Roles for p53 and p73 during oligodendrocyte development

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Summary

Oligodendrocytes make myelin in the vertebrate central nervous system (CNS). They develop from oligodendrocyte precursor cells (OPCs), most of which divide a limited number of times before they stop and differentiate. OPCs can be purified from the developing rat optic nerve and stimulated to proliferate in serum-free culture by PDGF. They can be induced to differentiate in vitro by either thyroid hormone (TH) or PDGF withdrawal. It was shown previously that a dominant-negative form of p53 could inhibit OPC differentiation induced by TH but not by PDGF withdrawal, suggesting that the p53 family of proteins might play a part in TH-induced differentiation.

As the dominant-negative p53 used inhibited all three known p53 family members – p53, p63 and p73 – it was uncertain which family members are important for this process. Here, we provide evidence that both p53 and p73, but not p63, are involved in TH-induced OPC differentiation and that p73 also plays a crucial part in PDGF-withdrawal-induced differentiation. This is the first evidence for a role of p73 in the differentiation of a normal mammalian cell.

Key words: Differentiation, Oligodendrocyte, p53, p63, p73, Rat

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 mitogen PDGF, for example, is one of these signals (Noble et al., 1988; Pringle et al., 1989; Raff et al., 1988). In the absence of PDGF, cultured OPCs prematurely stop dividing and differentiate into oligodendrocytes within 1-2 days (Noble and Murray, 1984; Temple and Raff, 1985). It seems probable that a lack of sufficient PDGF is responsible for the differentiation of some OPCs in vivo, especially early in development (Calver et al., 1998; van Heyningen et al., 2001). It is unclear, however, how PDGF withdrawal induces OPCs to differentiate.

Other extracellular signals are also required for the normal operation of the intrinsic timer in cultured OPCs, including hydrophobic signals such as thyroid hormone (TH) or retinoic acid (RA) (Ahlgren et al., 1997; Barres et al., 1994; Gao et al., 1998). When purified postnatal day 7 (P7) rat OPCs are

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 in vivo. p53-deficient mice have an unstable genome and an increased incidence of cancer, but they generally develop normally (Donehower et al., 1992), although a small proportion have defects in neural tube closure (Armstrong et al., 1995; Sah et al., 1995). In contrast, the *p63* and the *p73* genes are both critical for normal development. p63 is required for epidermal development. Mice lacking all forms of p63 have no skin or limbs (Mills et al., 1999; Yang et al., 1999), and anti-sense inhibition of ΔNp63 in developing zebrafish indicates that it is this isoform of p63 that is critical for epidermal and fin development in the fish (Bakkers et al., 2002; Lee and Kimelman, 2002). Mice deficient in all forms of p73 have severe defects in neural development, including hydrocephalus, hippocampal dysgenesis, loss of Cajal-Retzius neurons, and defects in pheromone sensory pathways; they also suffer from chronic infection and inflammation (Yang et al., 2000). The neuronal loss in these mice may be because of the loss of ΔNp73, which has been shown to prevent neuronal apoptosis by antagonizing p53 in both sympathetic and cortical neurons (Pozniak et al., 2002; Pozniak et al., 2000). A role for p73 in neural differentiation is suggested by the findings that RA-induced differentiation of a mouse neuroblastoma line depends on p73, and overexpression of p73 in these cells

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 and p73, but not p63, normally play a part in OPC differentiation.

Materials and methods

Purification and culture of rat OPCs

Sprague/Dawley rats were obtained from the breeding colony of University College London. All chemicals were from Sigma unless otherwise indicated. Optic nerves were removed from P7 rats and dissociated with papain as previously described (Barres et al., 1992). OPCs were purified to >99% purity by sequential immunopanning, as described previously (Barres et al., 1992). The purified cells were plated on poly-D-lysine (PDL)-coated tissue culture flasks (Falcon) and cultured in 8% CO_2 at 37°C in a modified Bottenstein-Sato medium (Bottenstein et al., 1979), based on Dulbecco's modified Eagles' medium and containing insulin (10 µg/ml), PDGF-AA (Prepotech, 10 ng/ml), neurotrophin 3C (NT3, Prepotech, 5 ng/ml), human transferrin (100 µg/ml), BSA (100 µg/ml), progesterone (60 ng/ml), sodium selenite (40 ng/ml), N-acetyl-cysteine (60 µg/ml), putrescine (16 µg/ml), forskolin (5 µM), biotin (10 ng/ml), and penicillin-streptomycin-glutamine (Gibco-BRL).

UV irradiation was performed with a UV cross-linker at 50 J/m^2. Cells were returned to the incubator for 8 hours before they were analysed.

RT-PCR analysis

Cells were harvested with trypsin and processed immediately for RT-PCR analysis. Total RNA was prepared using an RNeasy purification kit (Quiagen). cDNAs were synthetised using Superscript-II Reverse Transcriptase (Invitrogen) according to the supplier's instructions and were used as templates for the PCR reaction.

The following oligonucleotide primers were synthesised. For *glyceraldehyde-3-phosphate dehydrogenase* (*G3PDH*), the 5′ primer was 5′-ACC ACA GTC CAT GCC ATC AC-3′, and the 3′ primer was 5′-TCC ACC ACC CTG TTG CTG TA-3′; for *p53*, the 5′ primer was 5′-GCT TTG AGG TTC GTG TTT GTG CC-3′, and the 3′ primer was 5′-AGT CAT AAG ACA GCA AGG AGA GGG G-3′; for *TAp73*, the 5′ primer was 5′-AGG GTC TGT CGT GGT ACT TTG ACC-3′, and the 3′ primer was 5′-GGT TGT TGC CTT CTA CAC GGA TGA G-3′; for *ΔNp73*, the 5′ primer was 5′-CAC GAG CCT ACC ATG CTT TAC-3′, and the 3′ primer was 5′-GGT TGT TGC CTT CTA CAC GGA TGA G-3′; for *TAp63*, the 5′ primer was 5′-CTT ACA TCC AGC GTT TCA A-3′, and the 3′ primer was 5′-GTT AGG GCA TCG TTT CAC A-3′; for *ΔNp63*, the 5′ primer was 5′-TGG AAA GCA ATG CCC AGA CTC-3′, and the 3′ primer was 5′-CAA CCT GTG GTG GCT CAT AAG G-3′.

The RT-PCR reactions were performed as follows: 25 µl of reaction mixture contained 200 pg of template cDNA, 300 nM of 5′ and 3′ PCR primers, 0.2 mM dNTP, 1.25 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, and 1.25 units of Taq DNA polymerase (Promega). The reaction mixture was denatured for 3 minutes at 95°C. The PCR parameters were 95°C for 40 seconds for the denaturing step, 58°C (*p53*), 59.5°C (*TAp73* and *ΔNp73*) or 55°C (*TAp63*, *ΔNp63* and *GAPDH*) for 35 seconds for the annealing step, and 72°C for 35 seconds for the elongation step. The PCR products were electrophoresed in a 1.6% agarose gel and stained with EtBr. The number of PCR cycles was 35 for *p53*, 37 for *TAp73* and *ΔNp73*, 39 for *TAp63* and *ΔNp63*, and 25 for *G3PDH*.

Retrovirus vectors

To identify infected cells, we used the pBird vector, which expresses the cDNA encoding enhanced green fluorescent protein (GFP), controlled by the CMV promoter (Tokumoto et al., 2001). To express human *p53*, *p53DN* (R175H) (Kern et al., 1992), *p53DD* (Shaulian et al., 1992), *TAp73* (De Laurenzi et al., 1998), *ΔNp73* (Grob et al., 2001), mouse *TAp63* (GeneBank accession y19234) and *ΔNp63* (GeneBank accession y1923) transgenes, we cloned the respective cDNAs into the pBird vector to create retroviral vectors that expressed the transgenes under the control of the Moloney murine leukemia virus long terminal repeat promoter, as well as the GFP transgene under the control of the CMV promoter. Recombinant retroviruses were produced and concentrated as previously described (Kondo and Raff, 2000).

Infection of rat OPCs with retroviral vectors

After 2 days in culture in the presence of PDGF, purified rat OPCs were infected with concentrated retroviral supernatant for 3 hours, washed, removed from the culture flask with trypsin, and replated on PDL-coated Nunc slide flasks in the presence of PDGF. After 1 day, the cells were either maintained in PDGF alone or switched to either PDGF plus TH (triiodothyronine, 40 ng/ml), PDGF plus RA (all-*trans*, 1 µM), or medium that did not contain PDGF. The latter three conditions were used to induce OPC differentiation. The percentage of GFP-positive cells that had differentiated into oligodendrocytes was determined at different time points, using a Leica inverted fluorescence microscope and morphological criteria (Temple and Raff, 1986). In some experiments, the percentage of oligodendrocytes was confirmed by fixing the cells and staining them with a monoclonal anti-galactocerebroside (GC) antibody (Raff et al., 1978; Ranscht et al., 1982), as described below.

Mouse optic nerve cells

$P53^{+/-}$ mice (Donehower et al., 1992) were bred at the animal facility at the Institute of Cancer Research, London. Mice were sacrificed at P7 and genotyped by RT-PCR. Optic nerves were removed, dissociated with papain, and the cells were cultured on PDL-coated Nunc slide flasks as previously described (Billon et al., 2002). They were cultured for 2 days in PDGF alone and then infected for 3 hours as described above. After another 2 days in PDGF alone, they were either maintained in PDGF alone or switched to either PDGF plus TH or to medium that did not contain PDGF for an additional 1-3 days. They were then fixed and stained for GC, as described below. In some cases, the optic nerve cells were counted in a haemocytometer, cultured on PDL-coated glass coverslips for 3-4 hours, before staining them for GC, NG2, or glial fibrillary acidic protein (GFAP), as described below.

Immunocytochemistry

All treatments were performed at room temperature. Cells were fixed in 2% paraformaldehyde in PBS for 5 minutes. After washing with PBS, they were incubated for 30 minutes in normal goat serum to block non-specific staining. They were then incubated for 1 hour in a mouse monoclonal anti-GC antibody (Ranscht et al., 1982) (supernatant, diluted 1/5) or rabbit anti-NG2 antibodies (Chemicon, diluted 1/100). Cells were then washed in PBS and incubated for 1 hour in Texas-Red-coupled goat anti-mouse IgG (GC) or goat anti-rabbit IgG (NG2) antibodies (Jackson Labs, diluted 1/100) and bisbenzamide (Sigma, 5 ng/ml) to stain the nuclei.

For GFAP, p53, p63 and p73 staining, the cells were fixed in paraformaldehyde as above and then permeabilised in 0.1% triton in normal goat serum for 30 minutes. The cells were then incubated for 1 hour in a mouse monoclonal anti-GFAP antibody (Sigma G3893, diluted 1/100), rabbit anti-p53 antibodies (CM5, gift from Alison Sparks, University of Dundee, diluted 1/10,000), a mouse monoclonal anti-p63 antibody (BD Pharmingen; diluted 1/100), or rabbit anti-p73 antibodies (P73N, raised against an N-terminal peptide in TAp73 that

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

nucleus of more than 20% of the cells using the same antibodies (not shown).

In contrast, both p63 and p73 could be detected in almost all OPCs and oligodendrocytes without prior UV irradiation (Fig. 2B,C), and, for p63, all of the staining was in the nucleus (see Fig. 2C). The intensity and location of the p63 staining did not change as oligodendrocytes matured over 5 days in PDGF+TH (not shown). In contrast, p73 staining was confined to the nucleus of OPCs (Fig. 2C) but could be detected in both the nucleus and the processes of oligodendrocytes, which could be readily identified by their morphology (Temple and Raff, 1986) and their staining with anti-GC antibody (Raff et al., 1978; Ranscht et al., 1982) (Fig. 2D). The cytoplasmic p73 staining became more intense as the oligodendrocytes matured (see Fig. 2D).

To determine whether p73 was expressed in OPCs in vivo, we purified OPCs from P7 rat optic nerves, cultured them on PDL-coated glass coverslips for 2 hours, and stained them for p73. As for the OPCs expanded for 10 days in PDGF, all of the freshly isolated OPCs expressed p73 in their nucleus (not shown).

Effects of *p53* or dominant-negative *p53* transgenes in rat OPCs

In a previous study (Tokumoto et al., 2001), when purified OPCs were infected with a retroviral vector encoding a dominant-negative form of human p53 (R175H, which we shall refer to as p53DN) (Kern et al., 1992), the infected cells failed to stop dividing and differentiate in response to TH or RA, although they stopped dividing and differentiated normally in response to PDGF withdrawal. This mutant form of p53, however, has been shown to inhibit the transcriptional activity of p63 and p73, as well as of p53 (Gaiddon et al., 2001; Strano et al., 2000).

We therefore repeated these experiments with a dominant-negative form of p53 (p53DD) (Shaulian et al., 1992) that lacks the central core domain, which is thought to mediate the interaction of wild-type p53 with p73 (Gaiddon et al., 2001). This form of p53 therefore should specifically inhibit p53 and not p73. We cultured purified rat OPCs in PDGF without TH and RA for 2 days and then infected them with either a control retroviral vector (pBird), which encodes GFP only, or a retroviral vector that encodes both GFP and either p53DN (pBird-p53DN) or p53DD (pBird-p53DD). After a further day in culture in PDGF without TH and RA, we either maintained the cells in these conditions or induced them to differentiate by switching them to PDGF plus TH, PDGF plus RA, or medium that did not contain PDGF. After three days, we determined the percentage of GFP-positive cells with morphological features characteristic of oligodendrocytes. In some experiments, we confirmed the oligodendrocyte identity of these cells by staining for GC (not shown). As shown in Fig. 3, both the p53DN and the p53DD vectors completely blocked TH- and RA-induced differentiation. Neither of them, however, inhibited spontaneous differentiation in the presence of PDGF without TH or RA or the differentiation induced by PDGF withdrawal, as shown previously for P53DN (Tokumoto et al., 2001). These findings suggest that p53 is involved in OPC differentiation induced by TH or RA in culture, but not in OPC differentiation induced by PDGF withdrawal.
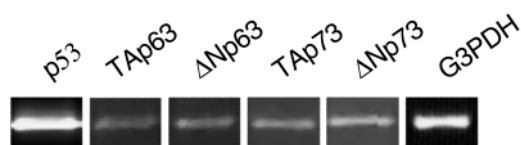
To test whether overexpression of wild-type p53 would

Fig. 1. Expression of *p53*, *p63* and *p73* mRNAs in OPCS. Purified P7 rat OPCs were cultured in PDGF without TH and RA. After 10 days, RNA was extracted and processed for RT-PCR.
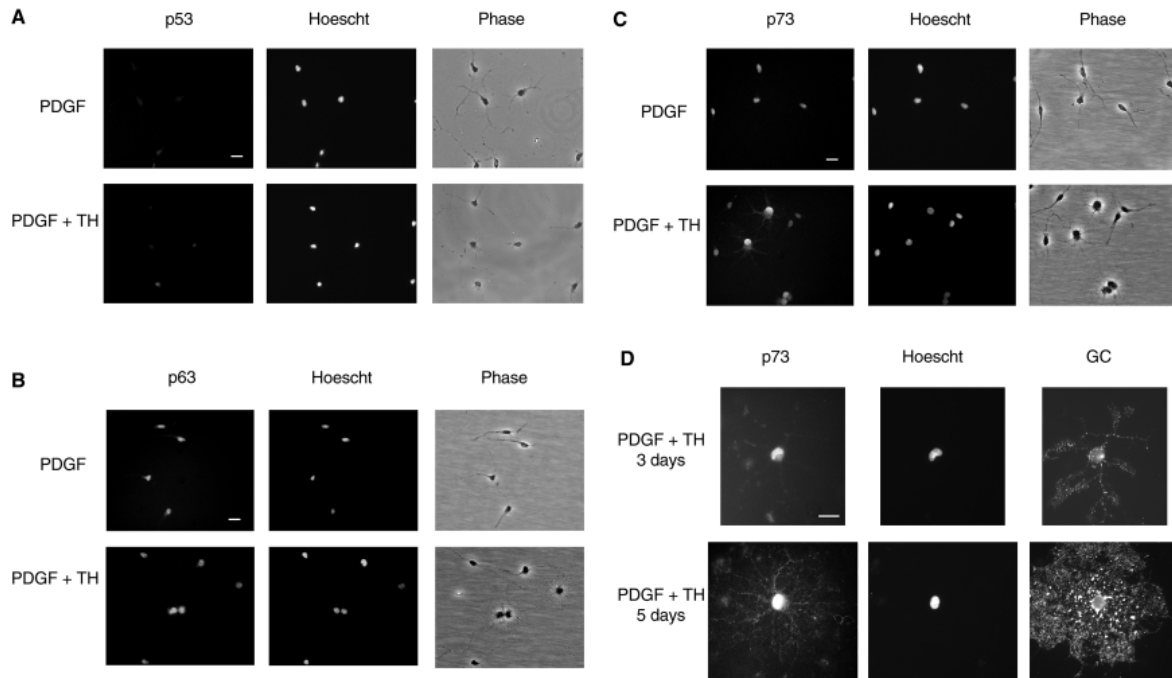
Fig. 2. Expression of p53, p63 and p73 proteins in oligodendrocyte lineage cells. Purified P7 rat OPCs were cultured in PDGF without TH and RA for 10 days. They were then cultured for 3 or 5 days in either PDGF alone or PDGF and TH to induce differentiation. (A-C) After 3 days, the cells were fixed and 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 μm.
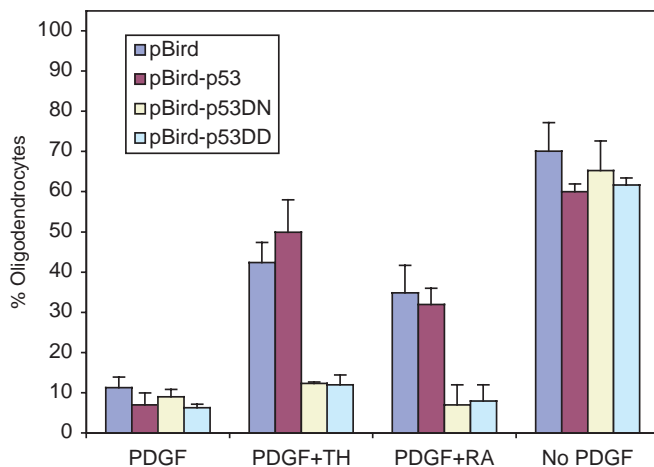

Fig. 3. Effect of the expression of a wild-type or two dominant-negative forms of *p53* transgenes on OPC differentiation. Purified P7 rat OPCs were cultured for 2 days in PDGF without TH and RA. They were then infected for 3 hours with either the pBird control vector encoding GFP alone or a vector encoding GFP and either wild-type (pBird-p53) or a dominant-negative p53 (pBird-DNp53 or pBird-DDp53). After a further day in PDGF alone, the cells were either left in PDGF alone or switched to PDGF and TH, PDGF and RA, or medium without PDGF for a further 3 days to induce differentiation. The percentage of GFP+ cells that had acquired the characteristic morphology of oligodendrocytes was scored in an inverted fluorescence microscope. Similar results were obtained when oligodendrocytes were identified by staining with anti-GC antibody (not shown). In this and the following three Figures, the results are shown as mean±s.d. of at least three experiments.

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.
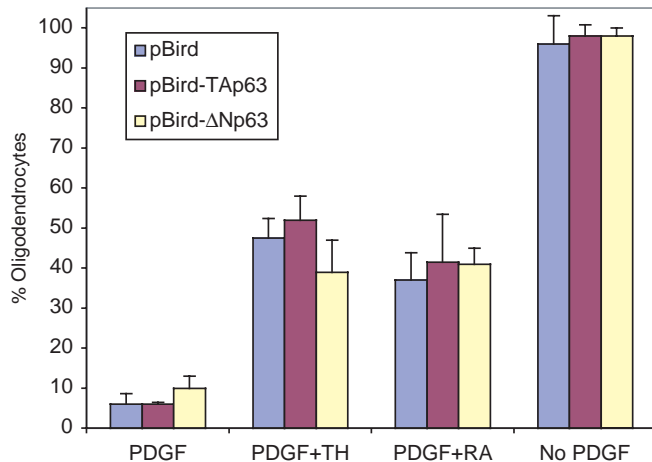
Fig. 4. Effect of the expression of *TAp63* or *ΔNp63* transgenes on OPC differentiation. Purified P7 rat OPCs were cultured and analysed as in Fig. 3, except that they were infected with either pBird, pBird-TAp63 or pBird-ΔNp63 vectors. The differences between the pBird and the pBird-ΔNp63 results in PDGF+TH are not statistically significant when analysed by Student's *t*-test (*P*>0.07).

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
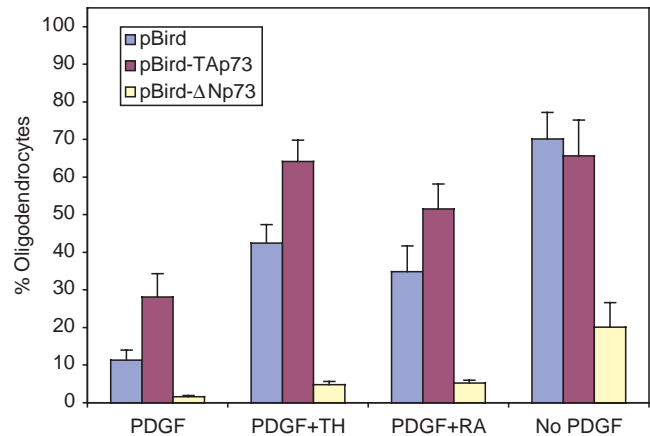

Fig. 5. Effect of the expression of *TAp73* or *ΔNp73* transgenes on OPC differentiation. Purified P7 rat OPCs were cultured and analysed as in Fig. 3, except that they were infected with either pBird, pBird-TAp73 or pBird-ΔNp73 vectors. The differences between the pBird and the pBird-TAp73 results in PDGF, PDGF+TH and PDGF+RA are statistically significant when analysed by Student's t-test (*p*=0.03, 0.03 and 0.02, respectively). The differences between the pBird and the pBird-ΔNp73 results in PDGF, PDGF+TH, PDGF+RA and without PDGF are also statistically significant (*p*=0.04, 0.004, 0.03 and 0.001, respectively).

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 with wild type (Fig. 7A). Thus the total number of oligodendrocytes was decreased in the *p53^−/−* nerve compared with wild type.

To determine whether the decrease in oligodendrocytes in the *p53^−/−* optic nerve was accompanied by a change in the number of OPCs or astrocytes, the two other major cell types in the optic nerve, we also analyzed the proportion of NG2-positive cells, which are mainly OPCs (Stallcup, 2002), and GFAP-positive cells, in wild-type and *p53^−/−* nerves at P7. The proportion of NG2-positive cells was significantly increased in *p53^−/−* optic nerves compared with wild type (Fig. 7B), whereas the proportion of GFAP-positive astrocytes was not significantly different in the two genotypes (Fig. 7C). The findings that oligodendrocytes are decreased and OPCs are increased in p53-deficient nerves are consistent with the possibility that p53 normally plays a part in OPC differentiation.
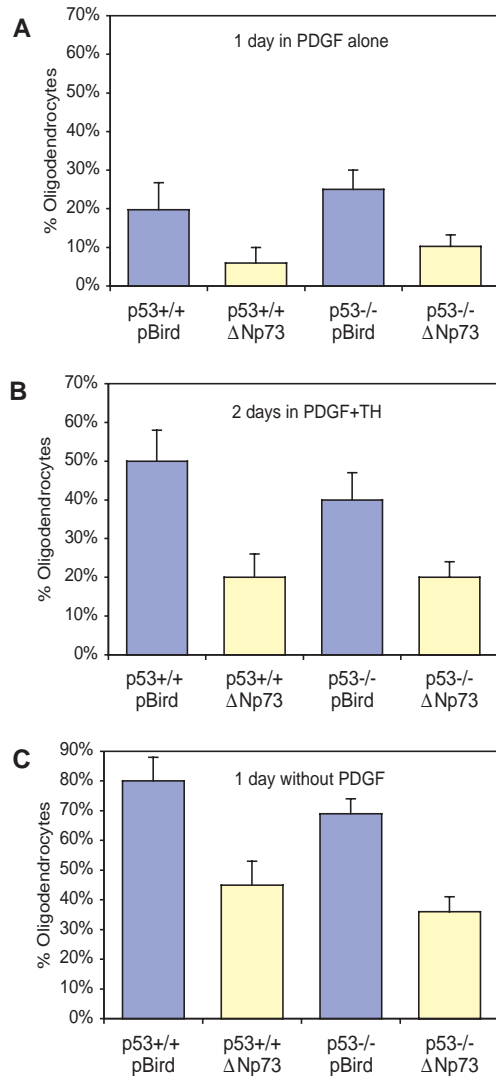
Fig. 6. Effect of ΔNp73 transgene on the differentiation of wild-type and *p53*^{−/−} OPCs. Cells were dissociated from P7 mouse optic nerve and cultured in PDL-coated Nunc slide flasks for 3 days in PDGF alone. They were then infected for 3 hours with either the pBird control vector or the pBird-ΔNp73 vector. After a further day in PDGF alone, they were then either kept in PDGF alone (A) or switched to either PDGF and TH (B) or medium without PDGF (C). After various times, they were fixed and stained for GC. The differences between the pBird and the pBird-ΔNp73 results in all cases are statistically significant when analysed by Student's *t*-test (in A, *p*=0.02 for *p53*^{+/+} and 0.05 for *p53*^{−/−}; in B, *p*=0.002 for *p53*^{+/+} and 0.01 for *p53*^{−/−}; in C, *p*=0.03 for *p53*^{+/+} and *p*=0.02 for *p53*^{−/−}).

Fig. 7. Comparison of the proportions of oligodendrocytes, OPCs and astrocytes in the P7 optic nerve of *p53*^{+/+} and *p53*^{−/−} mice. P7 optic nerve cells were isolated, counted and cultured on PDL-coated coverslips for 3-4 hours. The cells were then fixed and stained for GC to identify oligodendrocytes (A), for NG2 to identify OPCs (B) or for GFAP to identify astrocytes (C). Between 5 and 15 pups were analysed for each genotype. Each pair of optic nerves was processed separately, and 2 coverslips were counted for each pair. The total number of cells isolated per nerve was not significantly different in the two genotypes (not shown). The results are expressed as the mean±s.e.m. The differences between the *p53*^{+/+} and *p53*^{−/−} results in Fig. 7A and 7B are statistically significant when analysed by Student's *t*-test (*p*=0.02 and 0.04, respectively).

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 of proteins, but it was unclear which family members are involved. In the present study, we have dissected the contribution of 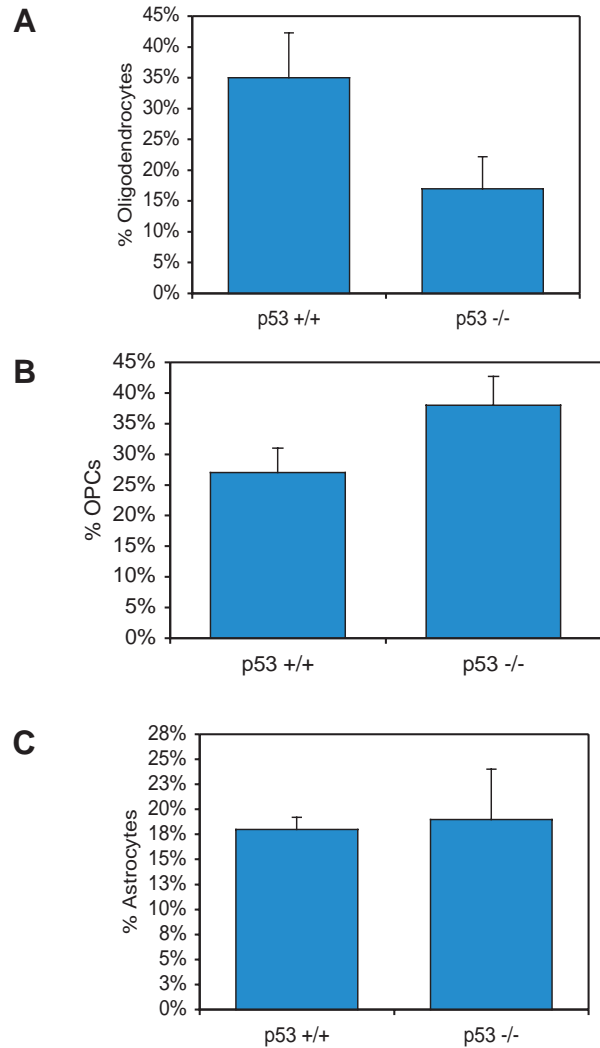the three family members. Our findings suggest that both p53 and p73, but not p63, have key roles in oligodendrocyte development.

We can detect mRNAs for each of the three families in purified OPCs by RT-PCR, raising the possibility that all three may be involved in oligodendrocyte development. However, although we can detect mRNA for both p63 and ΔNp63 in OPCs and p63 protein in the nucleus of almost all OPCs and oligodendrocytes in culture, it seems unlikely that p63 is involved in OPC differentiation. The expression of transgenes that encode either TAp63 or dominant-negative ΔNp63 in

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 RA and is p53-family-dependent and another that is activated by PDGF withdrawal and is p53-family-independent (Tokumoto et al., 2001). Our present findings are consistent with this hypothesis for p53 but suggest that p73 may be a key player in both pathways. As discussed previously, it is probable that oligodendrocytes develop by both pathways in vivo (Tokumoto et al., 2001): whereas a lack of sufficient PDGF may be responsible for the differentiation of some OPCs, especially early in development (Calver et al., 1998; van Heyningen et al., 2001), TH is more likely to influence OPC differentiation postnatally, when TH levels are rising and OPCs are becoming more responsive to TH (Gao et al., 1998).

More work on p53-deficient mice and p73-deficient mice will be required to establish roles for p53 and p73 in OPC differentiation in vivo. It will also be important to determine how these proteins act to promote OPC cell-cycle withdrawal and differentiation. Both proteins promote the transcription of genes that encode cell-cycle inhibitory proteins such as

p21/Cip1 and p27/Kip1 (Blint et al., 2002; Fontemaggi et al., 2002), which probably help OPCs to exit the cell cycle and differentiate (Casaccia-Bonnefil et al., 1997; Durand et al., 1998; Durand et al., 1997; Tokumoto et al., 2001), but it is highly probable that p53 and p73 regulate the transcription of other genes involved in OPC cell-cycle withdrawal and differentiation. It will also be important to determine the relative contributions of TAp73 and ΔNp73 isoforms, especially as OPCs are the first normal mammalian cells in which p73 has been implicated in differentiation.

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