3\%$  (n=83) of

cells within clones. This is a clear indication that p27 dramatically reduces the rate of oligodendrocyte precursor cell division. In pBird.p27 infected cultures, when individual clones were followed in real time, the GFP<sup>+</sup> cells, which were over-expressing p27, divided more slowly than sister cells that were GFP<sup>-</sup>.

Given the proportion of infected cells ( $N_{p27}$ ) and control cells ( $N_{con}$ ), equation 4-2 can be rearranged to derive an expression for the ratio of the cell cycle times of infected ( $d_{p27}$ ) and control ( $d_{con}$ ) cells.

$$\frac{d_{p27}}{d_{con}} = \frac{\log_2 N_{con}}{\log_2 N_{p27}}$$

Equation 4-3

Using equation 4-3, the cell cycle of p27 over-expressing cells was slowed to 72% of control cells. Given that  $d_{con}$  is approximately 26 hours, the p27 over-expressing cells were dividing, on average, every 36 hours. In the most extreme case (see figure 4-5 and accompanying text), the cell cycle time of oligodendrocyte precursor cells over-expressing p27 was lengthened to four days. In light of this, we must question what it means for a cell to be ‘cell cycle arrested’. What is the difference between a cell that is arrested in G<sub>1</sub> and a cell that is progressing through a four-day long G<sub>1</sub>?

One possibility is that for a cell to be truly arrested, it must enter a modified G<sub>1</sub> state known as G<sub>0</sub>. G<sub>0</sub> is the state that cells enter when they quiesce following withdrawal of extracellular growth factors or mitogens. During G<sub>0</sub>, the cell cycle machinery is partially dismantled in that some of the CDKs and cyclins are destroyed and not re-synthesised. One of the first components to disappear are some of the D cyclins (Zetterberg *et al.*, 1995). G<sub>0</sub>

can be operationally defined as the state where cells have to re-synthesise the missing cell-cycle components before they can re-enter the cell cycle following re-addition of mitogens. It seems most likely that the p27 over-expressing oligodendrocyte precursor cells have not entered  $G_0$ , as mitogens are always present, but slowly progress through the cell cycle. These findings raise an interesting point concerning the definition of cell-cycle arrest: one must be extremely careful before branding a cell 'quiescent' as it may just be progressing through the cell cycle very slowly.

It is possible that oligodendrocyte precursor cells must enter  $G_0$  before they are able to differentiate. These high levels of p27 expression are able to slow the cell cycle, in some cases extending it to over four days, but are apparently not able to push the cells into  $G_0$ . Cells that do not contain any p27, although delayed with respect to wild-type cells, are able to stop dividing and differentiate. Clearly, other, as yet unidentified, intracellular changes are required for cells to stop dividing in the presence of a saturating concentration of mitogens. Perhaps, as is the case with the timely cell-cycle arrest of epidermal cells during *Drosophila* development, a concomitant fall in one of the cell-cycle promoters, such as cyclin E, is necessary (Knoblich *et al.*, 1994). Alternatively, the co-operation of additional CKIs may be involved, as is the case when certain cell lines are induced to stop dividing in response to treatment with TGB- $\beta$  (Reynisdottir *et al.*, 1995).

## **4.4 Methods**

### **4.4.1 Concentration of retroviral supernatant**

Aliquots of retroviral supernatant were removed from storage at  $-80^{\circ}\text{C}$  and thawed in a  $25^{\circ}\text{C}$  water-bath. The aliquots were pooled into 50ml centrifuge tubes, chilled on ice and the pH was adjusted to seven by addition of 1.0M sodium hydroxide solution. The tubes were spun for five minutes at 2500rpm in a bench-top centrifuge to remove cellular debris and the supernatant was sterilised by passing through a  $0.45\mu\text{m}$  filter into polyallomer ultra-centrifuge tubes (#326823, Beckman). The tubes were spun in a SW28 swing-out rotor in a Beckman L-70 preparative ultra-centrifuge for two hours at 20,000 rpm and  $4^{\circ}\text{C}$ . After centrifugation, the supernatant was aspirated away to leave a small, yellowish pellet. This was resuspended in 1.0ml B-S medium by prolonged but gentle trituration using a Gilson P1000 Pipette-man set at  $500\mu\text{l}$ .

### **4.4.2 Infection of mixed optic nerve cell cultures with recombinant retrovirus**

Excess culture medium was removed from cultures of mixed optic nerve cells leaving a final volume of 1.0ml. Concentrated retrovirus in B-S medium, without polybrene<sup>3</sup>, and fresh mitogens and growth factors were added. The cultures were returned to the incubator for three hours. After

infection, the entire medium was removed and either replaced with fresh B-S medium including mitogens and growth factors or the cultures were trypsinised from the flask for immunopanning.

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<sup>3</sup> Although polybrene has been found to enhance the efficiency of infection of some cell types (Themis *et al.*, 1998), it appears not to affect the efficiency of infection of oligodendrocyte precursor cell growing in culture and may indeed be toxic to these cells (my unpublished observations; Pierre-Alain Fernandez, personal communication).

**5 Does p27 over-expression, in the presence of thyroid hormone, advance the timer?**



## **5.1 Introduction**

In the previous chapter, I showed that over-expression of p27 in oligodendrocyte precursor cells, in the absence of TH, fails to trigger cell-cycle arrest and differentiation. However, artificially raising the level of p27 in precursor cells appeared to slow the rate at which they proliferate.

The accumulation of p27 as oligodendrocyte precursor cells proliferate in culture and the delay in differentiation of precursors derived from p27-deficient mice are consistent with the proposed role of p27 as part of the intrinsic timer. (Durand *et al.*, 1997; Durand *et al.*, 1998).

In this chapter, I have used the pBird.p27 retrovirus, which co-expresses human p27 and GFP, to infect oligodendrocyte precursor cells. I examine the effect of p27 over-expression in the presence of PDGF and TH, conditions where both the timing and effector components operate normally. The question I addressed is whether over-expression of p27 accelerates the timer, as might be expected if an increase in p27 is part of the timing mechanism.

## **5.2 Results**

### **5.2.1 Over-expression of p27 advances the progress of the timer**

To determine the effect of p27 over-expression on the oligodendrocyte precursor cell timer, we infected purified oligodendrocyte precursor cells with pBird.p27 and cultured them in the presence of PDGF and TH. Under these conditions, oligodendrocyte precursor cells will stop dividing and differentiate when the value of their internal timer reaches a threshold level. If the level of p27 is important for setting this value, over-expression should artificially advance the timer and result in precocious differentiation.

To reduce the background level of differentiation we used oligodendrocyte precursor cell from neonatal (P0) rats. This was aimed to ensure that the range of timer values within the population was relatively low with respect to the timer end-point (Gao *et al.*, 1998; Gao and Raff, 1997).

Oligodendrocyte precursor cells were purified by sequential immunopanning and were cultured in the absence of TH for two days prior to infection to permit recovery from the purification procedure. One day after infection, with either a p27-expressing retrovirus or an empty vector control, TH was added to cultures. The percentage of oligodendrocytes, relative to all oligodendrocyte lineage cells, was scored after five days.

The data show that over-expression of p27 in oligodendrocyte precursor cells, in the presence of TH, promotes precocious differentiation into oligodendrocytes (figure 5-1). Oligodendrocyte precursor cells over-expressing p27 were twice as likely to have differentiated than those expressing an empty vector control. Whereas the percentage of oligodendrocytes in the control

(expressing GFP alone) was  $41.4 \pm 3.8\%$  (n=690), in p27-infected cultures the number was  $75.2 \pm 5.8\%$  (n=299). The experiment was repeated three times with similar results. Infection with the empty vector and resultant expression of GFP alone did not affect the background level of oligodendrocyte differentiation.

To determine the effect of p27 over-expression on the endogenous timer as it runs its natural course, further experiments were conducted and the percentage of oligodendrocytes was scored at two time points following addition of TH. After two days culture in TH, there was more than four times the proportion of oligodendrocytes in the p27-infected cultures than in the controls. After a further three days, p27 over-expression maintained the increased probability of differentiation above the control, but the effect was reduced to two-fold (figure 5-2 and table 5-1). By the end of the experiment, nearly all of the pBird.p27 infected oligodendrocyte lineage cells, but only half of the non-infected cells, had stopped proliferating and differentiated into oligodendrocytes.

**p27 over-expression advances the OPC timer**

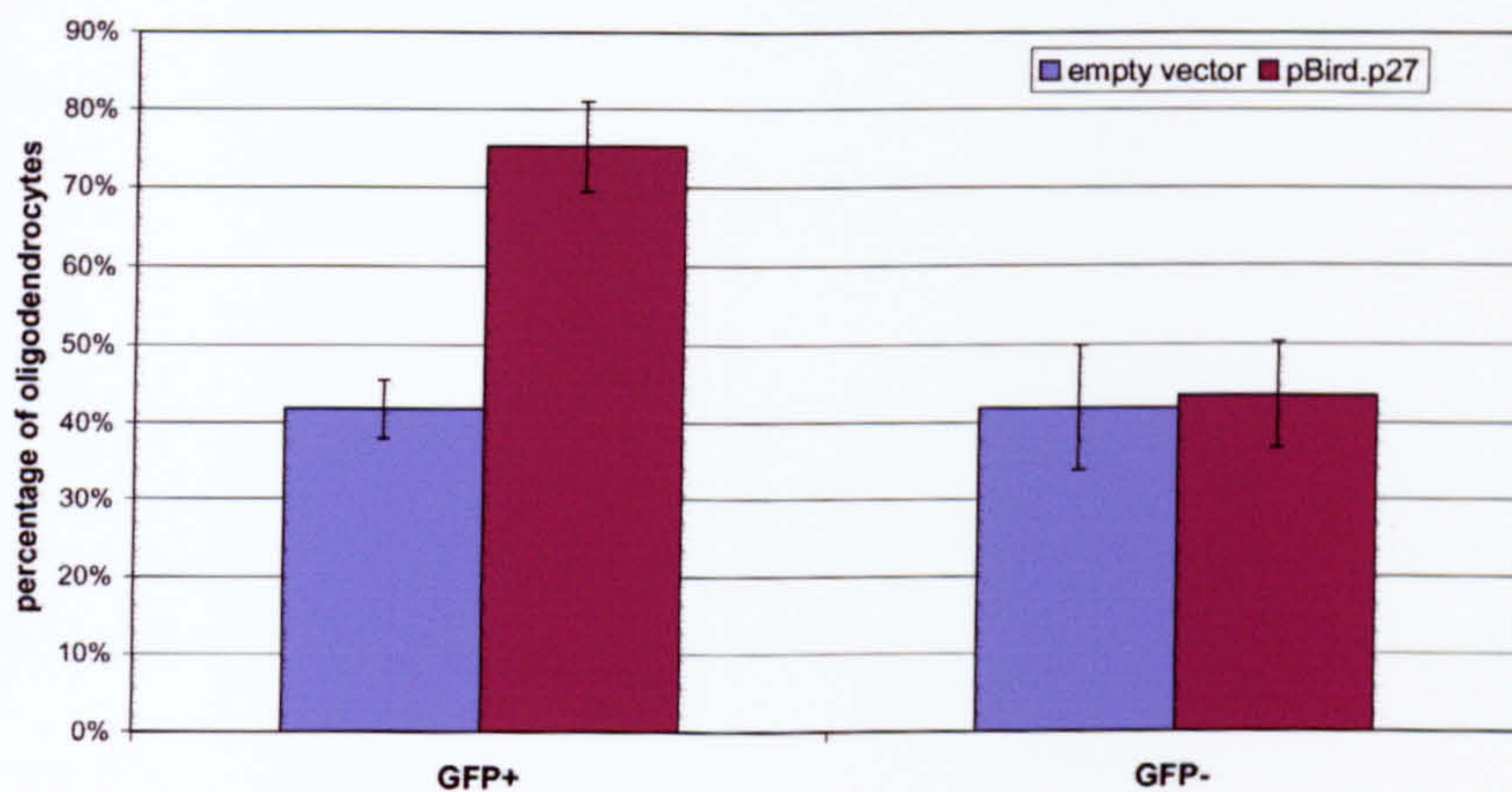


Figure 5-1: p27 over-expression advances the oligodendrocyte precursor cell timer. After five days in the presence of TH, twice as many oligodendroglial lineage cells differentiated if they were over-expressing p27 compared to those expressing an empty vector control.

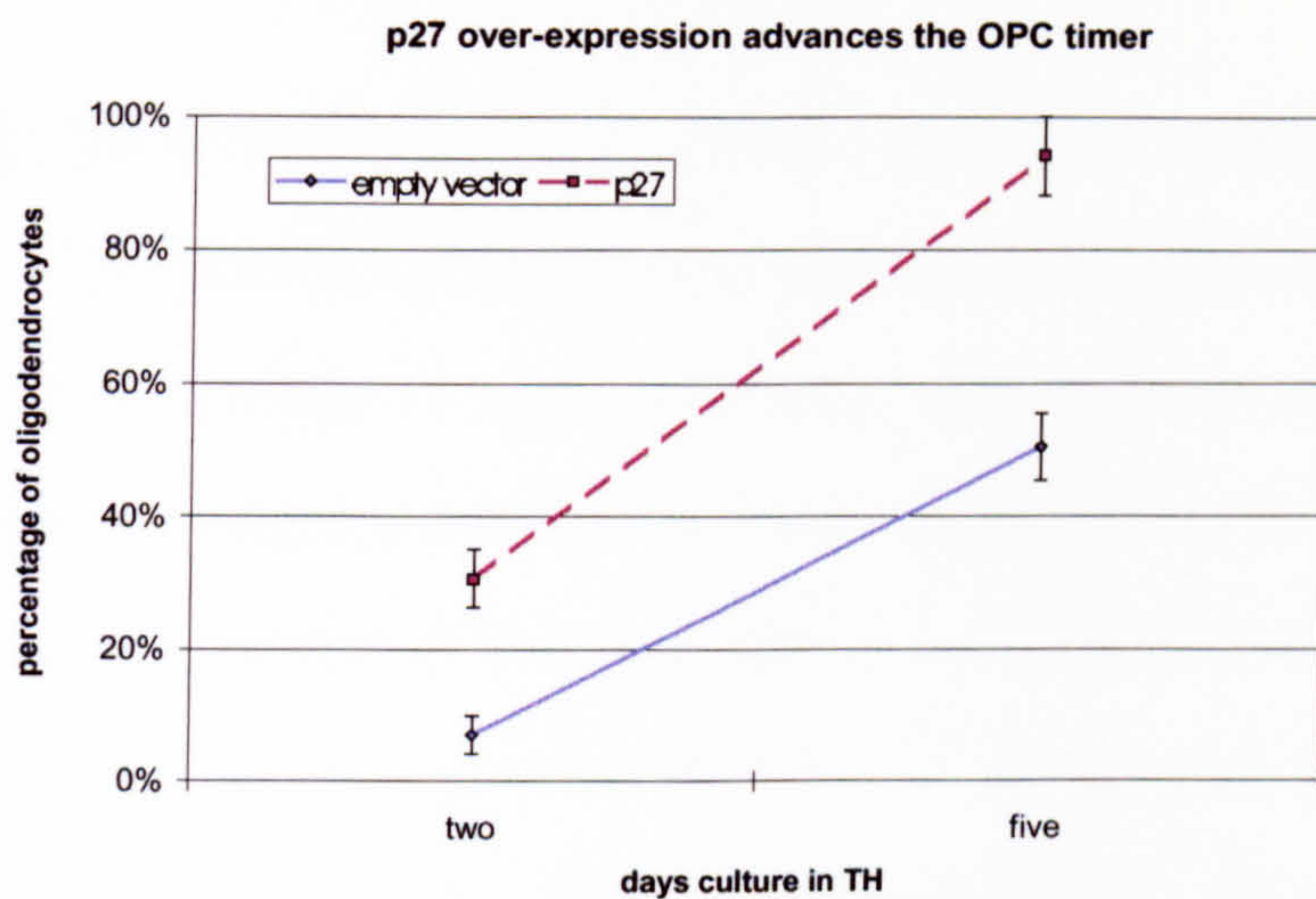


Figure 5-2: p27 over-expression advances the oligodendrocyte precursor cell timer. Purified oligodendrocyte precursor cells were infected with either pBird.p27 or an empty vector control. The percentage of oligodendrocytes, relative to all oligodendroglial lineage cells, was scored after two and five days in the presence of TH.

Table 5-1 – Over-expression of p27 continues to enhance the rate of oligodendrocyte precursor cell differentiation after several days in culture. However, the degree to which it advances the intrinsic timer becomes decreased as the timer runs its natural course.

days in culture	empty vector	p27	Enhancement
2	7.1 ± 2.9%	30.7 ± 4.3%	4.3
5	50.5 ± 5.1%	94.2 ± 5.9%	1.9

## **5.3 Discussion**

### **5.3.1 Over-expression of p27 advances oligodendrocyte differentiation by increasing the value of the timer**

The question of how the intrinsic timer controls oligodendrocyte development remains unanswered. It is likely that the timer does not operate by counting the number of precursor cell divisions (Gao *et al.*, 1997). The accumulation of the cell-cycle inhibitor p27 is commensurate with the progressive increase in the value of the timing component and plays a part in determining when the cells stop dividing and differentiate (Durand *et al.*, 1998; Durand *et al.*, 1997).

I wanted to determine whether over-expression of p27 in oligodendrocyte precursor cells would promote oligodendrocyte differentiation by artificially advancing the intrinsic timer in the presence of TH.

I found that oligodendrocyte precursor cells over-expressing a human p27 transgene differentiated sooner than oligodendrocyte precursor cells that contained an empty vector control. Over-expression of p27 did not induce immediate cell-cycle arrest and differentiation of all cells; rather it appeared to advance the normal timing of oligodendrocyte development. After five days in the presence of TH, oligodendrocyte precursor cells over-expressing p27 were twice as likely to have differentiated than control cells.

These data demonstrate that the amount of p27 expressed by oligodendrocyte precursor cells is important for determining the value of their endogenous timer. This is the first demonstration of a method for artificially advancing the timer as it runs its natural course.

Previously, we proposed that the timer has a distinct value in each oligodendrocyte precursor cell, which describes the probability that the cell will stop dividing and differentiate (Gao *et al.*, 1998). The value of the timer is 'early' in oligodendrocyte precursor cells that have divided for only a relatively short length of time, and are unlikely to differentiate, but becomes 'late' in cells that are near their timer endpoint. When a cell divides the value is inherited by each daughter cell and continues to rise during the time that they are proliferating. The value is further increased if TH is present and bound to its cognate receptor. When the value exceeds a certain threshold level the cells exit the cell cycle and differentiate.

In biochemical terms, the timer value presumably comprises the sum of activities of many different intracellular molecules. As the timer runs, some of these molecules, such as p27, increase whereas others decrease. In *Drosophila*, for example, a fall in cyclin E seems to be partly responsible for arresting proliferation of epidermal cells at the right time (Knoblich *et al.*, 1994).

In the experiments described here, I found that oligodendrocyte precursor cells over-expressing p27 differentiated sooner than cells expressing an empty vector control. This implies that the value of the timer in the cells over-expressing p27 has been advanced.

Expression of p27 from the constitutively-active retroviral promoter begins once the provirus has become integrated into the host chromosome. After a short lag, a steady-state level of p27 expression from the viral genome will be attained. The total amount of p27 protein ( $p27_{total}$ ) within infected cells will therefore comprise an endogenous component ( $p27_{timer}$ ), regulated as part

of the oligodendrocyte precursor cell timer, and a transgenic component ( $p27_{txg}$ ), that remains at a constant level (equation 5-1).

$$p27_{total} = p27_{timer} + p27_{txg}$$

Equation 5-1

The value of  $p27_{total}$  in the  $p27$  over-expressing cells is set above the value in uninfected or control-infected cells. As  $p27_{total}$  is important in determining the value of the timer, cells that would ordinarily be 'early' in their timer are advanced to a later value. Consequently, cells over-expressing  $p27$  reach their endpoint sooner than uninfected cells and differentiation occurs precociously (figure 5-3).

$p27$  was the first molecule to be identified that plays a role in governing the value of the oligodendrocyte precursor cell timer (Durand *et al.*, 1997). It is known that the expression levels of other intracellular molecules also change as oligodendrocyte precursor cells proliferate and their timers run (Yasuhito Tokumoto, unpublished observations; Gao *et al.*, 1998). However, the individual contribution of these components to the value of the timer has never been determined. It is not known whether all components contribute equally to the overall value, or if different molecules are more or less important at various times during precursor cell maturation.



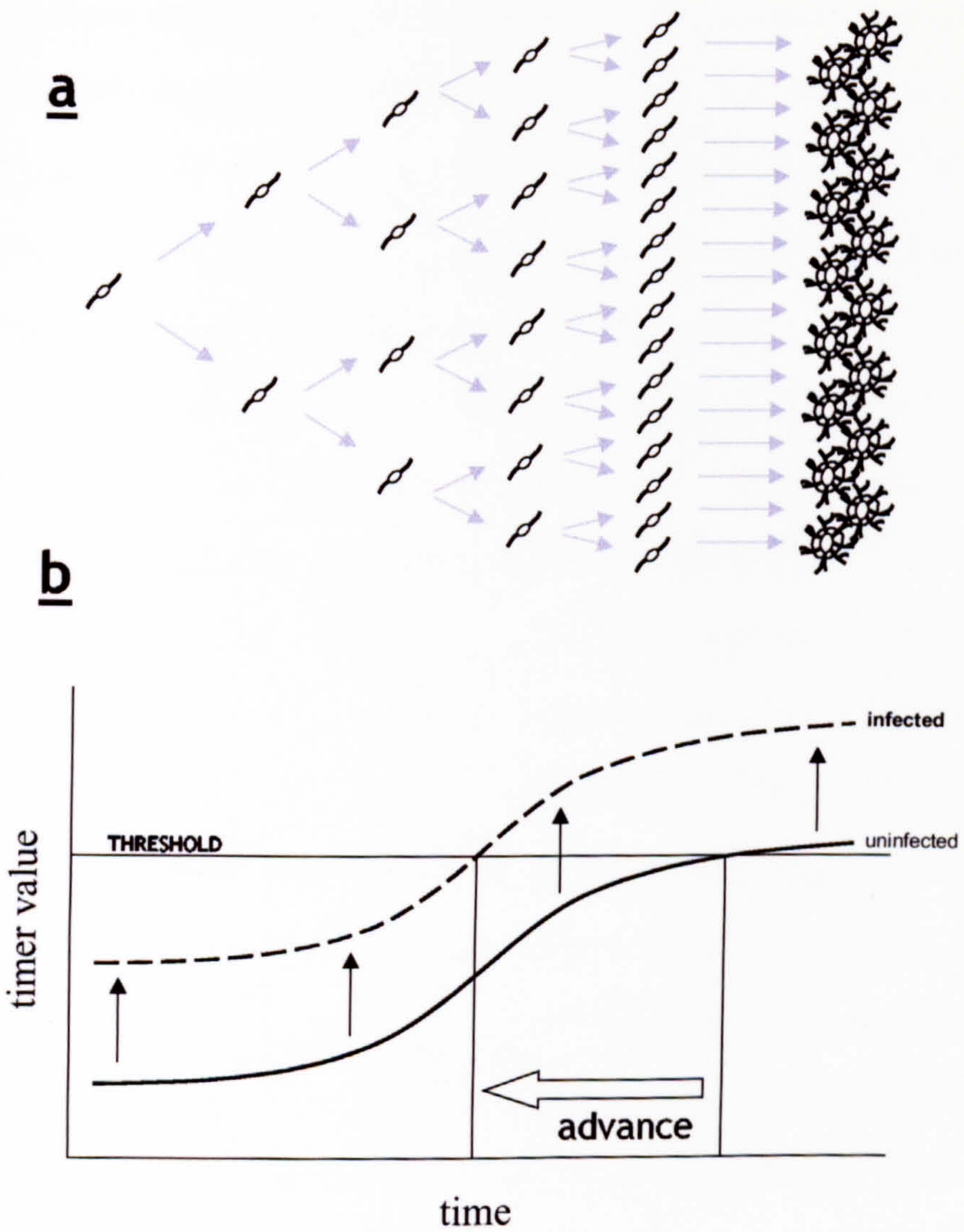


Figure 5-3: Over-expression of p27 in oligodendrocyte precursor cells advances the intrinsic timer. As oligodendrocyte precursor cells proliferate, the timing component of their timers increases in value and they become more likely to differentiate (a). Infection with pBird.p27 and over-expression of p27 raises the timer value by a set amount (b). Consequently, infected oligodendrocyte precursor cells express a timer value that is artificially advanced with respect to control cells and they differentiate sooner.

Time course experiments were conducted to assess the effect of p27 over-expression on the rate of oligodendrocyte differentiation. The proportion of oligodendrocytes was measured after two and five days culture, in the presence of TH. Although the proportion of oligodendrocytes was always greater in p27 over-expressing clones, I found that the rate of oligodendrocyte generation in these clones was the same as in the controls.

The timer value directly governs the likelihood that oligodendrocyte precursor cells will differentiate. Therefore, these experiments measure the contribution of the level of p27 to the overall value of the timer.

I assume that after a short lag following the activation of the retroviral genome, a steady-state level of  $p27_{txg}$  is achieved and the only change in  $p27_{total}$  is due to the accumulation of  $p27_{timer}$  (equation 5-1). If the contribution of the level of p27 to the overall timer value is constant, the amount by which the timer is advanced in cells that contain  $p27_{txg}$  should also remain constant. Consequently, the rate of oligodendrocyte generation should be the same in p27 over-expressing and control samples. If, however, the weight of the contribution of p27 to the timer value changes as the timer runs, or the retrovirus affects other components of the timer, then the rate of oligodendrocyte differentiation should change.

The results I obtained are consistent with the view that p27 directly contributes to the value of the timer and that the weight of that contribution remains constant (figure 5-4). Each molecule of p27 expressed appears to add the same increment to the timer value whether it is expressed at day two or day five, or whether there is a high or low level of p27 already present in the cells concerned. The only difference between cells that have been infected with

pBird.p27 and those that have not is that, in the former,  $p27_{total}$  is set at a higher due to the additional component provided by  $p27_{txg}$ .

Over-expression of p27 advances the intrinsic timer

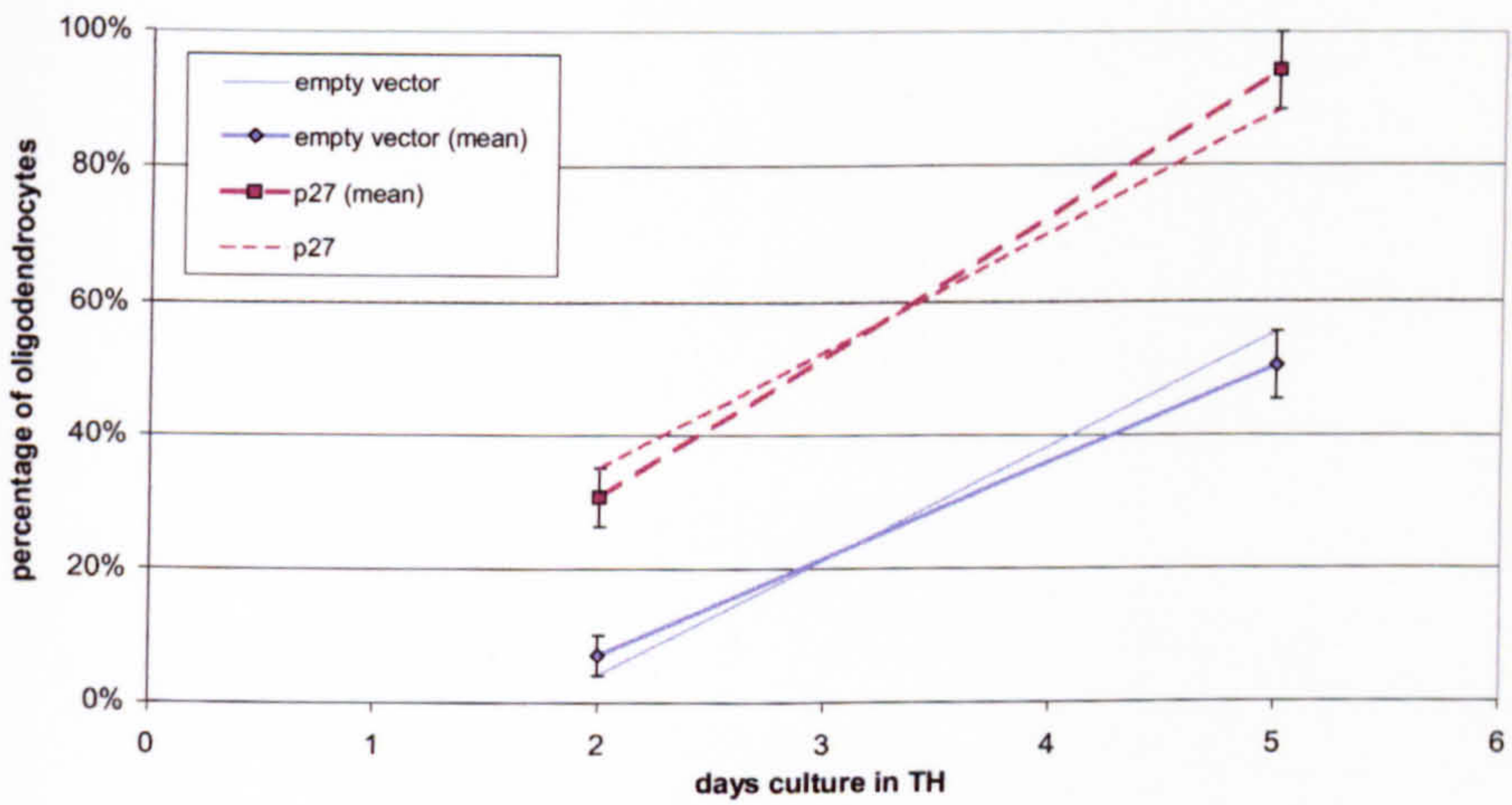


Figure 5-4: Over-expression of p27 does not significantly alter the rate of oligodendrocyte production over time. Within the range of experimental error (thin lines) the rate of oligodendrocyte production remains constant over three days.

## **5.4 Methods**

### **5.4.1 In vitro culture of oligodendrocyte precursor cells**

Oligodendrocyte precursor cells were prepared and cultured as described in chapters two and three (see §2.4.1, §2.4.2, and §3.4.3). In experiments employing thyroid hormone (TH) both triiodothyronine (T3; 30ng.ml<sup>-1</sup>) and thyroxine (T4; 40ng.ml<sup>-1</sup>) were added.

## **6 General Discussion**

The aim of this study was to elucidate the nature of the intrinsic timer that operates in oligodendrocyte precursor cells. I wanted to determine whether the timer primarily controlled the timing of cell-cycle exit, the timing of terminal differentiation, or both.

To help dissociate these alternative possibilities, I planned to inhibit the proliferation of precursor cells that were at an early stage of their timer, in the presence of PDGF and the absence of TH. If the cells stopped dividing and differentiated immediately, it is likely that that major influence of the timer is to control when precursors normally exit the cell cycle. In this case, which has been the favoured view (Gao *et al.*, 1998; Barres *et al.*, 1994), differentiation follows as a passive consequence of cell-cycle arrest. Alternatively, if precursors stopped dividing, but did not differentiate until the appropriate time, it is likely that the timer principally controls the timing of terminal differentiation. In this latter case, cell-cycle arrest may merely be part of the programme of differentiation, although it would still be possible that proliferation and differentiation were controlled separately, but in a co-ordinated way.

Two previous studies have attempted to address this question, using recombinant adenoviral vectors to force over-expression of the cell-cycle inhibitor p27 in proliferating precursor cells. Both groups showed that ectopic expression of p27 caused cell-cycle arrest, due to direct inhibition of cyclin E/CDK2 activity, but not did not cause differentiation of oligodendrocyte precursor cells. However, the inferences made by these groups seem to me to be seriously flawed: in particular, neither study examined the effect of p27 over-expression on the intrinsic timer (Tang *et al.*, 1998; Tikoo *et al.*, 1998).

First, the studies were conducted on cortical oligodendrocyte cells, rather than precursors derived from the optic nerve, that were cultured in conditioned medium for more than a week prior to experimentation. Cortical precursors are known to be more heterogeneous in the timing of their development than optic nerve precursor cells (Zhang and Miller, 1995; Vaysse and Goldman, 1990). Indeed, the major factor regulating the timely differentiation of oligodendrocytes in the brain and spinal cord may be competition for limiting amounts of PDGF, rather than an intrinsic timing mechanism (Calver *et al.*, 1998). Second, adenoviral vectors have severe cytopathic consequences when used to infect oligodendrocytes, and lead to dysmyelinating pathologies when used *in vivo* (Franklin *et al.*, 1999). In both studies, precursor cells died three days after infection with the recombinant vectors. Third, neither study looked at the effect of p27 over-expression on oligodendrocyte precursor cells in the presence of TH, and did not establish whether the p27 over-expressing cells had permanently withdrawn from the cell cycle or were just proliferating slowly. Fourth, and most perplexing, the experiments designed to examine the effects of p27 over-expression on the differentiation of oligodendrocyte precursor cells were conducted either in the presence of bFGF, or B104 conditioned medium, which contains bFGF (Louis *et al.*, 1992). bFGF is known to inhibit oligodendrocyte differentiation, therefore it is not surprising that oligodendrocytes failed to differentiate in these conditions (Bogler *et al.*, 1990; McKinnon *et al.*, 1990).

In my experiments, I developed a new retroviral vector, pBird, that co-expressed p27 and GFP, allowing the identification of infected precursor cells whilst they were growing in culture. I found that over-expression of p27 using



this vector was insufficient to induce cell-cycle arrest and differentiation into oligodendrocytes in the absence of TH. I was unable, therefore, to answer the main question that I posed at the outset of this study and, consequently, we still do not know what the intrinsic timer primarily controls.

However, two interesting findings did arise from my experiments. Over-expression of p27 in oligodendrocyte precursor cells, in the absence of TH, dramatically slows their rate of division, in the most extreme case extending the duration of the cell cycle to over four days, an increase of more than 370%. In the presence of TH, p27 over-expression advances the intrinsic timer causing oligodendrocytes to be produced earlier than normal, indicating that the timer is advanced if the level of p27 is artificially elevated.

The remarkable increase in the duration of the cell cycle in oligodendrocyte precursor cells that over-express p27 begs a question that has important ramifications for other studies of cell-cycle arrest. When is it valid to describe a cell as being cell-cycle arrested, when in fact it may be progressing through an unusually prolonged G<sub>1</sub>? Mature hepatocytes are often said to be quiescent, yet there is a constant, but very slow, renewal of these cells in adult liver (Goss, 1978). Maybe, like the p27-over-expressing oligodendrocyte precursor cells, they are merely in an extended G<sub>1</sub>? Similarly, adult stem cells, such as the adult oligodendrocyte progenitors that exist in the mature CNS (Wolswijk and Noble, 1989), divide much more slowly than their perinatal counterparts (Shi *et al.*, 1998). Do these cells enter the division cycle only when required to do so, such as in response to injury, or do they divide constantly, albeit very slowly?

The two previous studies, claiming that over-expression of p27 in oligodendrocyte precursor cells causes cell-cycle arrest, monitored division for three days after infection with recombinant adenovirus (Tang *et al.*, 1998; Tikoo *et al.*, 1998). In light of my findings, it is possible that they simply did not wait long enough to observe the slow proliferation of infected precursor cells.

The best-known function of p27 is to inhibit the cyclin/CDK complexes that operate in G<sub>1</sub>, particularly cyclin E/CDK2. However, matters are complicated because in addition to its inhibitory effect on G<sub>1</sub> progression, p27, and the other Cip/Kip CKIs, also help the assembly of functional cyclin D/CDK complexes (Sherr and Roberts, 1999), and active cyclin D/CDK holoenzymes contain Cip/Kip proteins (Cheng *et al.*, 1999). Sequestration of p27 by cyclin D/CDK complexes has been suggested as one mechanism by which proliferating cells can tolerate high levels of this CKI (Soos *et al.*, 1996), and this may explain why the cell-cycle time of oligodendrocyte precursor cells is not affected by the accumulation of endogenous p27 during normal timing (Durand *et al.*, 1997). Thus, to inhibit proliferation by over-expression of p27 it is not only necessary to inactivate all of the cyclin E/CDK2 in a cell, but to chelate all of the cyclin D/CDK as well. The rate of cyclin D synthesis, which is governed largely by signalling from mitogen receptors, establishes a threshold that must be overcome in order to arrest the cell cycle in G<sub>1</sub>. It is likely that the level of p27 expression achieved by pBird is insufficient to surmount this threshold, as retroviral LTR promoters are relatively weak, relative to other constitutive viral promoters used in current expression vectors (Guo *et al.*, 1996; Ray and Gage, 1992).

An alternative explanation for why over-expression of p27 failed to induce differentiation of precursor cells, in the absence of TH, is that differentiation may only take place once cells have entered a modified G<sub>1</sub> state, known as G<sub>0</sub> (Zetterberg *et al.*, 1995). A high level of p27, or any other CKI on its own, may not be sufficient by itself to induce precursors to enter G<sub>0</sub>. A second signal may be required in order for cells, which express high levels of p27, to enter G<sub>0</sub>, and this signal may normally be supplied by TH. In the absence of TH *in vivo* the timing of oligodendrocyte development is delayed, however, normal numbers of are produced ultimately (Ahlgren *et al.*, 1997; Ibarrola and Rodriguez-Pena, 1997). It seems likely that TH helps to co-ordinate development systemically by triggering events at the right time in several lineages, much as it co-ordinates events during amphibian metamorphosis (Gilbert and Frieden, 1981; Shi *et al.*, 1996; Tata, 1993).

Proliferating Mv1Lu mink lung cells contain high levels of p27 bound preferentially to cyclin D/CDK complexes (Polyak *et al.*, 1994a). Treatment with TGF- $\beta$  causes the cells to become quiescent. TGF- $\beta$  induces the expression of an INK4 family CKI, p15, which selectively inhibits cyclin D-dependent kinases by binding to the catalytic domain of the enzyme and displacing the cyclin subunit. This also liberates the sequestered p27, which subsequently binds to and inhibits cyclin E/CDK2, resulting in the firm cell-cycle arrest (Reynisdottir *et al.*, 1995).

TH does not induce the expression of p15 or other INK4 family proteins at the messenger RNA level (Tokumoto *et al.*, 1999), although the possibility remains that they are activated by post-transcriptional mechanisms. Interestingly, TH promotes a rise in the expression of cyclin D3 messenger

RNA and such an increase has been associated with the differentiation of some myoblast cell lines (Kiess *et al.*, 1995; Rao and Kohtz, 1995; Jahn *et al.*, 1994) and the human promyelocytic leukaemia HL60 cell line (Bartkova *et al.*, 1998). This may constitute a second signal that would be sufficient to force the oligodendrocyte precursor cells into G<sub>0</sub>.

The finding that over-expression of p27 in oligodendrocyte precursor cells causes precocious differentiation, in the presence of TH, supports the results of previous studies showing an important role for p27 in the intrinsic timer (Durand *et al.*, 1998; Durand *et al.*, 1997; Gao *et al.*, 1997). Over-expression of p27 in precursor cells more than quadrupled the proportion of oligodendrocytes that developed in culture, after two days in the presence of TH. The amount of p27 expressed by precursor cells, therefore, appears to contribute to the value of their intrinsic timers: cells that express high levels of p27 are more likely to stop dividing and differentiate than cells that express low levels.

How can the effects of p27 on the rate of precursor proliferation, in the absence of TH, and the advancement of differentiation, in the presence of TH, be reconciled? It is not difficult to understand how increasing the expression of p27 could reduce the rate of precursor cell division, given one function of p27 as an inhibitor of the cell cycle. Similarly, as p27 normally accumulates as part of the normal timing component of the timer, it is not hard to envisage how artificially raising the level of p27 advances oligodendrocyte differentiation. What is not clear is whether or not these two effects of p27 are related. The CKI function of p27 maybe separate from its role in setting the value of the oligodendrocyte precursor cells timer.

In *Xenopus*, a Cip/Kip homologue, p27<sup>Xicl</sup>, has been identified (Su *et al.*, 1995), which accumulates during retinal development and seems to control the timing of Müller glial cell differentiation (Ohnuma *et al.*, 1999). Overexpression of p27<sup>Xicl</sup> in the retina inhibits cell division and selectively induces the early differentiation of Müller glial cells, which are normally the last retinal cell type to be generated, with a compensatory loss of bipolar neurons. Antisense p27<sup>Xicl</sup> expressed in the developing retina leads to a decrease in the number of Müller cells, suggesting that p27<sup>Xicl</sup> is actively involved in Müller cell determination *in vivo*. The function of p27<sup>Xicl</sup> as a determinant of Müller cells is mediated by the N-terminal cyclin/CDK binding domain, a region which it shares with vertebrate p27. Given the apparent similarity of the roles of p27<sup>Xicl</sup> in *Xenopus* retinal development and p27 in oligodendrocyte development, it is likely that the timing function of p27 is mediated through its interaction with cyclin/CDK complexes.

I plan to elaborate on these studies and answer the question of what is the key event that the oligodendrocyte precursor cell timer is metering. It should be possible to find an efficacious means of arresting the precursor cell cycle and driving the cells into G<sub>0</sub>. For example, pBird could be used to express antisense RNA against the D cyclins (Schrump *et al.*, 1996) or *c-myc*, which is a central regulator of cell proliferation (Henriksson and Luscher, 1996; Evan *et al.*, 1994; Marcu *et al.*, 1992). Alternatively, a dominant-active form of pRb, from which the CDK target phosphorylation sites have been removed (Knudsen *et al.*, 1999), or a dominant-negative CDK2 (Hofmann and Livingston, 1996) could be tested.

The identification of molecular components of the intrinsic timing mechanism that controls oligodendrocyte developments raises several interesting questions. The accumulation of p27 appears to be controlled post-transcriptionally (Durand *et al.*, 1997) whereas the rise in the expression level of TH  $\beta$ -receptor is under transcriptional control (Toru Kondo, unpublished observations). If, as seems likely, transcriptional and post-transcriptional mechanisms are both involved in the timer, how are they co-ordinated such that they occur with commensurate time-scales (figure 1-4)?

Although the timing component of the timer is defective in p27-deficient animals, oligodendrocytes do develop after a delay of around one or two days (Durand *et al.*, 1998). These results suggest that other, as yet unidentified, molecules play a role in governing when precursor cells stop dividing and differentiate. A potential candidate is p18, an INK4 family CKI. The phenotype of mice in which both copies of the p18 gene have been ablated resembles that of p27-deficient mice: they are larger and contain more cells than do wild-type animals (Franklin *et al.*, 1998). Mice that lack both p27 and p18 have been generated and are even bigger than either of the single-gene mutants (Franklin *et al.*, 1998). p18 is rapidly induced during cell-cycle withdrawal and differentiation of muscle progenitors, by both transcriptional and translational mechanisms (Phelps *et al.*, 1998), however, it remains to be tested whether p18 normally accumulates during the proliferation of oligodendrocyte precursor cells and is part of the intrinsic timer.

Biological clocks and timers control events throughout the lifetime of all species, from the simplest bacteria (Golden *et al.*, 1998) to humans. The

problem of how biological systems are programmed with a timetable of forthcoming events is both intriguing and baffling. In some cases, the delay before the scheduled events occur can be considerable, for example, the flowering of some bamboo species happens with a period of 150 years.

The timing model of oligodendrocyte development has enlightened our understanding of how intrinsic changes can be hardwired into cellular physiology, however, this intrinsic timer is only one part of a more prolonged and complex maturation programme that changes several properties of the cells over time (Gao and Raff, 1997). This system offers significant advantages, such as the ability to grow and observe single cells in defined medium. Now, we are also able to experimentally manipulate the genome of oligodendrocyte precursor cells, and follow their behaviour over the course of many days *in vitro*. The future challenges in unravelling the molecular nature of the timing mechanism are not quite so daunting as they used to be.

## **Appendix: DNA sequences of vectors**



**pBabe.PURO: 5093bp**

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**pGimscript: 3446 bp**

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**pFoxIV: 6488 bp**

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**pFoxIV: sequenced 1820 bp**

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**pBird: 5483 bp**

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TC

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**The relationship between proliferation and differentiation  
during oligodendrocyte development**

**ADDENDUM**

## Introduction

How do precursor cells know when to stop dividing and differentiate? The phenomenon of lineage-specific progenitor cells undergoing a limited period of proliferation prior to terminal differentiation is a common theme in multicellular development. *Despite this, it is not known how cell-cycle withdrawal and differentiation are co-ordinated during development.*

I have studied the question of how proliferation and differentiation are co-regulated in the oligodendrocyte lineage in the rodent optic nerve. Oligodendrocytes are post-mitotic cells that myelinate axons in the vertebrate central nervous system (Bunge, 1968; Peters *et al.*, 1970). They develop from precursor cells whose maturation is controlled by a timer. The timer is an intrinsic property of the cells, and it seems to control the length of time during which divisions can occur, rather than the number of divisions the cell can undergo (Gao *et al.*, 1997).

Significant effort has been devoted to understanding how the intracellular timer works. It consists of two components that are modulated by distinct kinds of extracellular signals (Barres *et al.*, 1994; Bogler *et al.*, 1994). The mitogen PDGF drives a timing component, the value of which increases as precursor cells proliferate. Once this value exceeds a critical threshold, it triggers an effector component that promotes cell-cycle arrest and differentiation. The effector component is regulated by hydrophobic signalling molecules such as thyroid hormone (TH), while the timing component is not (Barres *et al.*, 1994).

The value of the timing component is thought to be determined by several intracellular proteins whose activities change as the timer runs. One of

these seems to be the cyclin-dependent kinase (Cdk) inhibitor p27/Kip1 (p27) - it accumulates in oligodendrocyte precursor cells as they proliferate in culture (Durand *et al.*, 1997). When p27 expression is high, the precursor cells are more likely to stop dividing and differentiate than when it is low. In oligodendrocyte precursor cells derived from mice that lack p27, the timer runs aberrantly, and both cell-cycle arrest and terminal differentiation are delayed (Durand *et al.*, 1998).

It is not known whether the timer serves to arrest the cell-cycle, with differentiation following by default, or whether it triggers differentiation, with cell-cycle arrest following by default. It is also possible that the two processes are controlled separately but in a co-ordinated way. These questions remain unanswered, at least partly because of difficulties in experimentally manipulating the genome of oligodendrocyte precursor cells and because attempts to arrest the cell cycle in the presence of PDGF have resulted in cell death (Martin Raff, personal communication).

The present study was an attempt to overcome these problems and had two aims: first, to devise a reliable system for transfecting oligodendrocyte precursor cells in a way that allows the transfected cells to be identified, and second, to determine whether over-expression of a cell-cycle inhibitor would be sufficient to induce oligodendrocyte differentiation in the presence of PDGF. I developed a new retroviral vector that co-expresses p27 and green fluorescent protein (GFP) in precursor cells. The use of GFP allows the identification of living precursor cells that over-express p27, which can then be followed over many days in culture.

My findings support previous work showing that p27 has a role in governing the timing of oligodendrocyte differentiation. They show that over-expression of p27 accelerates oligodendrocyte differentiation in the presence of TH, although it does not promote differentiation in the absence of TH, where the effector component is inoperative. The cell-cycle time of precursor cells that over-express p27 is dramatically extended, but it is not stopped. It therefore remains to be determined whether an experimentally-induced complete cell-cycle arrest and entry into a quiescent state ( $G_0$ ) is sufficient to elicit terminal differentiation in the presence of PDGF.



## Results

### *GFP expression faithfully reports co-expression of p27*

To validate the use of the pBird.p27 retrovirus for over-expression of p27 in normal oligodendrocyte precursor cells, I studied its expression in cells derived from mice that were homozygous null for p27 (p27<sup>-/-</sup> mice). I aimed to show that all GFP<sup>+</sup> cells co-expressed p27, and that all cells that expressed p27 were GFP<sup>+</sup>.

Cultures of mixed optic nerve cells were prepared from neonatal (P0) p27<sup>-/-</sup> mice and grown for two days *in vitro*. The cells were infected by addition of concentrated retroviral supernatant for three hours. Subsequently, fresh medium was added, and the cultures were returned to the incubator for a further five days. Infected cultures were fixed, permeabilised and stained by indirect immunofluorescence for both p27 and GFP.

In uninfected cultures, no cells were stained by the anti-p27 or anti-GFP antibodies, indicating that the antibodies did not react non-specifically with these cells (data not shown). In cultures infected with a control retrovirus, which expressed GFP alone,  $28.8 \pm 7.8\%$  cells were immunostained for GFP, and none of these cells co-stained for p27 (figure 1). Cultures infected with pBird.p27 were infected with an efficiency similar to the controls and, of the cells that expressed GFP,  $98.5 \pm 0.5\%$  were co-stained for p27 (figure 1).

The first cells to visibly express GFP were observed approximately 18 - 24 hours after infection. The number of cells expressing GFP, and the overall intensity of fluorescence, increased for another 24 hours thereafter. There was a variation in the intensity of green fluorescence detected, with some cells clearly expressing more GFP than others. In most cases, the intensity of the p27

staining, although not measured quantitatively, appeared to correlate with intensity of the GFP signal.

*Oligodendrocyte precursor cells that over-express p27 do not differentiate prematurely in the absence of thyroid hormone*

I aimed to determine whether over-expression of p27 in oligodendrocyte precursor cells would elicit premature cell-cycle arrest and concomitant differentiation in the absence of TH. Cultures of mixed optic nerves cells were prepared from P5 rats and grown for two days *in vitro* in the presence of PDGF and the absence of TH. They were infected with retroviruses that express either GFP alone (pBird), or in combination with p27 (pBird.p27). Oligodendrocyte precursor cells were purified after 24 hours by immunopanning and replated in the presence of PDGF and the absence of TH for four days. After this time, the proportion of GFP<sup>+</sup> oligodendrocytes, relative to the total number of GFP<sup>+</sup> oligodendrocyte lineage cells, was scored by identification of characteristic cellular morphologies .

Only  $17.5 \pm 5.4\%$  (n=4) of cells that over-expressed p27, as identified by direct GFP fluorescence, differentiated into oligodendrocytes. This did not differ significantly ( $p=0.5$ ) from GFP<sup>+</sup> cells that were infected with pBird or from the percentage of spontaneously differentiated oligodendrocytes observed in uninfected cultures (figure 2).

*Over-expression of p27 dramatically prolongs the cell-cycle time of oligodendrocyte precursor cells*

I aimed to determine if over-expression of p27 effected the cell-cycle time of oligodendrocyte precursor cells growing *in vitro* in the absence of TH. In the absence of cell death (which was readily quantifiable), the number of cells in a clone serves as a direct read-out of their average cell-cycle times. By comparing the size of clones infected with pBird.p27 to clones infected with a control virus it is possible to assess the effect of over-expressing p27 on the division rate of the constituent cells.

Mixed cell cultures were prepared from P5 rat optic nerves and grown for two days *in vitro* prior to infection with either concentrated pBird.p27 or pBird supernatant. The day after infection, oligodendrocyte precursor cells were purified by immunopanning, replated at clonal density and incubated for a further six days in the presence of PDGF and the absence of TH. Subsequently, the size and composition of clones were scored using characteristic morphological criteria to identify the cells.

Clones that were over-expressing p27 expanded more slowly than either uninfected clones or clones that expressed GFP alone (figure 3). The proportion of dead cells was similar in both cases (data not shown). After six days in culture, clones over-expressing p27 contained an average of 42 cells whereas control-infected clones contained an average of 60 cells. Clones infected with pBird.p27 never underwent more than six doublings during the time of the experiment, however, 92% of uninfected clones, growing in the same flask, doubled more than 6 times.

One concern when assessing the properties of clones within a population is that one may not be comparing like with like. Oligodendrocyte precursor cells isolated from perinatal optic nerves vary considerably in both their proliferative capacity (Barres *et al.*, 1994; Gao *et al.*, 1998) and the amount of endogenous p27 that they express (Durand *et al.*, 1997).

I exploited a particular feature of the retroviral life cycle to surmount this problem. Following endocytosis of the retroviral particle, the infected cell must undergo a round of DNA replication to allow the retroviral genome to integrate into genome of the host. If cells are replated at clonal density soon after infection, the first mitosis will yield two daughter cells that differ in a single respect: one will contain the integrated provirus and the other will not. Consequently, as the clone expands, a subset of cells within the clone will express the retroviral genome. I can therefore follow the fate of transfected and untransfected-sibling cells within the same clone in living cultures, as the transfected cells can be readily identified by their fluorescence.

The experimental procedure was modified to maximise the number of clones that expressed the retroviral genome in a subset of cells. Oligodendrocyte precursor cells were purified and replated three hours after infection, not 18 hours as in previous experiments. The data show that in cultures infected with a control retrovirus, which expressed GFP alone, the proportion of GFP<sup>+</sup> cells varied in a range from 33% to 70%, with a mean of  $47.6 \pm 2.3\%$  (n=83). The generally equal number of infected and uninfected cells implies that GFP-expression does not affect the rate of clonal expansion. In cultures infected with pBird.p27, however, the subset of cells within clones over-expressing p27 comprised from 3% to 32%, with a mean of  $16.4 \pm 2.1\%$

(n=77; figure 4). Thus, in clones infected with pBird.p27, infected cells represented only around one-sixth of the total cells in the clone. As the proportion of dead cells was not affected by either pBird.p27 or the control virus, increasing the amount of p27 in oligodendrocyte precursor cells clearly reduced their rate of division, on average by 1.5 times. In the most extreme case that I observed, the p27 over-expressing cells divided 4.3 times slower than the uninfected cells within the clone.

*p27 over-expression accelerates the oligodendrocyte precursor cell timer and promotes precocious differentiation in the presence of thyroid hormone*

The accumulation of p27 as oligodendrocyte precursor cells proliferate in culture and the delay in differentiation of precursors derived from p27<sup>-/-</sup> mice are consistent with the proposal that p27 is part of the intrinsic timer. To assess the effect of artificially raising the level of p27 in the presence of TH, oligodendrocyte precursor cells were purified from the optic nerves of P0 rats and cultured for two days *in vitro* prior to infection, in the absence of TH. After incubation with concentrated retroviral supernatant the cultures were returned to the incubator overnight before TH was added to the medium. The proportion of GFP<sup>+</sup> oligodendrocytes, relative to the total number of GFP<sup>+</sup> oligodendrocyte lineage cells, was scored at several time points.

The percentage of GFP<sup>+</sup> oligodendrocytes in cultures infected with the control retrovirus rose steadily, reaching 95.1 ± 6.0% of all GFP<sup>+</sup> oligodendroglial cells after eight days (the equivalent of P11 *in vivo*; figure 5). The proportion of GFP<sup>+</sup> oligodendrocytes in these cultures did not differ significantly from the proportion of GFP<sup>-</sup> oligodendrocytes (data not shown),

indicating that expression of GFP did not, by itself, influence the timing of differentiation.

Over-expression of p27 in oligodendrocyte precursor cells accelerated the onset of differentiation. Two days following addition of TH there were more than four times the proportion of oligodendrocytes in p27-infected cultures than in the control cultures. After a further three days, the difference was reduced to two-fold, and by the end of the experiment almost all of the cells, irrespective of whether they were over-expressing p27, had differentiated into oligodendrocytes (figure 5).

I next studied the effect of p27 over-expression in the presence of TH on clones in which only a subset of cells had been infected. If the level of p27 expressed by an oligodendrocyte precursor cell is important for determining the value of its timer, one would predict that the subset of cells infected with pBird.p27 would differentiate before their uninfected siblings.

Mixed cell cultures were prepared from P0 rat optic nerves and grown for two days *in vitro* in the absence of TH. Subsequently, the cultures were infected with either concentrated pBird.p27 or pBird supernatant for three hours. Oligodendrocyte precursor cells were purified by immunopanning, replated at clonal density and cultured for four days in the presence of TH. After this time, clones in which only a subset of cells was GFP<sup>+</sup> were scored for their fluorescence and the number of oligodendrocytes and precursors.

I found that in cultures infected with pBird.p27, GFP<sup>+</sup> cells within mixed GFP<sup>+</sup>/GFP<sup>-</sup> clones tended to differentiate first (figure 6). I observed that in 56% (18/32) of these clones all GFP<sup>+</sup> cells were oligodendrocytes and all precursors were GFP<sup>-</sup>. Only 2/32 clones were found in which the reverse was

true. Furthermore, in 81% (26/32) of clones, all GFP<sup>+</sup> cells were oligodendrocytes and only some oligodendrocytes GFP<sup>-</sup>. In control cultures infected with pBird, none out of 24 clones were found in which all GFP<sup>+</sup> cells were oligodendrocytes (figure 6).

## Discussion

It has been suggested previously that p27 is part of the timer that regulates oligodendrocyte differentiation. The level of p27 expressed by oligodendrocyte precursor cells increases progressively as they proliferate *in vitro*, and this rise parallels an increasing probability of differentiation (Durand *et al.*, 1997). In oligodendrocyte precursor cells isolated from p27<sup>-/-</sup> mice the timing of oligodendrocyte development is delayed by one or two days relative to wild-type cells (Durand *et al.*, 1998). In the absence of TH the effector component of the timing mechanism is inoperative, and the majority of oligodendrocyte precursor cells fail to stop dividing and differentiate, even after several weeks in culture (Barres *et al.*, 1994; Gao *et al.*, 1998). Conversely, in the presence of TH the effector component serves to limit proliferation, and, once the timing component signals that 'time is up', it initiates cell-cycle arrest and terminal differentiation (Barres *et al.*, 1994).

In this study I have over-expressed p27 in oligodendrocyte precursor cells and determined its effect on the timing of oligodendrocyte differentiation with and without TH.

My findings show that over-expression of p27 in the absence of TH is, by itself, insufficient to promote cell-cycle arrest and differentiation of oligodendrocyte precursor cells. In conditions where the effector component of timing mechanism is inoperative because TH is absent, cells that over-express p27 continue to proliferate, albeit more slowly than their normal siblings. However, if infected cells are cultured in the presence of TH, the timing of oligodendrocyte development is markedly accelerated in cells that are over-expressing p27.



### *Identification of infected cells by GFP fluorescence*

I have constructed a dual-promoter retrovirus, pBird, which can be used to introduce both a transgene of interest and GFP into oligodendrocyte precursor cells. In cultures infected with pBird.p27, fluorescence detection of GFP is more than 98% reliable as a marker for cells that co-express the p27 transgene. The overall level of expression varies from cell to cell, which may reflect secondary effects due to the site of proviral insertion in the host genome. I found that the intensity of GFP fluorescence generally seems to report the level of p27 expression. This system should prove an invaluable tool to extend studies on oligodendrocyte development, not only because it permits the efficient transfection of living oligodendrocyte precursor cells, but also because it allows one to follow the transfected cells as they proliferate in culture over several days.

### *Over-expression of p27 in the absence of TH*

In the presence of PDGF and the absence of TH over-expression of p27 did not promote differentiation of oligodendrocyte precursor cells. After four days in these conditions, the proportion of oligodendrocytes that differentiated was approximately 17% of the total number of cells and was not significantly different from the controls that were untransfected or expressed GFP alone. The majority of these oligodendrocytes were present as single cells, indicating that they had not divided prior to differentiation. This strongly suggests that they had committed to differentiation either prior to or immediately after replating, perhaps as a result of becoming insensitive to mitogens following trypsinisation. It has been shown previously that up to 10% of perinatal

precursor cells differentiate spontaneously during prolonged culture *in vitro* in the absence of TH (Gao *et al.*, 1998; Ibarrola *et al.*, 1996). The frequency of such spontaneous differentiation events increases with the absolute age of the precursor cells and is probably a result of stochastic events that operate independently of the TH-dependent effector mechanism (Gao *et al.*, 1998).

Although over-expression of p27 in oligodendrocyte precursor cells in the absence of TH does not promote differentiation, it does reduce the rate of precursor cell division. When I observed clones of cells that were over-expressing p27 and compared them to uninfected or control-infected clones, their rate of expansion was significantly reduced. Clones that over-expressed p27 contained fewer cells than control clones. The proportion of dead cells observed in these experiments was very small and was not different between p27 over-expressing and control cultures. When I looked at clones in which only a subset of the cells over-expressed p27, the GFP<sup>+</sup> component comprised, on average, one-sixth of the total number of cells, implying that p27 slows the division rate of these cells. In the controls, the GFP<sup>+</sup> component comprised around half of the clone, implying that expression of GFP alone did not affect the rate of cell division. In the most extreme case that I observed, a precursor cell that was over-expressing p27 did not divide until the seventh day of the experiment; in the same time, its uninfected siblings doubled more than six times. This is an important finding, as it indicates that one cannot assume that a cell has stopped dividing just because it does not divide during a week of observation. For this reason, previous experiments by other workers, which have been interpreted to indicate that arresting the cell cycle does not on its own induce oligodendrocyte precursor cell differentiation, need to be

reassessed (Tang *et al.*, 1998; Tikoo *et al.*, 1998). It remains to be determined whether an experimentally-induced complete cell-cycle arrest and entry into a quiescent state ( $G_0$ ) is sufficient to elicit terminal differentiation in the presence of PDGF. This may not be achievable by over-expression of Cdk inhibitors alone, although it would be worth testing other Cdk inhibitors, both alone and in combination.

It seems likely, therefore, that a major effect of artificially elevating the level of p27 is to extend the time it takes for oligodendrocyte precursor cells to go through the cell cycle. This finding is consistent with the known function of p27, which is to inhibit the Cdks that drive cells through the division cycle (Polyak *et al.*, 1994; Sherr *et al.*, 1995).

#### *Over-expression of p27 in the presence of TH*

As increasing p27 expression does not induce precursor cells to differentiate in the absence of TH, I was able to test its effects in the presence of TH. Artificially increasing the level of p27 in oligodendrocyte precursor cells in the presence of TH had a dramatic effect on the timing of oligodendrocyte differentiation. Precursor cells that over-expressed p27, which were readily identifiable because they were also GFP<sup>+</sup>, differentiated sooner than uninfected or GFP<sup>+</sup> control cells. Oligodendrocyte development in cultures that were infected with pBird.p27 was accelerated with respect to control cultures by one or two days. After five days in the presence of TH, cells that over-expressed p27 were more than twice as likely to have differentiated than the controls.

In further experiments, in which mixed GFP<sup>+</sup>/GFP<sup>-</sup> clones were studied, I found that cells over-expressing p27 in the presence of TH tended to differentiate before uninfected cells in the same clone. In control cultures, expression of GFP alone in a subset of cells within clones did not influence the timing of oligodendrocyte differentiation.

These data support the hypothesis that the amount of p27 expressed by an oligodendrocyte precursor cell is important for determining the value of its endogenous timer. The p27 expressed from the transgene presumably adds to the endogenous p27 in a cell and thereby increases the likelihood that the cell will engage the effector component of the timer. Initially, few cells in a population of perinatal precursor cells, even if they are expressing the transgene, have a high enough timer value to trigger cell-cycle arrest and differentiation. As the timing component runs in proliferating precursor cells, the level of endogenous p27 rises, and a greater proportion of cells reach the threshold value at which point the cells stop dividing and differentiate. A cell that expresses additional p27 from the retroviral genome reaches this critical point ahead of its normal siblings and therefore differentiates sooner.

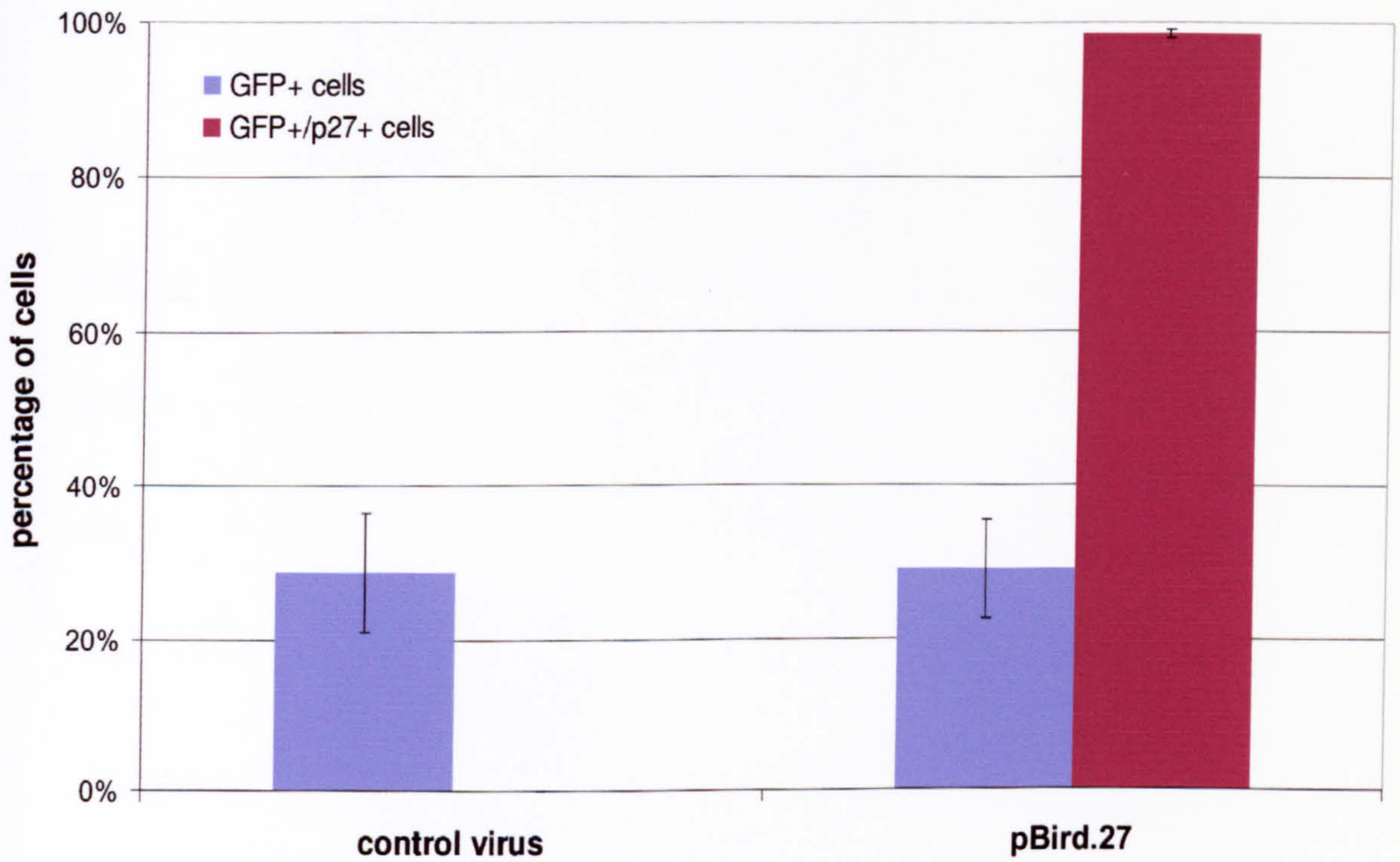
Thus, both the previous loss-of-function experiments (Durand *et al.*, 1998) and the present gain-of-function experiments together provide very strong evidence that p27 is a component of the timer that regulates the timing of oligodendrocyte development. It is, however, only one such component, as the timer runs quite well, if a little inaccurately, without it. The challenge now is to identify the remaining components and study their roles in the timing of oligodendrocyte development.

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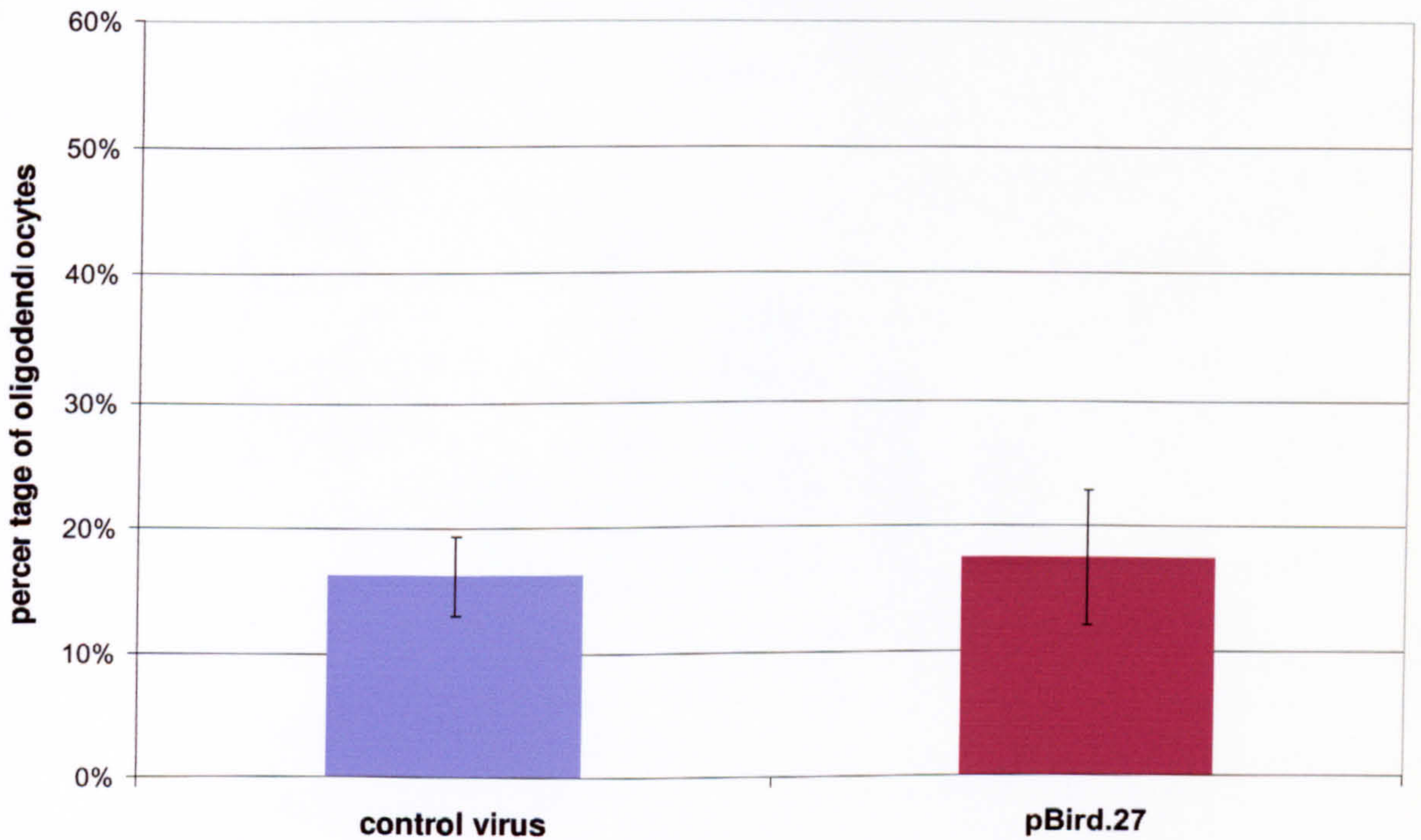
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### Expression of GFP faithfully reports co-expression of p27



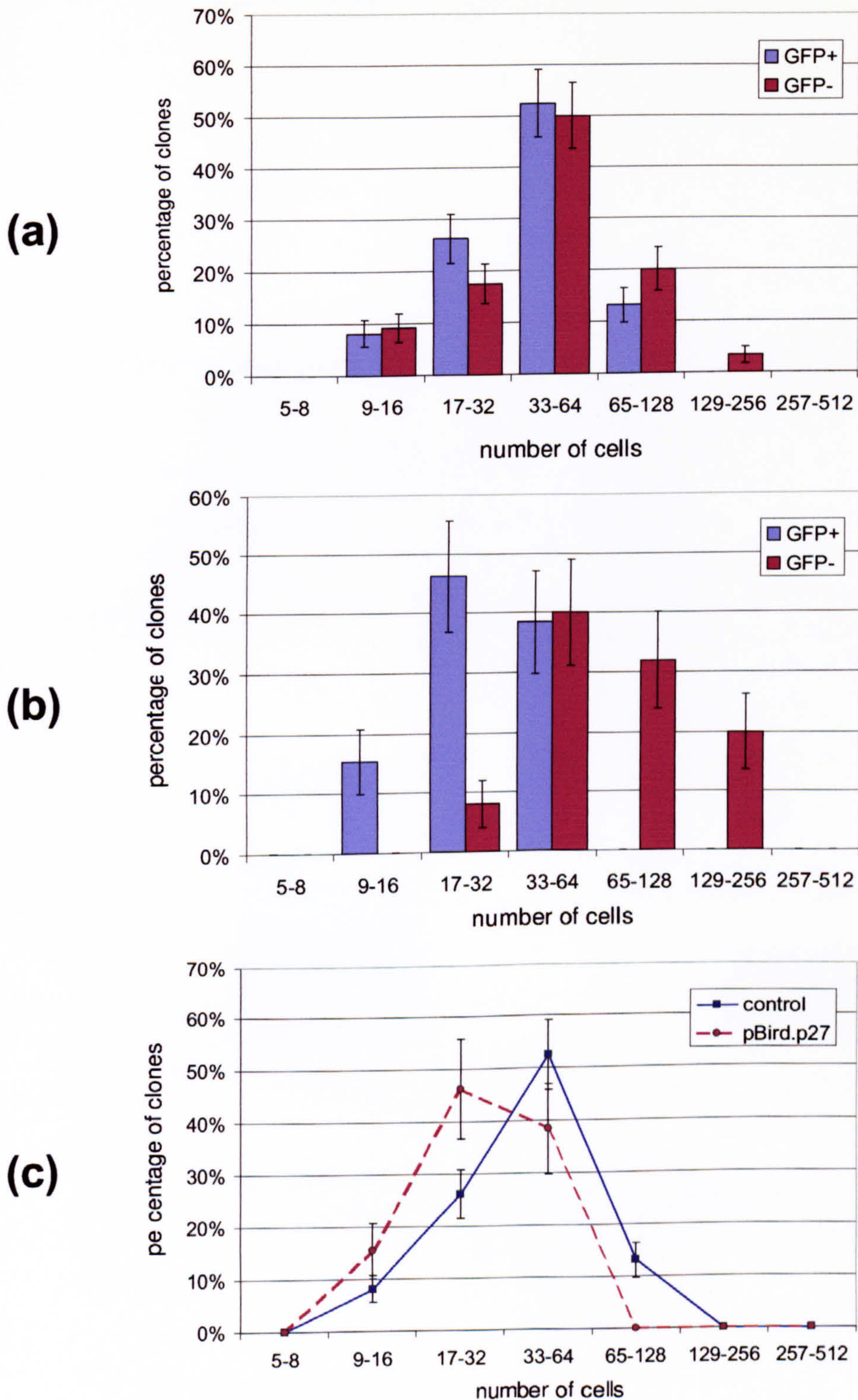
**Figure 1:** Expression of GFP faithfully reports co-expression of p27. Oligodendrocyte precursor cells from p27<sup>-/-</sup> mice were infected with either pBird.p27 or a control virus were immunostained for expression of GFP and p27.

**p27 over-expression in the absence of TH does not promote oligodendrocyte differentiation**



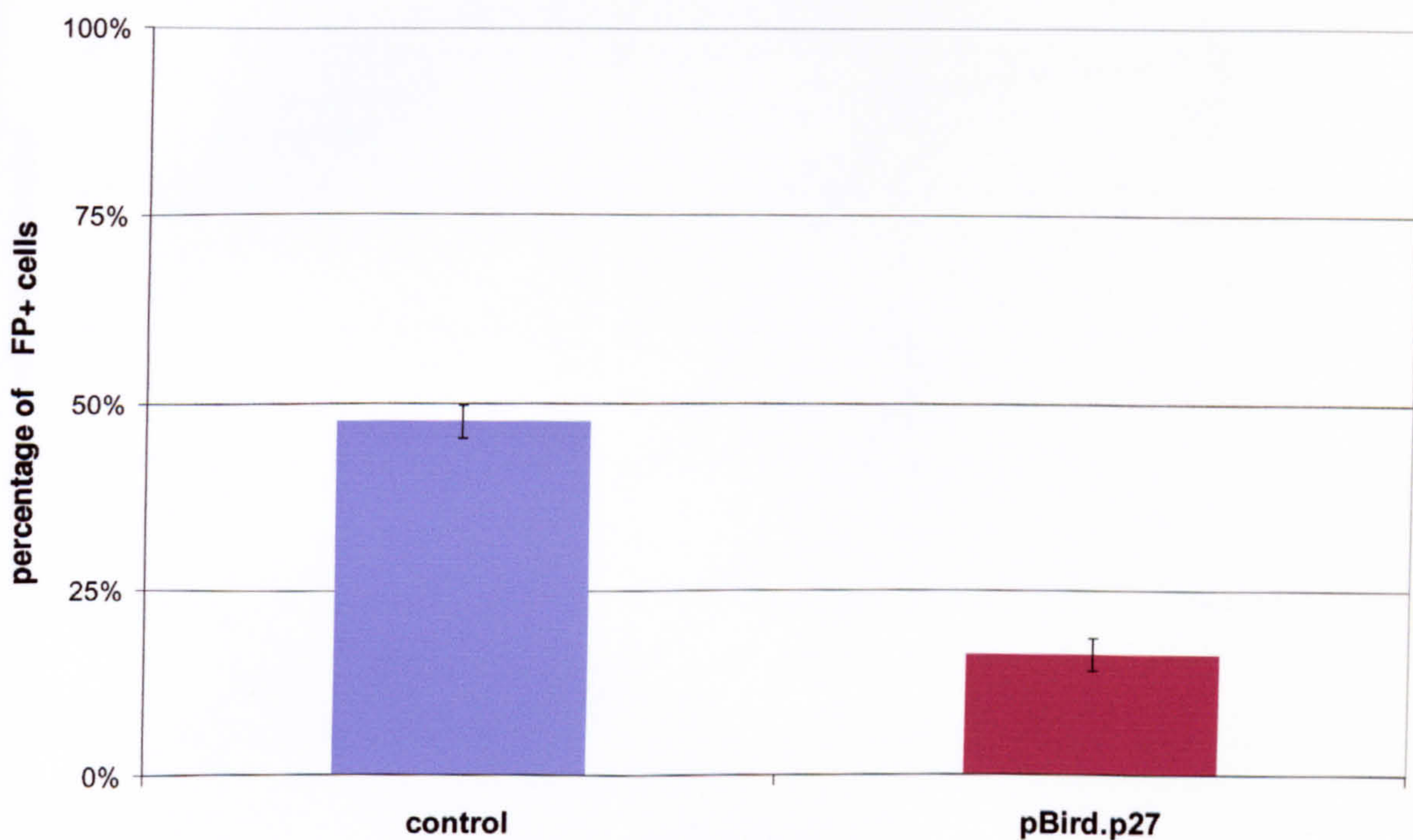
**Figure 2:** Over-expression of p27 in oligodendrocyte precursor cells does not elicit oligodendrocyte differentiation in the absence of TH. Using Student's t-test, there is no significant difference between the means ( $p=0.5$ ).





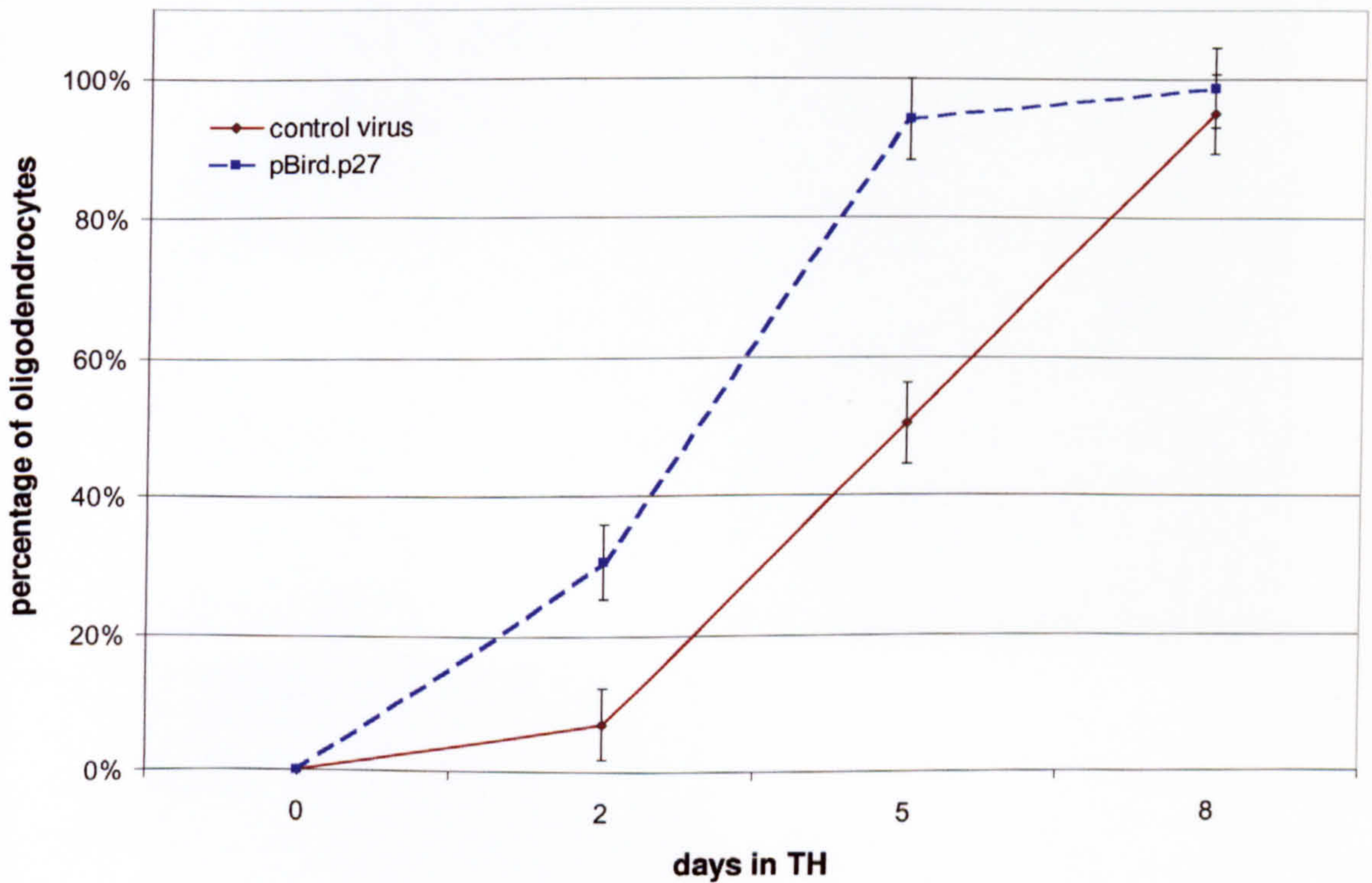
**Figure 3:** Over-expression of p27 in oligodendrocyte precursor cells dramatically reduces the rate of proliferation in the absence of TH. (a) In cells infected with a control virus, there was no significant difference in size between GFP<sup>+</sup> and GFP<sup>-</sup> clones. (b) In clones infected with pBird.p27, the GFP<sup>+</sup> clones contained fewer cells than GFP<sup>-</sup> clones. (c) After five days in culture, p27 over-expressing clones contained, on average, 18 fewer cells than control-infected clones.

### Over-expression of p27 decreases the rate of precursor cell division

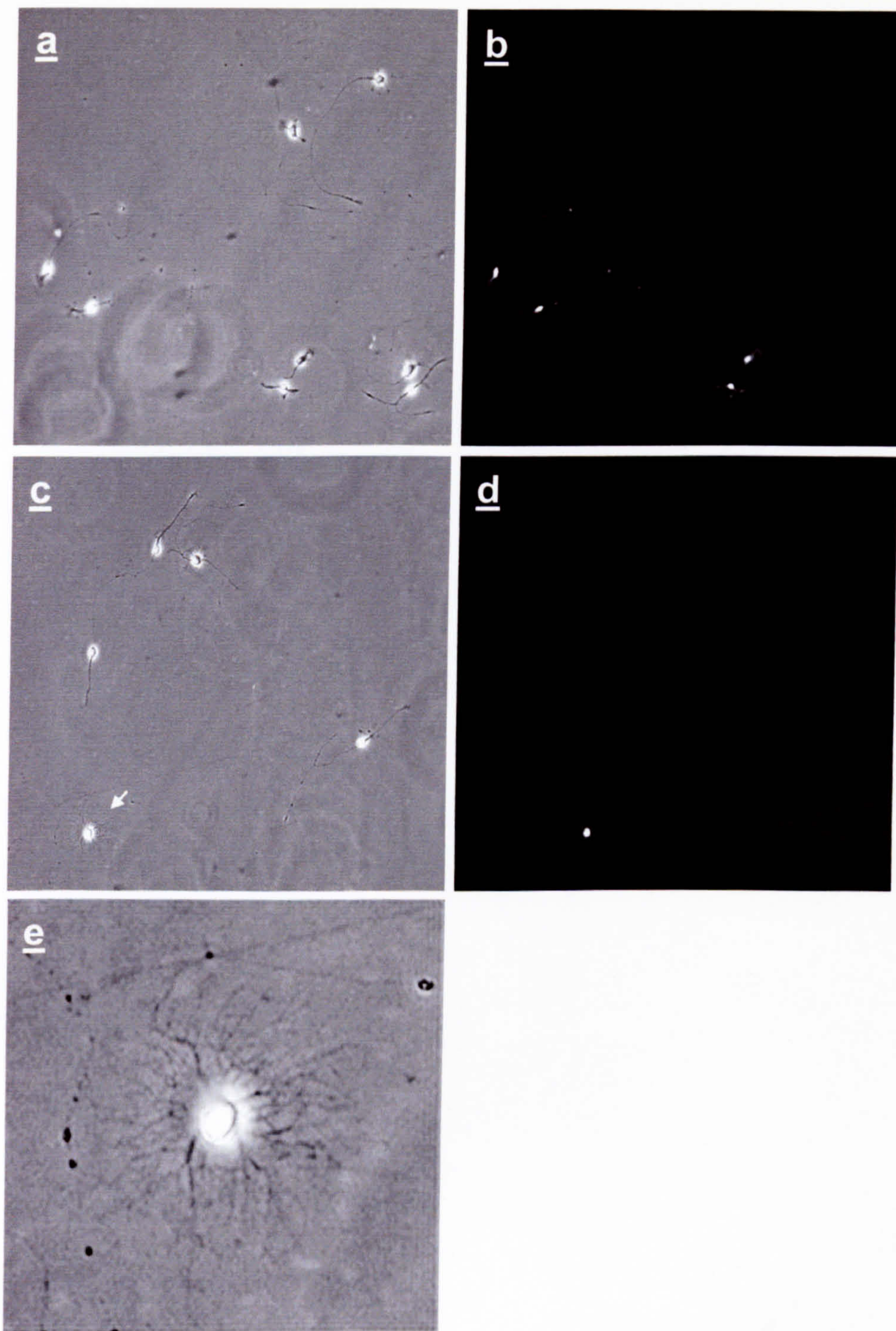


**Figure 4:** Over-expression of p27 reduces the rate of oligodendrocyte precursor cell proliferation. The histogram shows the mean percentage of GFP<sup>+</sup> cells with standard deviations compiled from three independent experiments.

### p27 over-expression in the presence of TH advances the timer



**Figure 5:** Over-expression of p27 accelerates the oligodendrocyte precursor cell timer and promotes precocious differentiation in the presence of TH. Cultures were infected with either pBird.p27 or a control retrovirus, and the percentage of GFP<sup>+</sup> oligodendrocytes, relative to total GFP<sup>+</sup> oligodendrocyte lineage cells, was scored at several time points following addition of TH.



**Figure 6:** Over-expression of p27 within clones containing mixed GFP<sup>+</sup>/GFP<sup>-</sup> oligodendrocyte lineage cells promotes early oligodendrocyte differentiation, in the presence of TH. (a-b) Phase-contrast and GFP fluorescence micrographs of a representative clone containing cells infected with a control virus and uninfected cells. (c-e) Clone containing cells infected with pBird.p27 and uninfected cells. Note that the cell which is over-expressing p27 [white arrow; enlarged in (e)] has differentiated before its uninfected siblings.