**Nmyc** plays an essential role during lung development as a dosage-sensitive regulator of progenitor cell proliferation and differentiation

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**Summary**

Understanding how lung progenitor cells balance proliferation against differentiation is relevant to clinical disorders such as bronchopulmonary dysplasia of premature babies and lung cancer. Previous studies have established that lung development is severely disrupted in mouse mutants with reduced levels of the proto-oncogene **Nmyc**, but the precise mechanisms involved have not been explored. We show here that Nmyc expression in the embryonic lung is normally restricted to a distal population of undifferentiated epithelial cells, a high proportion of which are in the S phase of the cell cycle. Overexpression of **Nmyc**EGFP in the epithelium under the control of surfactant protein C (**Sftpc**) regulatory elements expands the domain of S phase cells and upregulates numerous genes associated with growth and metabolism, as shown by transcriptional microarray. In addition, there is marked inhibition of differentiation, coupled with an expanded domain of expression of Sox9 protein, which is also normally restricted to the distal epithelial compartment. By contrast, conditional deletion of **Nmyc** leads to reduced proliferation, epithelial differentiation and high levels of apoptosis in both epithelium and mesenchyme. Unexpectedly, about 50% of embryos in which only one copy of **Nmyc** is deleted die perinatally, with similarly abnormal lungs. We propose a model in which **Nmyc** is essential in the developing lung for maintaining a distal population of undifferentiated, proliferating progenitor cells.

Key words: Lung development, **Nmyc**, Conditional inactivation, Progenitor cells, Sox proteins, Growth, Differentiation

**Introduction**

The mammalian lung develops from two primary buds consisting of an inner epithelial layer surrounded by mesenchyme. These buds undergo repetitive outgrowth and branching to give rise to the respiratory tree containing different specialized epithelial cell types organized along the proximodistal axis. These include the ciliated, secretory and neuroendocrine cells of the proximal bronchi, and the type II and type I cells of the distal alveoli (for reviews, see Cardoso, 2000; Warburton et al., 2000). As with other organ systems, lung development involves a balance between expansion in the number of undifferentiated progenitor cells and withdrawal of cells from this pool by terminal differentiation. Understanding how this balance is achieved is relevant to a number of clinical problems; for example, the pulmonary dysplasia of premature babies, lung repair after injury, and the progression of lung cancer. Previous studies had pointed to a crucial role in lung development for the gene **Nmyc1** (hereafter **Nmyc**), which is specifically expressed in the epithelium; **Myc** (previously **c-Myc**) is transcribed only in the mesenchyme. **Nmyc** is amplified in some lung tumors, and analysis of mutant mouse embryos with reduced levels of **Nmyc** activity suggested a role in cell proliferation and branching morphogenesis (Moens et al., 1992; Moens et al., 1993; Sawai et al., 1993; Stanton et al., 1992). However, since these initial studies there has been no new information about the precise role of **Nmyc** in lung development.

**Nmyc** is a member of a small family of proto-oncogenes (**Myc, Lmyc** and **Nmyc**) encoding basic helix-loop-helix-leucine zipper (bHLHZ) proteins. Myc proteins can either activate or repress transcription by interacting with specific binding partners, and by recruiting cofactors, including histone and chromatin protein modifying enzymes, to the vicinity of a very large number of target genes (for reviews, see Eisenman, 2001; Levens, 2003; Patel et al., 2004; Zeller et al., 2003) (see also http://www.myccancergene.org/site/mycTargetDB.asp). Myc proteins appear to coordinate many interdependent processes, including cell growth (increase in cell mass), cell proliferation (DNA replication and cell cycle progression), differentiation and apoptosis. Recent gain- and loss-of-function studies in the embryonic nervous system have highlighted roles for **Nmyc** both in promoting cell cycle progression in undifferentiated progenitor cells and in inhibiting their differentiation in response to specific signaling pathways. In mouse cerebellar granule neuron progenitors, for example, **Nmyc** is directly upregulated by sonic hedgehog acting as a mitogen, and **Nmyc** overexpression in the same cells stimulates cyclin D1 accumulation and cell cycle progression (Kenney et al., 2003). Moreover, recent studies have shown that Nmyc protein is partially stabilized in neuronal cells by activity of the PI3K pathway (Kenney et al., 2004). By contrast, conditional
deletion of Nmyc in the embryonic nervous system results in a decrease in the pool of granule cell precursors, largely due to an increase in their expression of cell cycle inhibitors and premature differentiation (Knoepfler et al., 2002).

As indicated earlier, it is well established that Nmyc is required for lung development (Moens et al., 1992; Moens et al., 1993; Sawai et al., 1993; Stanton et al., 1992). Embryos homozygous for a hypomorphic mutation (Nmyc<sup>9a/9a</sup>) die at birth with lungs that are about half the normal size, and that contain fewer branches and highly enlarged air spaces. Compound mutants between Nmyc<sup>9a/9a</sup> and a null allele (Nmyc<sup>B/R1</sup>) have an even more severe reduction in lung development. To further explore how Nmyc functions in the lung, we have exploited transgenic and conditional gene deletion techniques to overexpress Nmyc, or, conversely, to remove one or both copies of the gene specifically in the epithelium of the developing lung.

**Materials and methods**

**Transgenic and Nmyc<sup>floxed/floxed</sup> mice**

To generate NmycEGF fusion mouse, mouse Nmyc<sup>1</sup> cDNA was inserted into the Smal site of pEGFP (BectonDickinson), excised by Sal<sup>I</sup> and EcoRI, and inserted into a vector containing a 3.7 kb promoter/enhancer of the human SFTP gene (Wert et al., 1993). Transfection of 293 cells shows that the fusion protein localizes to the nucleus (see Fig. S1 in supplementary material). Four transgenic embryos were generated by pronuclear injection into (C57BL/6<sup>DBA</sup>)<sup>F2</sup> fertilized eggs. They were collected at E18.5, so embryos were generated by pronuclear injection into (C57BL/6<sup>DBA</sup>)<sup>F2</sup> fertilized eggs. They were collected at E18.5, so embryos were generated by pronuclear injection into (C57BL/6<sup>DBA</sup>)<sup>F2</sup> fertilized eggs.

The results of this study confirm and extend previous findings regarding the importance of Nmyc in lung development. Nmyc overexpression results in a dramatic increase in the number of epithelial cells in the developing lung. Conversely, Nmyc deficiency leads to a reduction in the number of epithelial cells, consistent with the phenotype observed in homozygous Nmyc<sup>9a/9a</sup> mice. These results provide additional evidence for a key role of Nmyc in lung development, and suggest that Nmyc may function as a transcription factor that regulates the expression of genes involved in epithelial cell differentiation.

**β-Gal staining**

Lungs were fixed in 4% paraformaldehyde in PBS (pH 7.4), permeabilized in 2 mM MgCl<sub>2</sub>, 0.01% NaDeoxycholate, 0.02% NP-40, stained with X-gal overnight, embedded in paraffin wax, sectioned at 7 μm, and counterstained with Eosin. BrdU incorporation

BrdU (Amersham Bioscience, UK) was injected intraperitoneally into pregnant females at a dose of 10 μl per gram bodyweight. After 1 hour, embryonic lungs were fixed in 4% paraformaldehyde in PBS (pH 7.4). For immunohistochemistry, BrdU monoclonal antibody (Sigma, St Louis, MO, USA) was used, in combination with with MOM-blocking solution (Vector Laboratories, Burlingame, CA, USA).

**RT-PCR**

Total RNA was extracted using the RNeasy Kit (Qiagen, Valencia, USA). The cDNA was synthesized from 1 μg total RNA using SuperScript<sup>TM</sup> First-strand Synthesis Kit (Invitrogen). Primer sets were:

- claudin 6, 5′-ATGGCCTACTCTTGCTGCAAAATC-3′ and 5′-GCATACACAAATTCTTGTGGG-3′;
- cyclin D2, 5′-TGAAGACATCTTTGAGAACTG-3′ and 5′-CTCAGGTCACACAATCCCG-3′;
- Nmyc, 5′-CGAATTGGCTAGGGATGCT-3′ and 5′-TGTGGCTGCTGAGGATGG-3′;
- Nol5a, 5′-TGAAGAAGCTTGGTGGTCAGG-3′ and 5′-CTAACCTCCTGTTTCTC-3′;
- Ppan, 5′-ATGGCCAGTGCGGGCGTC-3′ and 5′-GCTCAGTGTCTGAGTC-3′;
- Rod, 5′-CCCCACCTCAAGATCTTTTCGCC-3′ and 5′-AGGGTGCGACGAGGAGGTAG-3′;
- Scgb1a1, 5′-TGAAGATCGCCATCACAATC-3′ and 5′-ATCTGCGTTACACAGGAG-3′;
- Sox9, 5′-ACGTGTGATGTCGAGAACTG-3′ and 5′-ACTGTTGTCCCAGTGTCG-3′.

The primers for Sfip1, Sfipb, Sfipc, Aquaporin 5 (Aqp5) and βactin were as described (Okubo and Hogan, 2004).

**In situ hybridization**

Mouse Sfip1, rat Scgb1a1 and mouse Foxj1 cDNA have been described previously (Weaver et al., 1999). Digoxigenin (DIG) or fluorescein-labeled antisense cRNA probes were made using T7 or SP6 RNA polymerase. Briefly, paraffin wax-embedded sections were dewaxed, preheated with proteinase K and hybridized with one DIG-labeled and one fluorescein-labeled probe at 55°C overnight. Slides were then washed sequentially in 5×SSC, 2×SSC (50% formamide), 0.1×SSC and blocking solution (supplied in TSA Plus Biotin System, Perkin Elmer). The DIG-labeled probe was detected using POD-coupled anti-DIG (Roche), followed by standard biotin-tyramide and streptavidin-HRP amplification, and visualized using Cy3-tyramide (Perkin Elmer). HRP activity was quenched by treatment with 1% H<sub>2</sub>O<sub>2</sub> in methanol (15 minutes). This was followed by detection of the fluorescein-labeled RNA probe with POD-coupled anti-fluorescein (Roche), and similar amplification and visualization with fluorescein-tyramide. Nuclei were counterstained with DAPI.

**Immunohistochemistry**

The following reagents were used: rabbit polyclonal antibody to Nmyc (SantaCruz); phosphohistone H3 (Upstate Biotechnology); cyclin D1 (clone AM29, Zymed); cleaved caspase-3 (Cell Signaling Technology, Beverly MA); Scgb1a1 (kindly provided by Barry Stripp, University of Pittsburgh); Sox2 (kindly provided by Larysa Pevny, UNC Chapel Hill); and hamster monoclonal anti-Gp38 (University of Iowa Hybridoma Bank). The rabbit Sox9 antibody was kindly provided by Dr Francis Poulat, CNRS, Montpellier, and was used at a dilution of 1:3000. It was raised against amino acids 408-504 of the human protein (Gasca et al., 2002), a region showing significant similarity only with sequences in mouse Sox10. However, RT-PCR analysis indicates that Sox10 is not expressed in either wild-type or transgenic embryonic mouse lung (data not shown). The specificity of the rabbit antibody for Sox9 in the chick was described previously (Moniot et al., 2004), and was confirmed in this study by specific staining of Sertoli cells in the mouse testis (E18.5; data not shown). The Nmyc antibody showed no staining of neurones in which Nmyc was deleted (Knoepfler et al., 2002), and only low-level staining of epithelial cells in conditional mutant lungs (Fig. 7F).

**Electron microscopy**

Tissue was fixed in 2% glutaraldehyde in PBS, post-fixed in osmium tetroxide, stained en bloc with uranyl acetate and embedded in Spur’s embedding medium (EM science). Thin sections were stained with uranylacetate/lead citrate before viewing with a Philips electron microscope.
DNA Affymetrix analysis

Total RNA (10 μg) was extracted from three different transgenic and two wild-type lungs (right caudal lobe) by using RNeasy kit. All subsequent reactions using Affymetrix GeneChip Mouse 430A arrays and statistical analyses were carried out in the Duke Center for Genome Technology Microarray core facility as detailed previously (Okubo and Hogan, 2004). All primary data files are freely available (see Tables S1 and S2 in supplementary material).

Results

Nmyc expression is restricted to distal epithelium containing a high proportion of cells in the S phase of the cell cycle

Levels of Nmyc RNA are highest in the early lung, decline significantly before birth and are very low in the adult (Fig. 1A). Immunohistochemistry revealed the previously unreported fact that Nmyc protein is preferentially localized to epithelial cells in the distal regions of the developing lung (Fig. 1B). This restriction is particularly evident at E12.5 to E16.5 as a boundary between the Nmyc-positive cells of the distal tubules and the mostly negative cells of the future proximal airways. Reactivity is both cytoplasmic and nuclear, as previously reported in neuronal cells (Wakamatsu et al., 1993). By E18.5, only low levels of Nmyc staining can be detected in the lung, in both presumptive type II cells and bronchial epithelium.

In the developing cerebellum, high levels of Nmyc expression coincide with distinct domains of the germinal neuroepithelium in which a large percentage of the progenitor cells are in S phase, as determined by BrdU incorporation (Knoepfler et al., 2002). Analysis of the embryonic lung revealed a similar overlap between Nmyc expression and BrdU-labeled cells in the distal epithelium (Fig. 1C for E12.5; Fig. 8M for E15.5). At E12.5, almost 75% of distal epithelial cells are labeled with BrdU over a 1-hour time period, whereas only 30% are positive in the proximal epithelium where Nmyc expression is low (Fig. 1C). Analysis of adjacent sections shows that the domain of high Nmyc expression also coincides with high levels of cyclin D1 expression. By contrast, phosphohistone H3-positive cells in late G2 and mitosis are not preferentially localized to either proximal or distal domains (Fig. 1C). These results suggest that the cell cycle kinetics of distal and proximal endoderm are already different at E12.5, and correlate with Nmyc expression.

Fig. 1. Nmyc expression and BrdU incorporation in the wild-type lung. (A) RT-PCR shows that Nmyc transcripts decrease during lung development, whereas those for differentiated epithelial cell markers (Sftpc, Sftpa and Aqp5) increase. (B) Immunohistochemistry at E12.5, E14.5 and E16.5 shows higher Nmyc expression in the distal compared with the proximal epithelium. Blue arrows mark the boundary between the two regions. At E18.5, low levels are seen in presumed type II cells (insets). (C) Immunostaining for Nmyc, BrdU, cyclin D1 and phospho-histone H3 (PHH3) in adjacent sections of E12.5 lung. (D) Percentage of BrdU-positive nuclei in Nmyc-high distal endoderm and Nmyc-low proximal endoderm of E12.5 lungs, as determined by counting nuclei in cross sections of tubules, where the identification of individual cells is unambiguous. *, difference is significant (P<0.001). Scale bars: 50 μm.
Overexpression of Nmyc disrupts lung development

For gain-of-function studies, we used a well-characterized human surfactant protein C (Sftpc) promoter to express an NmycEGFP fusion protein in the epithelium. Expression begins in primary lung buds from about E10.5 and continues throughout development with highest levels observed distally (Okubo and Hogan, 2004; Wert et al., 1993). The four transgenic lungs obtained at E18.5 resembled control lungs in overall dimensions and lobulation pattern (Fig. 2A). Internally, however, they had an abnormal phenotype that varied with the level of transgene expression, as determined by three independent criteria: fluorescence from NmycEGFP (Fig. 2B); RT-PCR (Fig. 2C); and transcriptional profiling by microarray (see Tables S1 and S2 in supplementary material). Control lungs had numerous primitive alveoli, with well-differentiated type II cells and flattened type I cells closely apposed to blood vessels (Fig. 2D,G,J). Such primitive alveoli were absent from transgenic lungs, which instead contained many distal epithelial tubules surrounded by abundant mesenchyme (Fig. 2E,F,H,I). In the two transgenic lungs with the highest levels of Nmyc expression (approximately 12- to 15-fold higher than normal from microarray), electron microscopy revealed large accumulations of

Table 1. Correlation between genotype and phenotype of conditional mutant lungs

<table>
<thead>
<tr>
<th>Mating genotype</th>
<th>cre(+) Nmyc&lt;sup&gt;fl&lt;sup&gt;ox&lt;/sup&gt;/+&lt;/sup&gt;</th>
<th>cre(+) Nmyc&lt;sup&gt;fl&lt;sup&gt;ox&lt;/sup&gt;/fl&lt;sup&gt;ox&lt;/sup&gt;&lt;/sup&gt;</th>
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<tr>
<td>*Sftpc-cre×Nmyc&lt;sup&gt;fl&lt;sup&gt;ox&lt;/sup&gt;/fl&lt;sup&gt;ox&lt;/sup&gt;&lt;/sup&gt; (F1)</td>
<td>28/52 (53.8%)</td>
<td>–</td>
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<tr>
<td>*cre(+) Nmyc&lt;sup&gt;fl&lt;sup&gt;ox&lt;/sup&gt;/fl&lt;sup&gt;ox&lt;/sup&gt;&lt;/sup&gt;×Nmyc&lt;sup&gt;fl&lt;sup&gt;ox&lt;/sup&gt;/fl&lt;sup&gt;ox&lt;/sup&gt;&lt;/sup&gt; (F2)</td>
<td>8/13 (61.5%)</td>
<td>12/12 (100%)</td>
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<td>†cre(+) Nmyc&lt;sup&gt;fl&lt;sup&gt;ox&lt;/sup&gt;/fl&lt;sup&gt;ox&lt;/sup&gt;&lt;/sup&gt;×Rosa26R(+) Nmyc&lt;sup&gt;fl&lt;sup&gt;ox&lt;/sup&gt;/+&lt;/sup&gt;</td>
<td>4/16 (25.0%)</td>
<td>9/9 (100%)</td>
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The Table shows the proportion of embryos/newborn pups from different crosses with an abnormal phenotype (number of lungs with abnormal phenotype/total number).

*Results of the mating scheme shown in Fig. 6A.
†Results of the mating scheme used to generate the data in Fig. S2 in supplementary material.
glycogen typical of undifferentiated endoderm (Ten Have-Opbroek, 1981; Ten Have-Opbroek, 1991), but no lamellar bodies characteristic of differentiated type II cells (Fig. 2L). However, some lamellar bodies were present in the cells and lumina of lungs with lower Nmyc expression (up to 8-fold higher than normal) (Fig. 2K).

BrdU incorporation and phosphohistone H3 staining showed a significantly higher number of proliferating cells in both the endoderm and mesenchyme of transgenic lungs when compared with wild type (Fig. 3A-D). Staining with anti-cleaved caspase 3 antibody also revealed many apoptotic epithelial cells in the transgenic lungs (Fig. 3E,F).

**Increased levels of Nmyc expression inhibit epithelial differentiation**

To explore the effect of Nmyc overexpression on endoderm differentiation, we first examined transcripts for Scgb1a1 and Foxj1, markers for Clara and ciliated cells, respectively (Fig. 4A,B, and data not shown for Foxj1). At E18.5, Scgb1a1 is highly expressed in proximal epithelium, with a sharp boundary between the terminal bronchioles and the future alveoli, which contain type II cells and their precursors expressing high levels of Sftpc. This boundary corresponds to the future bronchioalveolar duct junction (BADJ). It is also seen in sections of E18.5 lungs stained with antibody to Sox2, an HMG box transcription factor specifically expressed in proximal lung endoderm from E11.5 (Fig. 4C, and data not shown). By contrast, in E18.5 transgenic lungs, there is no distinct boundary between Scgb1a1 and Sftpc domains (Fig. 4B). Instead, there is an intermediate zone in which expressing cells are interspersed with non-expressing cells. A similar absence of a sharp boundary was seen with Sox2 (Fig. 4D). Taken together, these results suggest that Nmyc overexpression significantly reduces the probability that progenitor cells will differentiate into proximal or distal cell types. This conclusion was supported by RT-PCR analysis that showed a dramatic reduction in the expression of Aqp5, SftpA and SftpB, late differentiation markers for alveolar type I and type II cells (Fig. 4E).

**High-level expression of Sox9 in Nmyc overexpressing lung**

The idea that Nmyc overexpression inhibits the differentiation of progenitor cells was further supported by immunohistochemistry to Sox9. This HMG box transcription factor is specifically expressed in the distal progenitor cells from E11.5-E16.5 (Fig. 5A,C) (Liu and Hogan, 2002). By E18.5, however, the protein is almost undetectable in normal lungs (Fig. 5E,G). Significantly, in transgenic lungs the downregulation of Sox9 expression does not occur and high levels of nuclear protein are present throughout the E18.5 epithelium, including the bronchiolar region in which Scgb1a1-expressing cells are present (Fig. 5G,H). Double immunohistochemistry shows that a few transgenic cells express both Sox9 and Scgb1a1 (Fig. 5H). Elevated levels of Sox9 protein are seen in transgenic lungs even though RNA levels are not apparently different from wild type, as judged by RT-PCR and microarray (Fig. 4E and see Tables S1 and S2 in supplementary material). This suggests that posttranscriptional mechanisms are involved in maintaining the levels of Sox9 protein.

**Microarray analysis of gene expression in Sftpc-NmycEGFP transgenic lungs**

Previous studies have identified many genes that are directly or indirectly upregulated by Nmyc or Myc in mammalian cell lines or tumors, or in normal mouse skin (Boon et al., 2001; Frye et al., 2003; Patul et al., 2004; Raetz et al., 2003; Shiio et al., 2002; Zeller et al., 2003). The majority of these genes are associated with the general machinery of cell growth (RNA and protein synthesis and energy metabolism) and proliferation. To see whether a similar set of genes is upregulated by Nmyc in embryonic lung epithelium, we used mouse MOE430 Affymetrix gene arrays to analyze transcripts in E18.5 transgenic and wild-type lungs. We found 381 genes (excluding ESTs) that were upregulated twofold or more in transgenic lungs (P<0.05), and 391 that were similarly
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Fig. 4. High levels of Nmyc inhibit epithelial cell differentiation. (A,B) Sections of normal and severely affected E18.5 transgenic lungs hybridized with riboprobes for Scgb1a1 (a marker for differentiated Clara cells) and SftpB (a marker, when expressed at high levels, for type II cells). (A,C) In wild type, a sharp boundary is present (arrowheads) at the BADJ between bronchioles lined with Clara cells (red) and the future alveoli, lined by type II cells (green). In the transgenic lung the boundary is not sharp (white bracket). (C,D) Sections of the same lungs after staining with antibody to Sox2. In normal lungs (C), Sox2 is restricted to nuclei of proximal epithelial cells, with a sharp boundary at the BADJ (arrowheads). In the transgenic lung (D), the boundary is not sharp (white bracket). Nuclei are stained with DAPI. (E) Gene expression assayed by RT-PCR of total RNA extracted from two normal and two transgenic lungs. Representative genes are lung differentiation markers (Aqp5, SftpA, SftpB, SftpC, and Scgb1a1), upregulated genes from microarray data (Ppan, Nol5a, Rog, cyclin D2 and claudin 6), and Sox9. Scale bars: 50 µm.

Regulators is the primary mechanism by which Nmyc controls cell differentiation. Finally, analysis showed that there was an upregulation of genes encoding proteins with a proapoptotic function, for example Bid and Siva. This correlated with the presence of apoptotic epithelial cells in the transgenic lungs (Fig. 3F).

The genes downregulated in SftpB-NmycEGFP lungs also fell into several categories (see Table S2 in supplementary material). One of the largest (12% of total) encodes specialized products characteristic of type I and II alveolar cells, numerous genes encoding transmembrane proteins involved in the directed movement of ions and molecules into and out of cells, and proteins involved in cellular immunity (complement, immunoglobulin). Another category includes proteins involved in cell adhesion, extracellular matrix production and cell structure (26%). This grouping may reflect the simpler cuboidal morphology of the distal epithelium in transgenic lungs compared with the more complex three-dimensional organization of the primitive alveoli in the non-transgenic lungs. However, a number of the genes in this category are similar to those downregulated in other epithelial cells, such as keratinocytes (Arnold and Watt, 2001), and/or are direct targets of Myc repression (http://www.myccancergene.org/site/mycTargetDB.asp). In the transgenic lungs, we saw no evidence for epithelial cells detaching from the basal lamina, although branching morphogenesis was severely disrupted. RT-PCR confirmed the up- and downregulation of a number of the genes detected by microarray analysis (Fig. 4E).

Conditional deletion of Nmyc leads to abnormal lung development

To study the effect of conditional disruption of Nmyc, we used a SftpB-cre transgene, driving cre recombinase specifically in lung endoderm from about E10.5 (Okubo and Hogan, 2004), in combination with a previously described floxed allele of Nmyc (Knoepef et al., 2002).

To generate cre(+);Nmyc<sup>flox/flox</sup> mice, we initially crossed SftpB-cre and homozygous Nmyc<sup>flox/flox</sup> mice (Fig. 6A). As expected, about 50% of the F1 offspring were cre(+);Nmyc<sup>flox</sup>. It was verified repeatedly that the Nmyc<sup>flox</sup>
allele was deleted in the lung epithelium of embryos inheriting Sftpc-cre (Fig. 6B and data not shown). Previous studies had shown that mice heterozygous for both a germline null allele of Nmyc, and a conditional null allele in the nervous system (Knoepfler et al., 2002), have only a mild phenotype of reduced body or brain mass. It was therefore surprising to observe that about half of the cre(+)::Nmyc<sup>flox/+</sup> pups died at or shortly after birth with a very severe lung phenotype (Fig. 7A,B; Table 1). This result was obtained whether the cre transgene was inherited from the mother or father. The remaining pups (both males and females, in normal ratio) developed normally and had no obvious defects in lung morphology when analyzed as adults.

Surviving F<sub>1</sub> cre(+)::Nmyc<sup>flox/+</sup> mice were mated with Nmyc<sup>flox/flox</sup> mice (Fig. 6A) giving offspring in approximately Mendelian ratios: cre(–)::Nmyc<sup>flox/+</sup>, 29.6%; cre(+)::Nmyc<sup>flox/+</sup>, 24.1%; cre(–)::Nmyc<sup>flox/flox</sup>, 25.9%; and cre(+)::Nmyc<sup>flox/flox</sup>, 20.4%. All cre(+)::Nmyc<sup>flox/flox</sup> embryos died at birth or had very severe lung phenotypes when examined prenatally. Again, unexpectedly, a significant proportion (8/13; 62%) of the cre(+)::Nmyc<sup>flox/+</sup> pups or embryos had abnormal lung phenotypes (Table 1). In these affected lungs there were regions that appeared normal by external morphology and histological analysis (Fig. 7B). In some cases, an entire lobe was normal, but more usually the lung consisted of a mosaic of normal and highly abnormal areas. By contrast, serial sectioning of cre(+)::Nmyc<sup>flox/+</sup> lungs that were scored as normal showed a uniform histology throughout, resembling wild-type lungs (data not shown).

To confirm that the cre transgene is active, we crossed cre(+)::Nmyc<sup>flox/+</sup> mice with mice compound heterozygous for Nmyc<sup>flox/+</sup> and the Rosa26R reporter allele. These compound heterozygotes were generated by crossing Nmyc<sup>flox/flox</sup> mice (129/11003 C57BL/6) with Rosa26R mice (C57BL/6) (see Materials and methods). This showed that recombination had indeed taken place throughout the epithelium in both cre(+)::Nmyc<sup>flox/flox</sup> and cre(+)::Nmyc<sup>flox/+</sup> lungs at E16.5 and E18.5 (see Fig. S2 in supplementary material). In this set of experiments, 25% (4/16) of the cre::Nmyc<sup>flox/+</sup> lungs were abnormal (Table 1).

**Increased apoptosis, reduced proliferation, and evidence for depletion of progenitor pool in conditional mutant lungs**

At E18.5, the lobulation pattern and tracheal morphology of cre(+)::Nmyc<sup>flox/flox</sup> and affected cre(+)::Nmyc<sup>flox/+</sup> lungs were normal. However, they were composed of numerous large fluid-filled sacs, containing cellular debris, lined by highly attenuated epithelial cells, and separated by a thin layer of mesoderm (Fig. 7A,B; Fig. 8F; and data not shown).

When examined earlier (E14.5-E16.5), the external dimensions of the lungs of cre(+)::Nmyc<sup>flox/flox</sup> and affected cre(+)::Nmyc<sup>flox/+</sup> lungs were normal. However, they were composed of numerous large fluid-filled sacs, containing cellular debris, lined by highly attenuated epithelial cells, and separated by a thin layer of mesoderm (Fig. 7A,B; Fig. 8F; and data not shown).

We have observed that the number of Sox9-positive cells is greatly reduced (B.D). By contrast, there are numerous Sox9-positive epithelial cells, including small clusters (arrowheads), in the E18.5 Sftpc-NmycEGFP lung (F). (G,H) Double immunohistochemistry for Sox9 and Scgb1a1. Note the sharp junction between proximal and distal domains (arrowhead in G), and low Sox9 expression, in wild-type lung. In transgenic lung (H), note the absence of a clear boundary and the high Sox9 expression, even in bronchiolar regions (br) where Scgb1a1 is expressed. DAPI staining marks nuclei. Scale bars: 50 µm.
**Fig. 6.** Strategy for conditional deletion of *Nmyc* in the developing lung.

(A) Breeding strategy to generate *Stfpc-cre* transgenic mice either homozygous or heterozygous for *Nmyc*<sup>flox</sup>.

(B) Typical result of genotyping lung and liver DNA. Note that the recombined *Nmyc*<sup>flox</sup> allele was detected in the lung and not the liver. The unrecombined *Nmyc*<sup>flox</sup> allele in the cre(+) lungs presumably derives from the mesoderm in which cre is not expressed.

**Fig. 7.** Conditional deletion of *Nmyc* results in severely abnormal lung development. (A) Gross morphology of lungs at E18.5. The lung that is cre(+);*Nmyc*<sup>flox/+</sup> (middle panel) has a mosaic phenotype with some normal tissue (white box). By contrast, all cre(+);*Nmyc*<sup>flox/flox</sup> lungs (right panel) had a much more uniform abnormal phenotype. (B) Sections of E18.5 lungs after staining with Hematoxylin and Eosin. In this case, two examples of cre(+);*Nmyc*<sup>flox/+</sup> lungs are shown (middle panels): the one on the left has a normal morphology and resembles a cre(−) lung (far left), whereas the one on the right is severely abnormal and resembles a cre(+);*Nmyc*<sup>flox/flox</sup> lung (far right), except that regions of normal tissue are present (black box enlarged in inset). (C,D) Sections of wild-type and abnormal heterozygous cre(+);*Nmyc*<sup>flox/flox</sup> or *Nmyc*<sup>flox/+</sup> lungs at E14.5 (C) and E15.5 (D). (E) Level of *Nmyc* RNA in conditional mutant lungs as measured by RT-PCR of total RNA. Note the significant decrease in RNA levels at E14.5-E16.5. Asterisk indicates abnormal cre(+);*Nmyc*<sup>flox/+</sup> lung at E15.5. (F) Nmyc antibody staining of wild-type and cre(+);*Nmyc*<sup>flox/flox</sup> lungs at E18.5. Note there is only weak staining of cells in which *Nmyc* has been deleted. Scale bars: in B, 200 µm; in C,D, 100 µm; in F, 50 µm.
the conditional mutant lungs showed very reduced branching, with a few expanded tubes separated by abundant mesoderm. RT-PCR analysis showed that both the levels of \textit{Nmyc} RNA and protein were significantly reduced at these stages (Fig. 7E,F). Epithelial cell size was irregular, and dead cells were frequently observed in the lumen, suggesting a high level of apoptosis (Fig. 8D-F). This was confirmed by staining sections with an antibody to cleaved caspase 3 (a marker for apoptosis), which revealed many positive cells in both the epithelium and mesenchyme (Fig. 8G-L). BrdU labeling at E15.5 and E16.5 (Fig. 8M-O) showed a striking reduction in the proportion of proliferating cells in the mutant lung endoderm (17% compared with 65% for wild type at E15.5). These results indicate that

Fig. 8. Evidence for increased apoptosis and reduced proliferation in conditional mutant lungs. (A-F) Sections of cre(–) and cre(+) lungs at E14.5, E15.5 and E18.5. Note the presence of cellular debris (arrowheads), and the irregular shape and smaller size of the epithelial cells in the conditional mutant lungs (D-F). (G-L) Immunofluorescence for cleaved caspase 3 and DAPI (nuclei). Note abundant apoptotic cells in the lumen, epithelium and mesenchyme of cre(+)\textit{Nmyc}^{+/-} and cre(+)\textit{Nmyc}^{+/+} lungs at E15.5, E16.5 and E18.5 (arrowheads). The faint fluorescence seen in J is from red blood cells. (M-O) BrdU labeling. Most distal epithelial cells in the E15.5 wild-type lung are positive for BrdU after a 1-hour pulse. By contrast, note the reduced epithelial labeling of mutant lungs at E15.5 and E16.5 (arrowheads). Scale bars: 50 µm.
Nmyc deletion severely inhibits both cell proliferation and survival.

Finally, we explored the differentiation of epithelial cells in the conditional mutant lungs. There was a clear reduction in the number of Sox9-positive cells in cre(+)::Nmyc<sup>flox/flox</sup> lungs at E14.5 and E16.5 (Fig. 5B,D), suggesting that the pool of progenitor cells is not maintained but is lost through apoptosis or differentiation. The presence in E18.5 conditional mutant lungs of both Sftpc-positive type II cells and Gp38 (T1α)-positive, attenuated type I cells shows that differentiation does occur (Fig. 9B,D). Evidence that some differentiation may be premature comes from the analysis of lung differentiation markers at E16.5 by RT-PCR. As shown earlier in Fig. 1A, expression of Aqp5 (a marker for type I cells) is normally not upregulated until E17.5-E18.5. However, Aqp5 was detected in cre(+)::Nmyc<sup>flox/flox</sup> lungs at E16.5, although it was absent from cre(−);Nmyc<sup>flox/+</sup> lungs at the same age (Fig. 9E).

**Discussion**

Nmyc was shown over 10 years ago to be expressed at high levels in the epithelium of the embryonic mouse lung and to be required for its normal development. However, this paper provides the first detailed analysis of the temporal and spatial distribution of the protein in relation to the proximodistal growth and patterning of the lung endoderm. This reveals a population of distal, immature epithelial cells that is characterized by high levels of Nmyc, Sox9 and cyclin D1 proteins, and contains a high proportion of cells in the S phase of the cell cycle. This population most likely represents a progenitor pool that normally gives rise to differentiated cell types along the proximodistal axis. At present, in the absence of detailed lineage analysis, it is not known whether the pool contains multipotent progenitors that can give rise to either proximal or distal cell types, a mixture of committed cells with a more restricted developmental potential, or progenitors that change their developmental potential from proximal to distal over time (Perl et al., 2002).

Independent of these models, our results suggest that high levels of Nmyc normally ensure that a progenitor cell in the distal endoderm (regardless of its potential) remains in a cell cycle program with a high probability of re-entering S phase, rather than exiting to a differentiated state with different cell cycle kinetics (Wartiovaara et al., 2002; Westbury et al., 2001).

How do high levels of Nmyc perform this function in the lung? Our array analysis provides evidence that overexpression of Nmyc leads to the upregulation of many well-documented Myc target genes that promote cell growth (increase in cell mass), RNA processing and nucleolar structure, DNA replication, and transit of the cell cycle. It is therefore tempting to speculate that Nmyc functions not only by controlling positive and negative cell cycle regulators but also by promoting a particular functional organization of the nucleus, including changes associated with S-phase, and/or elevated levels of nucleolar proteins such as fibrillarin and nucleostemin, which are characteristic of embryonic, stem and cancer cells (Newton et al., 2003; Tsai and McKay, 2002). Our results also suggest Nmyc functions in the lung through maintaining high levels the expression of the HMGbox protein Sox9. At present, we do not know the significance of this finding, as the effect of overexpressing or deleting Sox9 in the lung has not been reported. However, recent studies show that Sox9 protein is also localized in the proliferative compartment of the adult intestine and its overexpression in cell lines inhibits the expression of genes involved in epithelial cell differentiation (Blache et al., 2004).

The increased proliferation seen in Sftpc-Nmyc transgenic lungs was also accompanied by apoptosis. The induction of both apoptosis and hyperproliferation in response to Myc overexpression has been well documented in other systems, particularly under conditions in which the availability of cell survival factors becomes rate limiting (for reviews, see Hipfner and Cohen, 2004; Hueber and Evan, 1998). Recent studies have suggested a mechanism by which cells undergoing
apoptosis are extruded from an epithelial layer (Rosenblatt et al., 2001), and this might account for the dead cells seen in the lumen of both transgenic and conditional mutant lungs.

**Nmyc is essential for the proliferation and survival of embryonic lung epithelial cells and for normal epithelial-mesenchymal interactions**

Previous studies on embryos carrying germline mutations in *Nmyc* had suggested that lung development is particularly sensitive to reductions in Nmyc levels. Our results here support this conclusion and show that conditional deletion of *Nmyc* leads to inhibition of cell proliferation and to extensive cell death. Dying cells drop out of the epithelial layer and the survivors that fill the vacated space may possibly undergo premature differentiation, so that, by E18.5, only attenuated type I cells and a few type II cells line the large sacs. The extensive apoptosis seen in the conditional mutant lungs is in marked contrast to the effect of deleting *Nmyc* in neuronal progenitors in the developing brain. In this organ, there is little cell death of the neuroblasts in which *Nmyc* is deleted, and they appear to undergo cell cycle arrest and precocious differentiation (Knoopfeler et al., 2002). There are at least two possible reasons for this discrepancy between lung and brain. First, in the lung, other members of the myc gene family apparently cannot compensate for the absence of an essential function normally provided by *Nmyc*. By contrast, in the brain Myc can presumably compensate for the absence of *Nmyc* (Knoopfeler et al., 2002). Another reason may relate to the different organization of the cell layers in the two organs, and to the recent proposal that absence of Myc places cells at a disadvantage when competing with their neighbors for limited access to growth factors or necessary substrates (de la Cova et al., 2004; Moreno and Basler, 2004). In the case of the developing cerebellum, the progenitor cells in the germinal layer that undergo neurogenic (asymmetric) cell divisions rather than proliferative (symmetric) division generate daughter cells that move into the developing brain and differentiate. There may therefore be relatively little selective pressure in the germinal epithelium against cells with reduced or absent *Nmyc* expression. By contrast, in the lung, all the daughters of epithelial cells normally remain attached to a common, continuous basal lamina. Therefore, since recombination takes place asynchronously, the cells that are the first to lose *Nmyc* will be at a growth disadvantage compared with their neighbors, and may be competed or forced off the substrate on which they depend for their survival. Restriction in access to essential factors for cell growth and survival may also account for the high level of apoptosis seen in the mesenchyme of conditional mutant lungs at E15.5 and E16.5, even though *Nmyc* is not expressed in this cell population. We and others have shown that, in the developing lung, the endoderm produces factors, such as sonic hedgehog, that are necessary for the survival and proliferation of the mesoderm (Gebb and Shannon, 2000; Weaver et al., 2003).

A general conclusion from our gain- and loss-of-function studies is that Nmyc plays a key role in controlling the flow of cells into and out of a distal progenitor pool during lung development. It will therefore be important to determine how factors such as Wnts, TGFβs and IGFs regulate the expression of *Nmyc* RNA, and the phosphorylation and turnover of the protein, at different stages of embryogenesis (Frederick et al., 2004; Kenney et al., 2004) (our unpublished observations suggesting that *Nmyc* is a direct target of Wnt signaling in the embryonic lung).

**Effects of acute Nmyc hemizygosity on lung development**

A striking finding of this study was the failure in lung development in half (40/81) of the F1 generation from crossing Sftpc-cre;*Nmyc*+/− with *Nmyc*box/box mice, to 62% (8/13) in the F2 generation (Fig. 6), and 25% (4/16) when Sftpc-cre(+) *Nmyc*box/− mice were crossed with *Nmyc*box/+;Rosa26R mice (Table 1). There are two possible explanations for these results, neither of which can be excluded without further extensive experimentation. The first possibility is that the (129×C57BL/6) background on which the *Nmyc*box allele is maintained is segregating for a modifier gene that decreases the probability that a lung epithelial cell in which only one *Nmyc* allele is active will continue to proliferate. The second, more speculative, model proposes that the level of *Nmyc* transcription is initiated is low and, once one allele is active in a cell, the transcription of the other allele is inhibited by the feedback loop. This essentially monoallelic expression is then maintained, possibly by epigenetic modification. Consequently, the epithelium of the primary buds of heterozygous cre(+) *Nmyc*box/− embryos will be a mosaic of cells in which either one or the other of the *Nmyc* alleles is active. Those cells in which the *Nmyc*box allele is active will become functionally null after recombination. Depending on the proportion of cells in the early lung primordium with each allele active, and on the level of competition between normal and mutant cells, the conditional mutant lungs will be either abnormal, a mosaic of normal and abnormal tissue, or completely normal. To further test this model will require analysis of the transcription or epigenetic modification of distinguishable *Nmyc* alleles at the single-cell level in the embryonic lung. Meanwhile, whichever model is correct, our findings raise the interesting possibility that, prior to amplification of Nmyc or aberrant activation of Myc, lung tumor cells should be particularly sensitive to agents downregulating Nmyc expression.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/6/1363/DC1

**References**


