p53 suppresses the self-renewal of adult neural stem cells

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There is increasing evidence that tumors are heterogeneous and that a subset of cells act as cancer stem cells. Several proto-oncogenes and tumor suppressors control key aspects of stem cell function, suggesting that similar mechanisms control normal and cancer stem cell properties. We show here that the prototypical tumor suppressor p53, which plays an important role in brain tumor initiation and growth, is expressed in the neural stem cell lineage in the adult brain. p53 negatively regulates proliferation and survival, and thereby self-renewal, of neural stem cells. Analysis of the neural stem cell transcriptome identified the dysregulation of several cell cycle regulators in the absence of p53, most notably a pronounced downregulation of p21 expression. These data implicate p53 as a suppressor of tissue and cancer stem cell self-renewal.

KEY WORDS: p53, Self-renewal, Stem cell, p21 (Cdkn1a), Adult, Cancer, Mouse, Trp53, Microarray data

INTRODUCTION
A hallmark feature of stem cells in the adult is their capacity to self-renew over the lifespan of the organism. The extensive self-renewal of tissue stem cells is in many ways similar to the expansion of cancer cells. Several studies indicate that the cell population in a tumor often is heterogeneous and that only a subset of the cells, which can be viewed as cancer stem cells, have the capacity to initiate new tumors and to form all other cells of the tumor (Pardal et al., 2003).

Several proto-oncogenes and tumor suppressors, such as Bmi1 (Molofsky et al., 2003), Gfi1 (Hock et al., 2004) and Pten (Groszer et al., 2003), control the self-renewal of normal untransformed tissue stem cells. This indicates that key components of the molecular regulation of tissue and cancer stem cell features may be shared, and that tumor formation, at least in some aspects, can be viewed as excessive stem cell expansion (Pardal et al., 2003). There is indeed evidence that some tumors may originate from tissue stem cells, whereas other may arise by the dedifferentiation of progenitor cells to a more stem cell like state (Daley, 2004). The self-renewal of tissue and cancer stem cells can be regulated by modulation of several distinct processes, including cell proliferation, cell death and differentiation (Molofsky et al., 2004). Understanding the molecular pathways controlling stem cell self-renewal may shed light on both tissue homeostasis and cancer development and progression.

p53 (Trp53 – Mouse Genome Informatics; TP53 – Human Gene Nomenclature Database) is the prototypical tumor suppressor gene and this pathway is inactivated in most human cancers (Vogelstein et al., 2000). In spite of its key role in tumor development, a potential function for p53 in tissue stem cells has not been addressed. p53 mutations are common in brain tumors, and are implicated in both tumor initiation and growth (Sidransky et al., 1992). The identification of cancer stem cells in brain tumors suggests that molecular mechanisms controlling neural stem cell proliferation and brain tumor initiation and growth may be shared (Singh et al., 2004b). We have addressed the function of p53 in neural stem cells in the adult brain, and report that it acts as a negative regulator of neural stem cell self-renewal.

MATERIALS AND METHODS
Mice
Male p53-null mice (Donehower et al., 1992) on a C57BL/6 background (8-12 weeks old) were used with their corresponding littermate wild-type controls. At least four animals of each genotype were used for all experiments.

Immunohistochemistry
All incubations were performed in a humidified chamber for 16 hours at 4°C overnight. Citrate antigen retrieval was used for p53 protein detection by boiling sections for 15 minutes in a 10 mM citrate solution at pH 6. For signal amplification, we used a combination of ABC staining (Vector Laboratories) and TSA (Perkin Elmer). The following primary antibodies were used: anti-phospo-histone H3 (1:2000, Upstate), anti-p53 (1:500, Novocastra), anti-Gfap (1:2000, DakoCytomation), anti-βIII-tubulin (TuJ1, 1:1000, Biosite), anti-O4 (1:100, Chemicon), anti-BrDU (1:1000, Chemicon), anti-Dcx (1:1000, Chemicon) and anti-musashi 1 (1:500, gift from H. Okano). Images were acquired on a Zeiss LSM510 Meta confocal microscope and a Zeiss Axioplan2 with Openlab software.

Apoptosis detection
Apoptotic cells with fragmented DNA were detected in tissue sections using the FragEL TUNEL kit (Calbiochem). In vitro apoptosis was detected by Annexin V (BD) labeling on single cells dissociated from small neurospheres 4 hours prior to staining or caspase activity using the Vybrant FAM assay (Molecular Probes), and both analyzed with a FACScalibur (BD) flowcytometer.

Neural stem cell cultures
The lateral walls of the lateral ventricles were dissected out and cells dissociated using papain (Worthington). Neurospheres were cultured as described (Johansson et al., 1999) in DMEM/F12 medium supplemented with B27 and EGF and bFGF (both 10 ng/ml). Cells were passaged every 4th day by dissociating neurospheres into single cells with papain. All experiments were performed between passage 2 and 5. Neural stem cell differentiation was induced on Biocoat polylysine/laminin culture slides with the addition of 1% calf serum for 7 days before fixing in 4% formaldehyde in PBS and processing for immunohistochemistry. Photos for size comparisons were taken 6 days after clonal expansion of a single cell.

Self-renewal assay
Neurospheres from p53⁻/⁻ and p53⁺/⁺ mice were dissociated into single cells with papain, cell profiles were compared by FACS, cell numbers counted and plated at clonal density. The number of secondary neurospheres...
generated from 2000 single cells in triplicate from four mice of each genotype was quantified after 7 days. Cells from the clonal expansion were used for assaying multipotency by in vitro differentiation and for photos of neurosphere size. Neurosphere photos for size comparisons were taken 6 days after clonal expansion of a single cell. Primary neurosphere formation from adult lateral ventricle wall was assessed by sorting single cells into 96-well plates using FACS (BD, FACS Vantage SE Diva) and counting neurospheres 7 days later.

**Proliferation assays**

BrdU labeling of proliferating cells in vivo was performed by three intraperitoneal injections of 50 mg/kg BrdU with 2 hours intervals and followed by sacrifice 2 hours after the last injection. Anesthetized mice were perfused transcardially with 4% formaldehyde in PBS and sectioned (25 μm) on a vibratome (Leica). Positive nuclei for BrdU or phosphorylated histone H3 were counted on serial coronal sections of the anterior horn of the lateral ventricle within a defined space of the subventricular zone. Sections were taken from coded animals and the genotype of the mice was revealed after statistical analysis.

For analysis in vitro, BrdU was added to cultures at a final concentration of 10 μM 30 minutes prior to fixation. BrdU detection was carried out with BrdU Flow (BD) on dissociated single cells and analyzed in a FACScalibur flowcytometer (BD).

**Microarray analysis**

Total RNA was prepared from triplicate samples on different occasions from neurospheres using the RNase purification system (Qiagen). Amplification of RNA was carried out using the RiboAmp total RNA amplification kit (Arcturus). Each sample was hybridized against the reference sample using two replicates hybridizations with the same dye assignments (reference sample always in Cy3). Every cDNA probe was printed twice on an array, there were therefore eight measurements in total for each genotype (two within-array replicates, two replicated hybridizations and two replicated neurosphere cultures). The reference sample consisted of Universal Reference RNA (Stratagene) amplified using the same approach as the neurosphere samples. Labeled material was hybridized to 16k mouse cDNA arrays using a reference-design approach (Siervetzton et al., 2005). All data analysis steps were carried out in the R environment for statistical computing and programming using several analysis packages as detailed in the supplementary material. Data corresponding to unreliable features and non-expressed genes were removed, and the remaining data normalized using an intensity-dependent print-tip lowess approach. Differentially expressed genes were identified using an empirical Bayes moderated t-test; a gene with a false-discovery rate adjusted P<0.05 and an M>±0.4 (corresponds to a fold-change value of greater than 1.4) was considered differentially expressed. Additional details regarding the sample preparation, array hybridization and subsequent data analysis are available on request. Additional information on the cDNA microarray used can be obtained from ArrayExpress microarray data repository (Accession number E-MEXP-483). Further details on the array procedure can be provided on request.

**Real-time PCR**

RNA was isolated with Cells-to-Signal Lysis Buffer (Ambion) and cDNA was synthesized with Superscript and random hexamers (Invitrogen). Linear phase of logarithmic amplification was used for quantification and cycle number was compared between triplicate samples. SYBR green was used on an ABI machine according to the manufacturer’s instructions.

**Western blot**

The lateral wall from the lateral ventricle was dissected and prepared in LDS sample buffer (Invitrogen) solution with proteinase inhibitors (Roche). After homogenization and sonication, the protein extract was incubated with a reducing agent at 70°C for 10 minutes. Samples were loaded on a 12% Bis-Tris gel (Invitrogen). Membranes were incubated in blocking solution with anti-p21 antibody (BD, 1/500) at 4°C overnight and anti-β-actin (Sigma, 1/10000) at room temperature for 45 minutes, detected with horseradish peroxidase conjugated secondary antibody and visualized with ECL reagent (Amersham Biosciences). Quantification of band intensity was performed using a mean density measurement in Object-Image2.15.

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**RESULTS**

**p53 in a neurogenic niche in the adult brain**

We first analyzed the expression of p53 in the neurogenic niche in the lateral ventricle wall in adult mice. p53 mRNA expression can be detected in this brain region both during development and in adulthood (van Lookeren Campagne and Gill, 1998), but it has not been established which cell types express p53. Moreover, p53 is subject to post-translational regulation, so mRNA levels do not always accurately reflect protein levels (Bode and Dong, 2004).

Nuclear p53-immunoreactivity was evident in the majority of cells in the lateral ventricle wall neural stem cell niche (Fig. 1). Most cells in surrounding brain regions displayed much weaker or undetectable labeling. All labeling was abolished in sections from p53-null mice, corroborating the specificity of the antibody (data not shown). Multiciliated ependymal cells lining the ventricle appeared uniformly p53-immunoreactive (Fig. 1). The majority of cells in the subventricular zone, including most Gfap+ astrocytes and musashi 1+ progenitor cells displayed p53-immunoreactivity (Fig. 1A-E,G,H). p53 immunoreactivity was weaker or absent in the majority of differentiating cells, including Doublecortin+ neuroblasts (Dcx in Fig. 1F), in the migration path to the olfactory bulb (the rostral migratory stream). Thus, p53 protein is present at substantially higher levels in the neural stem cell lineage than in other cells in the adult brain, and is mainly detected in candidate stem cells and progenitor cells.

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**Fig. 1. p53 in a neural stem cell niche.** (A-C,E-G) Nuclear p53-immunoreactivity is seen in the majority of cells in lateral wall of the lateral ventricle (LV) and to a lesser degree in the rostral migratory stream (RMS), whereas levels are lower or undetectable in most cells in the surrounding areas of the adult mouse brain. (D,H) Three-dimensional reconstructions of confocal stacks demonstrate p53 in musashi 1 (Msi1) and Gfap-immunoreactive cells. Nuclei are labeled with DAPI and appear blue. The orientation of all sections is indicated in A, D, dorsal; M, medial. Scale bars: 10 μm.
Increased cell proliferation in the neural stem cell niche in the absence of p53

To assess a potential role for p53 in the adult neural stem cell lineage, we first analyzed the cell proliferation and apoptosis rate in the lateral ventricle wall in mice lacking p53 (Donehower et al., 1992). We found that p53−/− mice have a significantly higher number of proliferating cells, as assessed by the incorporation of the nucleotide analogue BrdU, in the lateral ventricle wall compared to wild-type littermates (Fig. 2A-C). This was paralleled by a similar increase in the number of cells immunoreactive to phosphorylated Ser10 on histone 3, a mitosis-specific marker (Wei et al., 1999), in the mutant mice (Fig. 2D-F). The distribution of proliferating cells in the subventricular zone along the lateral ventricle wall and in the rostral migratory stream was indistinguishable between wild-type and p53−/− mice.

An increase in the number of proliferative cells could in theory be due to a reduction in apoptosis of newborn cells. There is, however, very little apoptotic cell death normally in the subventricular zone (Biebl et al., 2000), and we could not detect any significant difference between wild-type and p53−/− mice (Fig. 2G), making it unlikely that the increase in proliferation is due to a decrease in cell death.

Negative regulation of neural stem cell self-renewal

Multipotent self-renewing neural stem cells can be propagated in vitro as clonal aggregates denoted neurospheres (Reynolds and Weiss, 1992). Although these cells display hallmark stem cell properties in vitro, it is unclear whether the neurosphere-forming cells act as stem and/or progenitor cells in vivo. We asked whether the increased proliferation in the lateral ventricle wall of p53−/− mice was accompanied by an alteration in the number of cells capable of forming neurospheres. Indeed, a significantly larger proportion of lateral ventricle wall cells from p53−/− mice formed neurospheres compared with wild-type cells (Fig. 2H).

Cells from p53−/− animals generated significantly larger neurospheres compared with wild-type cells (Fig. 3A-C). This was not due to a difference in cell size (Fig. 3L) but to a larger number of cells in the p53 null neurospheres.

Neurospheres are initiated by multipotent neural stem cells, but as the clone expands, an increasing heterogeneity will ensue with many cells committing to specific fates. Therefore, it cannot be directly inferred that an increase in cell number in a neurosphere is due to increased stem cell self-renewal. To directly assay the number of stem cells in the neurospheres, we analyzed the number of cells that were capable of forming new secondary neurospheres. This revealed a higher number of neurosphere-initiating cells in p53−/− null neurospheres, establishing that there was an increased generation of cells with in vitro neural stem cell potential in the absence of p53. By analyzing the relative frequency of neurosphere cells that were capable of reinitiating a clone, we found that this was not only due to the larger number of cells in these neurospheres, but the proportion of cells that were able to reinitiate neurosphere formation was significantly higher in p53−/− compared with wild-type neurospheres (Fig. 3D). The increased number of neural stem cells generated in the absence of p53 establishes p53 as a negative regulator of neural stem cell self-renewal.

p53 regulates neural stem cell proliferation and apoptosis

Stem cell self-renewal can be regulated by the modulation of any process that affects the number of progeny from a stem cell maintaining stem cell properties, including for example cell proliferation, survival or differentiation (Mikkers and Frisén, 2005; Molofsky et al., 2004). To address by which mechanism p53 regulates neural stem cell self-renewal, we assessed these parameters in p53 null and wild-type neurospheres. Owing to the rapidly emerging size difference between p53 null and wild-type neurospheres, we performed all analyses on small secondary neurospheres (2 days after passage when the neurospheres contain ~4-20 cells), to minimize differences secondary to variation in neurosphere size. We first analyzed proliferation by BrdU incorporation in neurospheres. As in the lateral ventricle wall in vivo, BrdU incorporation was significantly increased in vitro in neurospheres in the absence of p53 (Fig. 3J,K).
We next assessed apoptotic cell death. In contrast to the in vivo situation, where very few apoptotic cells are seen (Biebl et al., 2000), there is a substantial proportion of apoptotic cells in neurospheres, which can be detected by flow cytometric analysis of caspase activity or AnnexinV immunoreactivity. We found that significantly fewer cells labeled with these markers in p53–/– compared with wild-type neurospheres (Fig. 3E,F), indicating decreased apoptosis in the absence of p53. The phenotype appears more pronounced in vitro than in vivo, with a larger increase in proliferation as well as reduced apoptosis in p53–/– neural stem cells compared with wild-type mice (K) and more cells from p53–/– mice have a DNA content corresponding to S and M phases (J). There is no size difference between wild-type and p53 mutant cells (L). All bars in graphs indicate means±s.d. *P<0.05, **P<0.01, ***P<0.001. Scale bar: 100 μm.

p53 regulation of the neural stem cell transcriptome
To gain insight into how p53 impinges on the molecular program controlling self-renewal, we analyzed the transcriptome of p53–/– and wild-type neurospheres using 16k cDNA arrays enriched for genes expressed in murine stem cell populations (Sievertzon et al., 2005). Cultured cells are likely to differ from their in vivo counterparts as p53 regulation may be affected, but allow for a rather homogeneous population to be analyzed. We used the empirical Bayes moderated t-test to identify differential expression. The contribution of within-array duplicate features was downweighted and genes with a false-discovery rate adjusted P<0.05 combined with an M-value cut-off (|M|>0.4) were considered differentially expressed (details can be provided on request). Using these criteria, 325 genes were differentially expressed and 98 of these had P<0.001 (Fig. 4A, Table 1; see Table S1 in the supplementary material for a complete list). The expression of seven of these genes was previously reported to be p53 regulated (Harms et al., 2004), validating the approach. However, the majority of dysregulated genes have not been implicated as p53 targets. Functional classification of dysregulated genes revealed significant enrichment of several categories related to cell proliferation (gene ontology themes with an Ease-score<0.05, Fig. 4B; see Table S3 in the supplementary material).
The most strikingly dysregulated gene was p21 (p21cip1; Cdkn1a–Mouse Genome Informatics). Quantitative RT-PCR confirmed a reduction of p21 mRNA in p53-null neurospheres, and to a lesser degree p27 (p27kip1; Cdkn1b–Mouse Genome Informatics), another member of the cip/kip family of CDK inhibitors (Fig. 4C). Both p21 and p27 have been implicated as regulators of stem cell self-renewal (Kippin et al., 2005), and the decreased expression of these genes in concert with altered expression of other modulators of proliferation and apoptosis (see Table S1 in the supplementary material) is likely to contribute to the observed phenotype in the neurospheres. Western blot analysis of p21 protein levels in the lateral ventricle wall revealed that p53–/–mice had 77±37% (average±s.d., n=6 of each genotype) of the level seen in wild-type mice (Fig. 4D). Several pathways implicated in stem cell self-renewal regulate p53 expression or activity (Fig. 4E), suggesting that p53 may be an important modulator of self-renewal in multiple lineages.

**DISCUSSION**

Uncovering the molecular control of stem cell self-renewal is an important step in understanding tissue homeostasis as well as tumor development and growth. In this study, we describe the effects of p53 inactivation in adult mouse neural stem/progenitor cells. Mice lacking p53 display an elevated proliferation rate in the neurogenic niche of the adult lateral ventricle wall, as well as an increase in self-renewal of in vitro propagated p53–/– neural stem cells. The number of stem cells generated, i.e. the degree of self-renewal, is a complex feature that can be modulated at several levels. We find that p53 control neural stem cell self-renewal by regulating both their proliferation and survival.

The analysis of the transcriptional effects of loss of p53 in neurospheres unveiled downstream candidates potentially responsible for this phenotype. Although redundancy between different pathways often can mask a role of an individual gene in the analysis of mutant mice, we found several dysregulated genes in p53–/– neurospheres. There is also a risk of a developmental phenotype in a mutant giving secondary effects in adulthood. With the exception of a subset of p53-null mice developing neural tube defects and dying at birth (Sah et al., 1995), the nervous system appears normal in adult p53-null mice. The most prominent dysregulation was the reduction of p21 in p53–/– neurospheres. p21 is a well-established actor in p53-mediated cell cycle arrest and through its negative effects on CDKs, p21 inhibits both the G1-to-S and the G2-to-mitosis transitions (Gartel and Radhakrishnan, 2005). p21 negatively regulates self-renewal of hematopoietic stem cells (Cheng et al., 2000) and the precipitous decrease in expression in p53–/– neurospheres may contribute to the increased self-renewal. Indeed, proliferation is increased in the lateral ventricle wall and in neurospheres in the absence of p21 in young adult mice (Kippin et al., 2005). Another study found increased proliferation in p21–/– mice only after ischemia and not in the intact brain (Qiu et al., 2004). Moreover, p27 has been established as a negative regulator of proliferation in the lateral ventricle wall in the adult brain (Doetsch et al., 2002). The decreased expression of cip/kip family genes in conjunction with the upregulation of a large number of genes positively regulating cell proliferation and downregulation of several pro-apoptotic genes (see Table S1 in the supplementary material) in p53–/– neural stem cells is likely to contribute to the observed phenotype.
Kippin et al. recently demonstrated an exhaustion of neural stem cells in old p21−/− mice (Kippin et al., 2005). This suggests that increased proliferation may result in premature senescence and that it may be important to suppress self-renewal for the long-term maintenance of a stem cell population. The limited life span of p53-null mice because of their predisposition to tumors precludes an analysis of the role of p53 in the aging of stem cells.

A recent study demonstrated that p53 promotes the differentiation of embryonic stem cells by suppressing Nanog expression (Lin et al., 2005). This suggests that p53-mediated suppression of stem cell self-renewal may be general. However, the mechanisms by which p53 regulate self-renewal are different, as Nanog expression is restricted to embryonic stem cells and germ-line stem cells.

Several recent studies have described the existence of a subpopulation of cells in brain tumors that has characteristics of stem cells (Singh et al., 2004a; Singh et al., 2004b). The concept that a cancer stem cell is maintaining the growth of a tumor has important implications for our understanding of tumorigenesis and design of cancer therapy. The cancer stem cell hypothesis suggests that cells in a tumor have different abilities regarding proliferation, self-renewal and differentiation (Singh et al., 2004a). The influence of p53 inactivation on these processes is an important factor to understand as p53 is mutated in the majority of tumors found throughout the human body (Vogelstein et al., 2000). The mutations that inactivate p53 function in cancer cells almost all appear to localize to the DNA-binding domain of the p53 protein and produce a protein that fails to transcribe p53-responsive genes (Harris and Levine, 2005). We have in this study chosen to analyze the p53 pathway in adult neural stem and progenitor cells in vivo and in vitro in order to gain insight into the molecular mechanisms regulating proliferation and self-renewal. Focussing on the proliferative zones of the adult brain for the study of p53 is due to the suggested connection between tumorigenesis and sites of immature cells. Furthermore, neural progenitor cells retain the ability to proliferate and self-renew throughout adulthood and may be susceptible to convert into a malignant phenotype and may serve as an interesting model for the cancer stem cell hypothesis. The increased proliferation rate in the neural stem cell niche may elevate the risk of acquiring mutations, which could contribute with other previously described mechanisms (Vogelstein et al., 2000) to the increased predisposition to cancer in the absence of p53.

There is accumulating evidence for the role of tumor suppressors and oncogenes in the maintenance and regulation of stem cells. Our array analysis of neural stem cells lacking p53 provides an overview of the pathways that are important for the increased self-renewal phenotype observed in p53−/- neurospheres. When compared with previous array studies of neural stem cells (Fortunel et al., 2003), we find that few of the genes with enriched expression in neural stem cells are dysregulated in the absence of p53 (see Fig. S1 in the supplementary material). Instead, many of the dysregulated genes control cell cycle progression and apoptosis without affecting differentiation. Our molecular description of a self-renewal phenotype using a cDNA array based analysis of the transcriptome opens up pathways for the discovery of genes that regulate this fundamental property of stem cells. The functional analysis of other known oncogenes and tumor suppressors in stem cell maintenance, together with the present data, suggests that several molecular pathways in stem cell biology may converge on p53 for the control of stem cell self-renewal (Fig. 4E).

This study was supported by grants from the Swedish Cancer Society, IDRF, the Swedish Research Council, the Swedish Childhood Cancer Fund, the Karolinska Institute, the Göran Gustafsson Foundation, the Tobias Foundation, the Wallenberg Consortium North (WCN), the Cancer Society in Stockholm, the EU and the Foundation for Strategic Research. We thank members of the Frisén laboratory for valuable discussions and comments on the manuscript, Xiaobing He for help with mouse breeding and genotyping, Marie Carlén for microarray expertise, and Hideyuki Okano for the kind gift of anti-musashi 1 antibodies.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/2/363/DC1

**References**

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RESEARCH ARTICLE


