Introduction

In many vertebrate cell lineages, precursor cells divide a limited number of times before they stop proliferating and terminally differentiate. It is not known what causes the cells to stop dividing and differentiate when they do. The stopping mechanisms are important because they influence both the timing of cell differentiation and the number of differentiated cells generated.

We have been studying the stopping mechanisms in the oligodendrocyte cell lineage in the rodent optic nerve. Oligodendrocyte precursor cells (OPCs) migrate from the brain into the developing rat optic nerve before birth (Small et al., 1987). After a period of proliferation, most OPCs stop dividing and terminally differentiate into oligodendrocytes (Temple and Raff, 1986), which then myelinate the axons in the nerve. The first oligodendrocytes appear in the rat optic nerve around birth and then increase in number for the next six weeks (Barres et al., 1992; Miller et al., 1985; Skoff et al., 1976).

The normal timing of oligodendrocyte differentiation can be reconstituted in cultures of perinatal rat optic nerve cells (Raff et al., 1985). Clonal analyses performed with single (Temple and Raff, 1986) or purified (Barres et al., 1994) OPCs show that the progeny of an individual OPC stop dividing and differentiate at approximately the same time, even if separated and cultured in different microwells, suggesting that a cell-intrinsic mechanism operates in the OPCs to help limit their proliferation and initiate differentiation after a certain period of time or number of cell divisions. The finding that OPCs cultured at 33°C divide more slowly but stop dividing and differentiate sooner, after fewer divisions, than when they are cultured at 37°C suggests that the intrinsic mechanism does not operate by counting cell divisions but instead measures time in some other way (Gao et al., 1997). The timing mechanism depends in part on the progressive increase in cyclic-dependent kinase (Cdk) inhibitor p27/kip1 (Casaccia-Bonnefil et al., 1997; Durand et al., 1998; Durand et al., 1997) and the progressive decrease in the inhibitor of differentiation 4 (Id4) protein (Kondo and Raff, 2000).

Although the timer is cell-intrinsic, it is not cell autonomous. It requires extracellular signals to operate normally. The

of PDGF, cultured OPCs prematurely stop dividing and differentiate into oligodendrocytes within 1-2 days (Noble and

cultured in the presence of PDGF but in the absence of TH and RA, most of the cells keep dividing and do not differentiate (Ahlgren et al., 1997; Barres et al., 1994; Tang et al., 2001). If TH is added after 8 days, however, most of the cells stop dividing and differentiate within 4 days (Barres et al., 1994). These findings and others (Bögler and Noble, 1994) suggest that the intrinsic timer consists of at least two components: a timing component, which depends on PDGF and measures time independently of TH or RA, and an effector component, which can be regulated by TH and RA and stops cell division and initiates differentiation when time is up. Thus, TH and RA can induce OPCs to differentiate only when the OPCs have reached a certain stage of maturation (Gao et al., 1998), whereas PDGF withdrawal induces OPCs to differentiate at any stage of maturation, whether TH or RA is present or not (Ahlgren et al., 1997; Barres et al., 1994; Gao et al., 1998).

Although it is unclear whether RA regulates oligodendrocyte development in vivo, it has long been known that TH does. Myelination, for example, is delayed in hypothyroid animals (Dussault and Ruel, 1987; Rodriguez-Pena et al., 1993) and accelerated in hyperthyroid animals (Marta et al., 1998; Walters and Morell, 1981). Moreover, perinatal hypothyroidism decreases the number of oligodendrocytes in the optic nerve of the rat (Ibarrola et al., 1996) and mouse (Ahlgren et al., 1997). Thus, it seems probable that the TH-regulated intrinsic timer is responsible for the differentiation of at least some OPCs in vivo, especially postnatally, when TH levels are rising and OPCs are becoming more responsive to TH (Gao et al., 1998). But TH is not required for oligodendrocyte development, as even in its absence some OPCs eventually differentiate into oligodendrocytes both in vivo (Ahlgren et al., 1997) and in vitro (Ibarrola et al., 1996; Ahlgren et al., 1997). Because TH influences the timing of differentiation in several cell lineages, it is probable that it plays a part in coordinating the timing of differentiation in different tissues during vertebrate development: TH coordinates the onset of myelination in the central and peripheral parts of the auditory nerve, for example (Knipper et al., 1998).

It remains unclear how TH or RA triggers OPC differentiation. Both act by binding to nuclear receptors that are members of the same superfamily of ligand-regulated transcription factors (Evans, 1988; Mangelsdorf et al., 1995). We showed previously that the TH receptor TRα1 is required for the normal timing of oligodendrocyte development in vitro and in vivo, but the downstream effectors of this differentiation pathway are still uncertain (Billon et al., 2002). One part of the pathway probably involves E2F-1, as TH rapidly inhibits the expression of E2F-1 in purified rat OPCs; the *E2F-1* promoter contains a negative TRE (called a Z-element) that binds thyroid hormone receptors (TRs), which directly activate *E2F-1* transcription in the absence of TH and repress it in the presence of TH (Nygård et al., 2003). As E2F-1 promotes progression from G1 into S phase of the cell cycle (Helin, 1998), its repression by TH is likely to contribute to the cell-cycle withdrawal and differentiation of OPCs in response to TH (Nygård et al., 2003). On a slower timescale, TH also influences the expression of other genes that would be expected to help induce OPC to exit the cell cycle and differentiate: by 16 hours, for example, it stimulates an increase in mRNAs that encodes various Cdk inhibitors, and, by 24 hours, it decreases the level of cyclin D1 and D2 proteins (Tokumoto et al., 2001).

There is also evidence that the p53 family of proteins plays a part in RA- and TH-induced OPC differentiation. This differentiation is blocked if purified OPCs are infected by a retroviral vector encoding a dominant-negative form of p53 that inhibits both p53 and other members of the p53 family (Tokumoto et al., 2001). It has also been reported that a dominant-negative form of p53 inhibits spontaneous oligodendrocyte differentiation in mixed cultures of neonatal rat brain cells (Eizenberg et al., 1996). It is still uncertain, however, which p53 family members are important for OPC differentiation or how they promote this differentiation.

p53, p63 and p73 proteins share considerable structural and functional homology (Yang et al., 2002). They all function as transcription factors and regulate the expression of similar groups of genes by directly binding to identical DNA response elements in the promoter. Whereas the *p53* gene encodes one major protein, both the *p63* and *p73* genes contain two separate promoters that direct the expression of two functionally distinct types of protein from each gene (reviewed by Irwin and Kaelin, 2001; Levrero et al., 2000; Melino et al., 2002). One type of protein, denoted TAp63 or TAp73, has an acidic N-terminus, which is homologous to the transactivation domain of p53. In the second type of protein, denoted ΔNp63 or ΔNp73, the N-terminus is truncated and lacks the transactivation domain. Alternative splicing at the 3′ end of the *p63* and *p73* RNA transcripts generates additional complexity by creating both TA and ΔN proteins with different C-termini. Whereas the TA isoforms are able to activate gene expression, the ΔN isoforms cannot and instead can exert a dominant-negative effect on p53 and the TA forms of p63 and p73 (Grob et al., 2001). In principle, this dominant-negative effect could involve competition between the ΔN and TA isoforms for DNA response elements, formation of non-functional oligomers between the ΔN and TA isoforms, or both (Melino et al., 2002).

Despite the functional similarities of p53, p63 and p73 proteins, deletion of the individual genes in mice has very different outcomes, suggesting that each gene has distinct roles ¢¢

induces their differentiation in the absence of RA (De Laurenzi et al., 2000).

In the present study, we have used RT-PCR, immunofluorescence, retrovirus-mediated gene transfer, and *p53*$^{-/-}$ mice to investigate the expression and function of the individual p53 family proteins in the developing oligodendrocyte lineage. Our findings suggest that both p53

is not present in ΔNp73; gift from Susan Bray, University of Dundee; diluted 1/10 000). Cells were then washed in PBS and incubated for 1 hour in fluorescein-coupled goat anti-mouse IgG (GFAP, p63), or anti-rabbit IgG (p53 and p73) antibodies (Jackson Labs, diluted 1/100) and bisbenzamide. In some experiments, cells were double stained for GC and p73. In this case, GC staining was performed first, followed by permeabilisation and p73 staining. Coverslips were mounted in Citifluor mounting medium (CitiFluor, UK) and examined with a Zeiss Axioskop fluorescence microscope. In all cases, no staining was seen when the fluorescent anti-IgG antibodies were used on their own.

Results

Previous findings suggested that p53 family proteins might play a part in OPC differentiation, but it was not determined which family members were involved. In the present study, we have dissected the contributions of p53, p63 and p73 by examining both their expression in developing oligodendrocyte lineage cells and the effects on OPC differentiation of overexpressing either full-length or dominant-negative isoforms of each family member.

Expression of p53, p63 and p73 in the rat oligodendrocyte lineage

We first used RT-PCR to examine the expression of *p53*, *TAp63*, *ΔNp63*, *TAp73* and *ΔNp73* mRNAs in cultured OPCs purified from the P7 rat optic nerve. The purified cells were expanded for 10 days in the presence of PDGF and the absence of TH and RA. As shown in Fig. 1, we could detect all of these mRNAs.

We next used immunofluoresence to examine the expression of p53, p63 and p73 proteins in both OPCs and oligodendrocytes. Purified P7 OPCs were expanded for 10 days in PDGF and the absence of TH and RA. They were then either cultured for an additional 3 days in the same conditions or were induced to differentiate into oligodendrocytes by either PDGF withdrawal or the addition of TH for 3 days. In some cases, the cells in PDGF and TH were maintained for an additional 2 days to allow the oligodendrocytes to mature further. The cells were then fixed and immunostained for p53, p63 or p73. The results for oligodendrocytes were the same whether the differentiation was induced by PDGF withdrawal or TH addition, and so only the results with TH addition will be illustrated. As shown in Fig. 2A, anti-p53 antibodies weakly stained the nucleus of less than 5% of the OPCs and oligodendrocytes, and no staining was observed in the cytoplasm of any of these cells. Thus, the great majority of both OPCs and oligodendrocytes did not contain detectable amounts of p53 protein. If, however, the OPCs were first irradiated with UV (50 J/m^2) and cultured for a further 8 hours before they were immunostained, p53 could be readily detected in the

Fig. 2. Expression of p53, p63 and p73 proteins in oligodendrocyte lineage cells. Purified P7 rat OPCs were cultured in PDGF without TH and RA for 10 days. They were then cultured for 3 or 5 days in either PDGF alone or PDGF and TH to induce differentiation. (A-C) After 3 days, the cells were fixed and stained by immunofluorescence for p53 (A), p63 (B) or p73 (C). (D) Cells in PDGF and TH for 3 or 5 days were fixed and double-stained for both GC and p73. In all cases, nuclei were counterstained with bisbenzamide. Scale bar: 20 μπΤφ /ΦσΤΔ 759∗ 0 Τχ 0 Τω ενζαμαΗ

influence OPC differentiation, we infected purified rat OPCs with a retroviral vector that encoded both GFP and p53 (pBird-p53) and then cultured the cells in the four conditions described above. As shown in Fig. 3, expression of the wild-type p53 transgene did not significantly affect either the spontaneous differentiation of OPCs cultured in PDGF without TH or RA or the differentiation induced by either TH, RA or PDGF withdrawal. As expected, the expression of the p53 transgene induced significant cell death in OPC cultures, but only live cells were included in the analysis. Although p53 was not detectable in more than 95% of untransfected OPCs and oligodendrocytes (see Fig. 2A), it was readily detected by immunostaining in the nucleus of most of the GFP^+ OPCs and oligodendrocytes transfected with wild-type p53 (not shown).

Effects of *TAp63* or *ΔNp63* transgenes in rat OPCs

To test whether the expression of transgenes encoding either the full-length TAp63 or dominant-negative ΔNp63 isoforms of p63 would affect OPC differentiation, we repeated the experiments just described but used retroviral vectors that encode either GFP and TAp63 (pBird-TAp63) or GFP and ΔNp63 (pBird-ΔNp63) and cultured the cells as described in Fig. 3. As shown in Fig. 4, the expression of either the TAp63 or ΔNp63 transgene did not significantly affect either the spontaneous differentiation of OPCs cultured in PDGF without TH or RA or the differentiation induced by TH, RA or PDGF withdrawal. Thus, p63 is unlikely to play a part in OPC differentiation, at least in culture.

Effects of *TAp73* or *ΔNp73* transgenes in rat OPCs

To test whether the expression of transgenes encoding either TA or ΔN isoforms of p73 would affect OPC differentiation, we used retroviral vectors that encode either GFP and TAp73 (pBird-TAp73) or GFP and ΔNp73 (pBird-ΔNp73) and cultured the cells as described above. As shown in Fig. 5, the expression of the *TAp73* transgene greatly increased the spontaneous differentiation of OPCs cultured in PDGF without TH and RA, as well as the differentiation induced by either TH or RA. Although the expression of the *TAp73* transgene induced significant cell death in OPC cultures, only live cells were included in the analysis.

Conversely, expression of the *ΔNp73* transgene completely inhibited the spontaneous differentiation of OPCs, as well as the differentiation induced by TH or RA (Fig. 5). Furthermore, in contrast to all the other dominant-negative transgenes we tested, *ΔNp73* also inhibited PDGF-withdrawal-induced differentiation (Fig. 5). These findings strongly suggest that p73 is involved in OPC differentiation induced by TH, RA or PDGF withdrawal in culture.

Effects of *p73ΔN* transgene on *p53*$^{-/-}$ mouse OPC differentiation

As ΔNp73 would be expected to inhibit the transcriptional activity of p53, as well as that of TAp73, it was important to determine whether ΔNp73 could inhibit OPC differentiation in the absence of p53. We therefore tested the effect of the *ΔNp73* transgene on cultures of P7 optic nerve cells prepared from wild-type or *p53*$^{-/-}$ mice. We infected the cells with the pBird-ΔNp73 retroviral vector and cultured them in either PDGF without TH and RA, in PDGF with TH, or without PDGF. After 1-3 days, we stained the cultures for GC to determine the proportion of GFP-positive cells that had differentiated into GC-positive oligodendrocytes.

As shown in Fig. 6, expression of the *ΔNp73* transgene significantly decreased both spontaneous differentiation (Fig. 6A) and the differentiation induced by either TH (Fig. 6B) or PDGF withdrawal (Fig. 6C) in both wild-type and *p53*$^{-/-}$ cultures. Thus, p53 is not required for the *ΔNp73* transgene to inhibit OPC differentiation in vitro. Interestingly, however, the level of induced differentiation in *p53*$^{-/-}$ cultures was slightly, but reproducibly, less than that in wild-type cultures (see Fig. 6B,C).

Oligodendrocyte development in *p53*$^{-/-}$ mouse optic nerves

To help determine whether p53 normally plays a part in oligodendrocyte development in vivo, we compared the number of oligodendrocytes in the optic nerves of wild-type and *p53*$^{-/-}$ mice at P7. We dissociated optic nerve cells and counted both the total number of cells and the proportion of GC-positive oligodendrocytes. Although the total number of cells in the nerve was not significantly different in the two genotypes (not shown), the proportion of oligodendrocytes was significantly reduced at P7 in *p53*$^{-/-}$ optic nerves compared

Discussion

OPCs are arguably the best understood precursor cells in the vertebrate central nervous system (CNS), but the intracellular mechanisms involved in their differentiation are still poorly understood. Previous evidence indicated that OPC differentiation, in culture at least, depends on the p53 family

purified rat OPCs has no detectable effect on OPC differentiation in culture – either on spontaneous differentiation or on differentiation induced by TH, RA or PDGF withdrawal.

In contrast, several lines of evidence suggest a crucial role for p73 in OPC differentiation. First, the only change in the three family member proteins that we observe when OPCs differentiate is in p73. Whereas p73 staining is seen exclusively in the nucleus in OPCs, it is seen in both the nucleus and the processes of oligodendocytes. The mechanism and functional significance of this change in p73 distribution remain to be determined. As the anti-p73 antibodies that we used recognise TAp73 isoforms but not ΔNp73 isoforms, it is probable that it is one or more TAp73 isoforms that redistributes when OPCs differentiate. As the antibodies do not distinguish between the various C-terminus isoforms of TAp73, which are generated by alternative splicing at the 3′ end of the p73 RNA transcript, we do not know which isoforms are expressed in OPCs or oligodendrocytes, or which ones redistribute upon OPC differentiation. The second line of evidence suggesting an important role for p73 in OPC differentiation is that the expression of a transgene encoding TAp73 in purified OPCs increases the spontaneous differentiation of OPCs in the presence of PDGF and the absence of TH and RA, as well as the differentiation of OPCs induced by treatment with TH or RA. This is not seen with transgenes encoding either p53 or TAp63. The third line of evidence is that the expression of a transgene encoding ΔNp73 in purified OPCs inhibits all forms of OPC differentiation in culture, including spontaneous differentiation and differentiation induced by either PDGF withdrawal or treatment with TH or RA. This is the only dominant-negative p53 family member that we tested that inhibits all OPC differentiation in culture. Although ΔNp73 would be expected to act as a dominant-negative inhibitor of all three p53 family members, it inhibits both spontaneous and induced OPC differentiation in cultures of *p53*$^{-/-}$ mouse optic nerve cells, indicating that the inhibition does not depend on the inhibition of p53. As ΔNp63 does not inhibit OPC differentiation, it is unlikely that the ΔNp73 inhibition of OPC differentiation depends on the inhibition of TAp63. Thus, we conclude that ΔNp73 inhibits all forms of OPC differentiation by blocking TAp73 isoforms and that one or more of these isoforms is required for normal OPC differentiation, at least in culture. It will be important to confirm this conclusion in *p73*-deficient mice, which have severe neurological defects, including congenital hydrocephalus, hippocampal dysgenesis, and abnormalities in pheromone sensory pathways (Yang et al., 2000). Oligodendrocyte development and myelination were not specifically addressed in the report on these mice (Yang et al., 2000).

Although we can only detect p53 by immunocytochemistry in a small fraction of OPCs and oligodendrocytes in culture, this does not necessarily exclude a role for p53 in oligodendrocyte development. Indeed, two lines of evidence suggest that p53 may be involved in OPC differentiation. First, the expression of either of two transgenes encoding mutant, dominant-negative forms of p53 in purified OPCs inhibits both TH- and RA-induced OPC differentiation, although not spontaneous or PDGF-withdrawal-induced differentiation, as reported previously (Tokumoto et al., 2001). Although one of these mutant forms of p53 (p53DN) would be expected to act as a dominant-negative inhibitor of all three family members, the other (p53DD) lacks the central core domain and would be expected to inhibit p53 specifically (Gaiddon et al., 2001; Shaulian et al., 1992). Second, we find a decrease in the number of oligodendrocytes and an increase in the number of OPCs in the P7 optic nerve of *p53*$^{-/-}$ mice compared with wild-type mice, consistent with the possibility that p53 plays a part in OPC differentiation in vivo. Similar results have recently been obtained independently in the developing *p53*$^{-/-}$ optic nerve by Dean Tang and his colleagues; in addition, they found that the numbers of oligodendrocytes and OPCs normalized in the *p53*$^{-/-}$ optic nerves by P21 (Lubna Patrawala and Dean Tang, University of Texas, personal communication). Together, these data strongly suggest that *p53*$^{-/-}$ OPCs have a delayed differentiation, at least in the optic nerve.

Although the CNS is thought to develop normally in most *p53*$^{-/-}$ mice (Donehower et al., 1992), a small proportion have defects in neural tube closure (Armstrong et al., 1995; Sah et al., 1995). A detailed study of oligodendrocyte development and myelination remains to be done in developing *p53*$^{-/-}$ mice. Interestingly, p53 has been shown to play an important part in the differentiation of neural and mesoderm cells in *Xenopus* embryos (Wallingford et al., 1997). It physically and functionally interacts with Smads in the activin and BMP signalling pathways to induce the expression of homeobox genes involved in mesoderm formation in *Xenopus* (Takebayashi-Suzuki et al., 2003).

In some respects, our results with p53 conflict with those of Eizenberg et al. (Eizenberg et al., 1996), who reported that p53 protein is highly expressed in brain-derived OPCs and translocates from the cytoplasm to the nucleus when these cells differentiate into oligodendrocytes in culture. Using three different antibodies, including the antibody used in their study (not shown), we see relatively little p53 staining in OPCs and oligodendrocytes and cannot detect any in the cytoplasm of either OPCs or oligodendrocytes. The reasons for these discrepancies are unclear. However, Eizenberg et al. did find that a dominant-negative form of p53 (p53DD) inhibited OPC differentiation in their culture system, consistent with our present and previous (Tokumoto et al., 2001) findings.

We previously suggested that there may be at least two independent intracellular pathways leading to cell-cycle arrest and differentiation in OPCs – one that is activated by TH and

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