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Original Article

Abnormal development of the diaphragm in *mdx:MyoD-/-* ^{9th} embryos leads to pulmonary hypoplasia

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ABSTRACT ***In vitro*** **studies have shown that mechanical factors play an important role in cell cycle kinetics and cell differentiation of the lung through an unknown mechanochemical signal transduction pathway. In this study we evaluated the** ***in vivo*** **role of mechanical factors due to fetal breathing movements (primarily executed by the diaphragm, which is the main respiratory muscle) on lung growth and development by using genetically engineered embryos. Lung growth and development of wild-type,** ***mdx:MyoD+/-*** ^{9th} **(in which the diaphragm develops normally) and** ***mdx:MyoD-/-*** ^{9th} **(in which the diaphragm muscle is significantly thinned and not functional) embryos were compared at embryonic day 18.5 using immunohistochemistry,** ***in vivo*** **TUNEL detection and morphometry. No abnormalities in lung organogenesis were observed in** ***mdx:MyoD+/-*** ^{9th} **term embryos, whereas lung hypoplasia was detected in** ***mdx:MyoD-/-*** ^{9th} **embryos. In the hypoplastic lung, the number of proliferating lung cells was lower in comparison to the wild-type and** ***mdx:MyoD+/-*** ^{9th} **embryos, while the gradient of thyroid transcription factor-1 (TTF-1) was not maintained. Surprisingly, no difference was observed in distribution and occurrence of apoptotic lung cells in** ***mdx:MyoD-/-*** ^{9th} **embryos. Together, it appears that mechanical forces generated by contractile activity of the diaphragm muscle play an important role in normal lung growth and development by affecting cell proliferation and TTF-1 expression.**

KEY WORDS: *MyoD, dystrophin, mouse, myogenesis, diaphragm, lung hypoplasia*

Introduction

Lung is composed of two main systems, the bronchial system and the respiratory system (Ten Have-Opbroek, 1991). The bronchial system is responsible for conducting air through conductive tubes, whereas the gas exchange takes place in the respiratory system. Proper lung growth and development seems to be dependent on hormonal factors and mechanical forces. Numerous *in vitro* and *in vivo* studies show that mechanical forces influence fetal lung development through pulmonary distension (Harding, 1991; Harding and Hooper, 1996; Kitterman, 1996; Liu *et al.,* 1999; Liu and Post, 2000). Pulmonary distension appears to play a role in lung growth and development by affecting cell differentiation and cell cycle kinetics through some unknown mechanochemical signal transduction pathways (Liu *et al.,* 1999; Liu and Post, 2000).

In vivo studies reveal that mechanical forces caused by fetal breathing movements (FBMs) play a role in lung growth and development (Wigglesworth, 1985; Harding, 1991; Kitterman, 1996). FBMs are intermittent respiratory-like movements caused by contraction in the respiratory muscle of mammalian embryos. The contractions are produced by rhythmic activation of the respiratory neurons in the brainstem (Harding, 1997; Jesudason *et al.,* 2000). The lack of FBMs causes a range of abnormalities in the lung organogenesis. For instance, spinal cord transection above the phrenic motor neurons in fetal rabbits and sheep leads to lung hypoplasia (Wigglesworth and Desai, 1979; Liggins *et al.,* 1981). In addition, many clinical case reports indicate that the infants with the absence of FBMs *in utero* suffer from pulmonary hypoplasia and almost all of them die in the neonatal period (Blott *et al.,* 1990; Roberts and Mitchell, 1995).

Diaphragm is the main respiratory muscle (Tortora and Grabowski, 1993) and plays an important role in producing FBMs (Maloney *et al.,* 1975). In Duchenne muscular dystrophy (DMD), progressive muscular degenerative changes occur in various muscles including the diaphragm (Stedman *et al.,* 1991; Blake *et al.,* 2002). The disease is caused by mutation in one gene on the X chromosome, which encodes a cytoskeletal protein named

Abbreviations used in this paper: FBM, fetal breathing movement; TTF-1, thyroid transcription factor-1.

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dystrophin (Blake *et al.,* 2002). Clinical reports show that respiratory complications due to respiratory muscle weakness are the main cause of death among the patients at the age of twenty (Blake *et al.,* 2002). Murine X-linked muscular dystrophy (*mdx*) is genetically homologous with DMD (Stedman *et al.,* 1991). Complete lack of dystrophin in DMD and *mdx* mice is the reason why *mdx* mice are used as an animal model for human DMD (Stedman *et al.,* 1991; Anderson *et al.,* 1998).

To study the dependence of lung development on the main respiratory muscle contractility during embryonic development, we employed double-mutant mouse embryos that in addition to dystrophin also lacked MyoD (a myogenic regulatory factor that plays a major role in muscle cell differentiation, as reviewed in Kablar and Rudnicki, 2000). Previous studies show that lack of MyoD leads to impairment of proper development of hypaxial musculature including the diaphragm (Kablar *et al.,* 1998). *mdx:MyoD-/-* mice were backcrossed for nine generations until no viable double-mutant embryos were obtainable. The term *mdx:MyoD-/-^{9th}* embryos showed approximately 70% reduction in the diaphragm only and all other muscles were normally developed (Inanlou and Kablar, unpublished data).

Lung development in the mouse is composed of four stages: pseudoglandular, canalicular, terminal sac and alveolar (Ten Have-Opbroek, 1991; Burri, 1997; Tseng *et al.,* 2000). Since the most prominent histological picture of pulmonary hypoplasia occurs from the beginning of the terminal sac stage on (Inanlou and Kablar, unpublished data), we analysed *mdx:MyoD-/-^{9th}* embryos at the embryonic day (E) 18.5 (or the terminal sac stage of the lung development). We investigated how impairment of the diaphragm, and therefore FBMs generated by the diaphragm, leads to lung hypoplasia by studying cell proliferation and programmed cell death in the lung and by examining the expression pattern of thyroid transcription factor-1 (TTF-1, a member of NKX2 family of homeodomain transcription factors involved in lung epithelial differentiation and lung organogenesis; Zhou *et al.,* 2001) in wild-type, *mdx:MyoD+/-^{9th}* and *mdx:MyoD-/-^{9th}* term (E18.5) embryos.

Together, our data show that insufficient FBMs can cause pulmonary hypoplasia by decreasing lung cell proliferation, disturbing TTF-1 expression gradient and without affecting lung cell apoptosis.

Results

mdx:MyoD-/-^{9th} *Embryos lack Spontaneous Breathing Movements*

All the embryos recovered by Caesarean section had normal pink colour and were able to move their limbs immediately after delivery. However due to the absence of the spontaneous breathing movements the *mdx:MyoD-/-^{9th}* embryos became cyanotic and died within minutes after birth. By contrast, normal breathing movements were observed in the wild-type and *mdx:MyoD+/-^{9th}* embryos. No abnormality in size and skeletal structure was observed among the embryos.

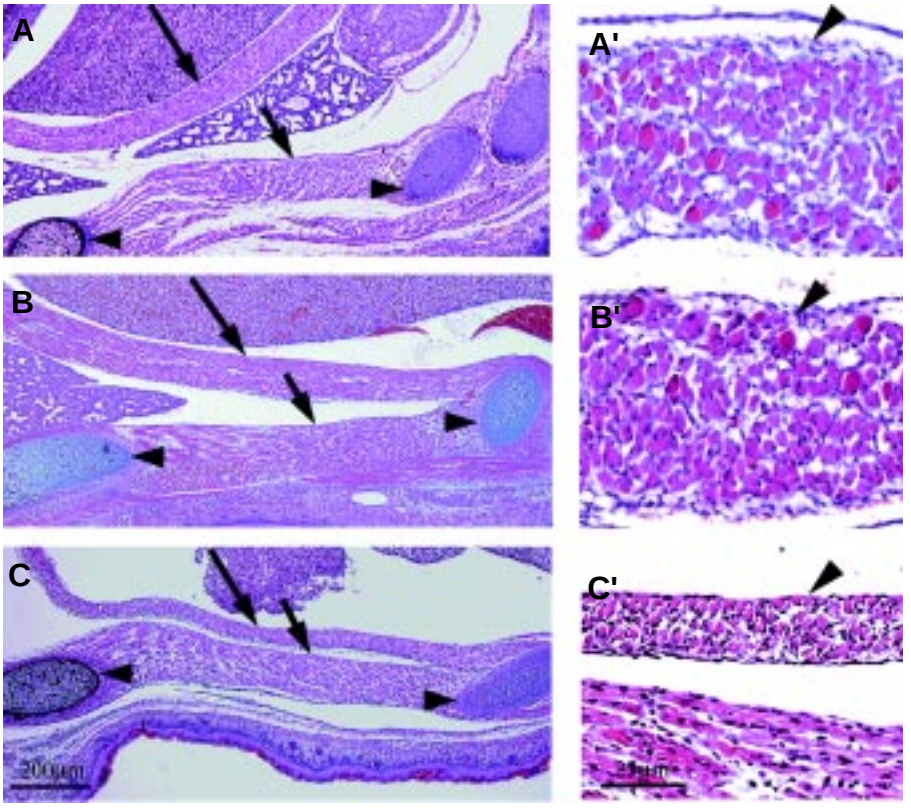

Fig. 1. A reduced size of the diaphragm is observed in *mdx:MyoD-/-^{9th}* embryos. *Intercostal muscles (short arrows in A-C), extending between the ribs (arrowheads in A-C), appear indistinguishable between wild-type* **(A)**, mdx:MyoD+/-^{9th} **(B)** *and* mdx:MyoD-/-^{9th} **(C)** *embryos, as compared in transverse paraffin H&E sections of the trunk. In contrast, the thickness of the diaphragm (long arrows in A-C) is significantly reduced in* mdx:MyoD-/-^{9th} *embryos (long arrow in C and arrowhead in C') in comparison to wild-type (long arrow in A and arrowhead in A') and* mdx:MyoD+/-^{9th} *(long arrow in B and arrowhead in B') embryos.*

Development of the Diaphragm is affected in mdx:MyoD-/-^{9th} *Embryos*

The thoraxes of all embryos were exposed under the stereomicroscope. The diaphragm was intact across the abdominal cavity and no diaphragmatic herniation was detected. The transverse H&E sections of the trunk showed that *mdx:MyoD-/-^{9th}* embryos had a very thin diaphragm in comparison to that of the *mdx:MyoD+/-^{9th}* and wild-type embryos (Fig. 1). The diaphragm in wild-type and *mdx:MyoD+/-^{9th}* embryos was approximately three times thicker in its thickest part and almost nine times thicker in its thinnest part in comparison to the respective parts of the *mdx:MyoD-/-^{9th}* diaphragm (Table 1). The gross anatomy and thickness of the intercostal musculature seemed to be normal in all embryos (Fig. 1).

mdx:MyoD-/-^{9th} *Embryos have Lung Hypoplasia*

The paraffin sections of the lung tissue stained with H&E revealed significant histological differences in the lung of *mdx:MyoD-/-^{9th}* embryos in comparison to

the lung of wild-type and *mdx:MyoD+/-^{9th}* embryos. The most prominent differences were observed in the respiratory and not in the conductive system of the lung. For instance, in the bronchial (or conductive) system, the bronchioles were lined up by semi-columnar and cuboidal epithelium similarly in wild-type, *mdx:MyoD+/-^{9th}* and *mdx:MyoD-/-^{9th}* embryos. In the respiratory system, the developing and expanding saccules occupied most of the lung tissue of wild-type and *mdx:MyoD+/-^{9th}* embryos (Fig. 2). The epithelium of the saccules in wild-type and *mdx:MyoD+/-^{9th}* embryos was semi-squamous and squamous. In *mdx:MyoD-/-^{9th}* embryos, the respiratory system seemed to be arrested in the canalicular stage, as almost no saccule was detectable. The lung tissue of *mdx:MyoD-/-^{9th}* embryos was composed of acinar tubules lined by the epithelium that was cuboidal (Fig. 2). The acinar tubular spaces in the lung of *mdx:MyoD-/-^{9th}* embryos were noticeably smaller than the saccular space in the lung of the wild-type and *mdx:MyoD+/-^{9th}* embryos. As their name implies, acinar tubules have a tubular appearance or look like more or less solid sprouts (Ten Have-Opbroek, 1981). Compared to acinar tubules, saccules have larger airspaces and more complex outlines (Collins *et al.,* 1986). Acinar tubules are composed of low columnar or coboidal epithelium, whereas saccules have a flatter epithelium, thinner and longer septal crests and narrower airspace walls with larger airspaces (Collins *et al.,* 1986).

In addition, we detected that the lung of *mdx:MyoD-/-^{9th}* embryos were noticeably lighter in comparison to the control (Table 2). Our comparisons of wet and dry lung weight versus the whole body weight of E18.5 embryos of all three genotypes revealed that only the lung of *mdx:MyoD-/-^{9th}* embryos had less than 4% ratio (i.e., 2.3 ± 0.1) between the wet lung weight and the whole body weight, indicating lung hypoplasia (Seegmiller *et al.,* 1986).

TABLE 1

COMPARISON OF THE DIAPHRAGM THICKNESS IN WILD-TYPE, *MDX:MYOD+/-^{9th}* AND *MDX:MYOD-/-^{9th}* TERM EMBRYOS

Diaphragm	Wild-type	*mdx:MyoD+/-^{9th}*	*mdx:MyoD-/-^{9th}*
thickest part	29 ± 1	31 ± 2	9 ± 1*
thinnest part	23 ± 2	26 ± 2	3 ± 1*

Note: The number of muscle fibres is presented as the mean ± SD; (*) significantly different number (ANOVA, $P < 0.05$).

TABLE 2

COMPARISON OF BODY WEIGHT AND LUNG WEIGHT IN WILD-TYPE, *MDX:MYOD+/-^{9th}* AND *MDX:MYOD-/-^{9th}* EMBRYOS AT E18.5

Genotype	Wild-type	*mdx:MyoD+/-^{9th}*	*mdx:MyoD-/-^{9th}*
BW (mg)	1135 ± 21	1136 ± 15	1123 ± 5
Wet LW (mg)	47.5 ± 3.5	46.6 ± 2.1	26.3 ± 0.5*
Dry LW (mg)	6.3 ± 0.2	6.7 ± 0.3	4.6 ± 0.4*
Percentage of wet LW/BW	4.2 ± 0.2	4.1 ± 0.4	2.3 ± 0.1

Note: BW, Body Weight; LW, Lung Weight.
(*) Statistically significant difference with ANOVA ($P<0.05$). In mice, pulmonary hypoplasia is determined by less than 4% wet LW/BW (Seegmiller *et al.,* 1986).

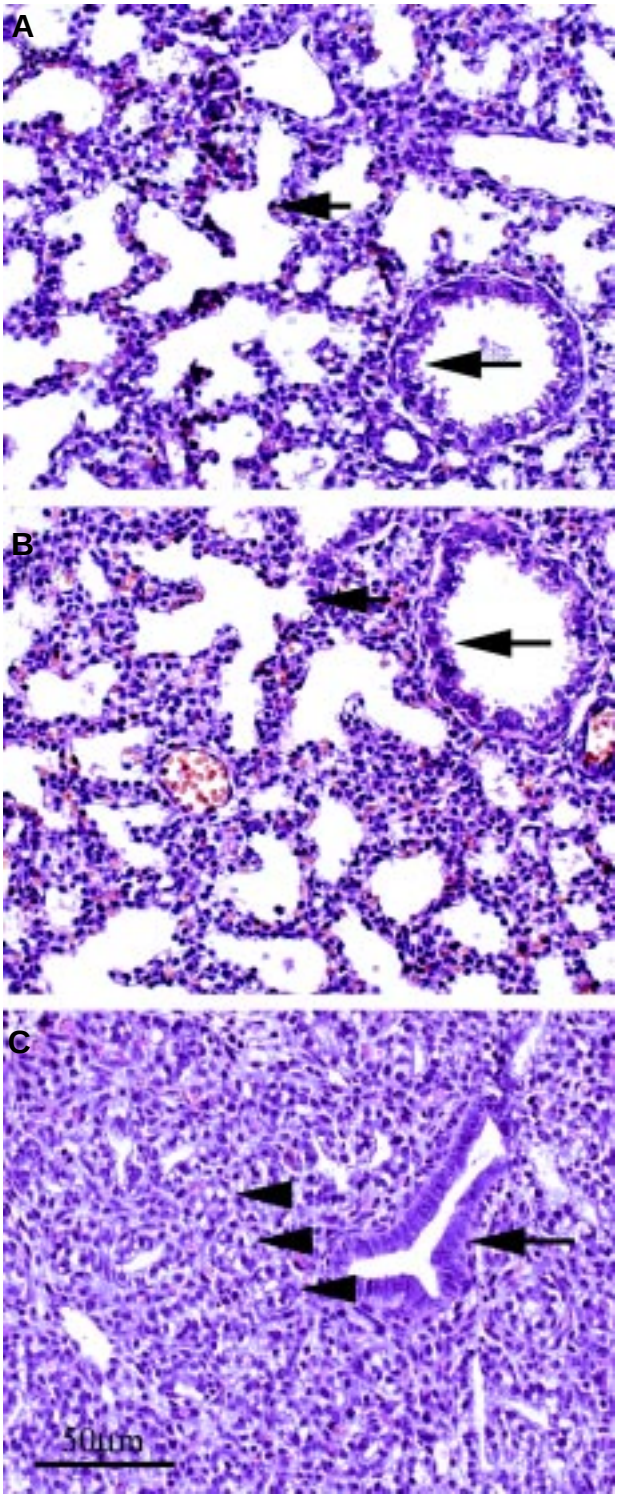

Fig. 2. *mdx:MyoD-/-^{9th}* embryos have pulmonary hypoplasia. *Paraffin embedded H&E sections of the wild-type* **(A)**, mdx:MyoD+/-^{9th} **(B)** *and* mdx:MyoD-/-^{9th} **(C)** *lung. Expanding saccules occupy most of the lung tissue in the wild-type and* mdx:MyoD+/-^{9th} *embryos (short arrows in A and B). Almost no saccule is detected in the lung of the* mdx:MyoD-/-^{9th} *embryos (C). Instead, the lung is composed of acinar tubules (arrowheads in C). The histological structure of bronchioles is similar among the embryos (long arrows in A-C).*

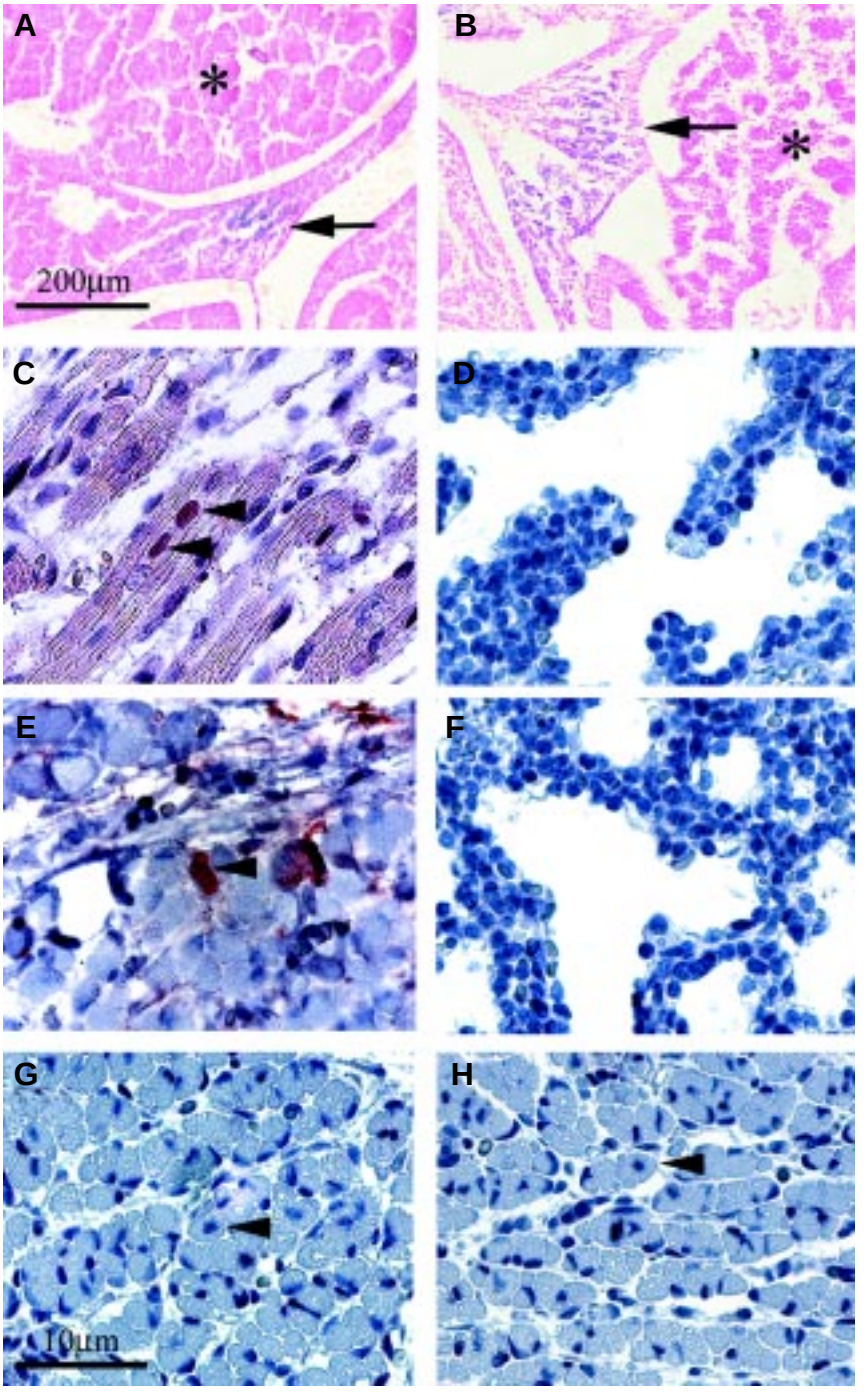

Fig.3. MyoD and dystrophin are not expressed in the lung tissue. *The expression of the* MD6.0-lacZ *transgene is clearly localized (the turquoise β-galactosidase staining in eosin counter-stained paraffin sections) (arrow in A) in the diaphragm of E12.5 embryos and not in the developing lung; see asterisk in* **(A)**. *Similarly, the* 258/-2.5lacZ *transgene is expressed in the E12.5 diaphragm (arrow in B) and not expressed in the lung tissue; see asterisk in* **(B)**. *In addition, neither MyoD* **(D)** *nor dystrophin* **(F)** *protein is detected in the E18.5 lung, as revealed employing an immunolabeling technique. Both of these proteins are observed in control longitudinal* **(C)** *and cross* **(E)** *sections of striated muscle of E18.5 wild-type embryos (nuclear MyoD staining is indicated by arrowheads in C, whereas cytoplasmic dystrophin staining is indicated by an arrowhead in E). Finally, E18.5* mdx:MyoD+/-^{9th} *skeletal muscle does not express dystrophin* **(G)**, *and E18.5* mdx:MyoD-/-^{9th} *skeletal muscle does not express MyoD* **(H)**. *Centrally located nuclei (arrowheads in G and H) in cross-sectioned muscle fibres indicating regeneration of the dystrophic muscle can be clearly observed.*

MyoD and Dystrophin Proteins are not expressed in the Lung

To examine the temporal and spatial pattern of *MyoD* expression in the mouse developing lung, mice carrying two different MyoD-*lacZ* transgenes were used: a) the *MD6.0-lacZ* transgene, containing 6 kb of *MyoD* upstream regulatory sequences including a potent muscle-specific enhancer located about 5 kb upstream from the *MyoD* transcription start site (Tapscott *et al.,* 1992; Asakura *et al.,* 1995) and b) the *258/-2.5lacZ* transgene, containing the –20 kb *MyoD* enhancer linked to 2.5 kb of *MyoD* promoter sequence and *lacZ* (Goldhamer *et al.,* 1995). The *258/-2.5lacZ* transgene is expressed earlier than *MD6.0-lacZ* transgene during the skeletal muscle development (Kablar *et al.,* 1997).

The expression of *MD6.0-lacZ* transgene was analyzed in serial transverse sections through the embryonic thorax and lung. *MD6.0-lacZ* transgene was clearly localized (e.g., the turquoise β-galactosidase staining in eosin counter-stained paraffin sections, as seen in Fig. 3A and data not shown) in the diaphragm of E11.5, E12.5 and E13.5 embryos. *MD6.0-lacZ* transgene was not found in any localization in the developing lung (Fig. 3A and data not shown). Similarly, the *258/-2.*5lacZ transgene was also expressed in the diaphragm and not expressed in the lung tissue at any analyzed age of embryonic development (Fig. 3B and data not shown).

In addition, neither MyoD nor dystrophin protein was detected in the lung of the examined embryos using immunolabeling technique (Fig. 3 D,F). Both of these proteins were observed in the striated muscle of the wild-type embryos (Fig. 3 C,E). Dystrophin was absent in the striated muscle of the *mdx:MyoD+/-^{9th}* embryos (Fig. 3G), while MyoD protein was detectable. Both dystrophin and MyoD (Fig. 3H) proteins were absent in the striated muscle of *mdx:MyoD-/-^{9th}* embryos. These data are in accordance with previous findings (Megeney *et al.,* 1996).

The Number of Proliferating Lung Cells decreases in mdx:MyoD-/-^{9th} *Embryos*

Immunohistochemistry against PCNA revealed that the number of proliferating lung cells decreased in both the epithelial and the mesenchymal compartments of *mdx:MyoD-/-^{9th}* embryos in comparison to that of wild-type and *mdx:MyoD+/-^{9th}* embryos (Fig. 4). The statistical data showed that the average percentage of the immunoreactive lung cells in the epithelial compartment of *mdx:MyoD-/-^{9th}* embryos was significantly lower (44±5%) than that of *mdx:MyoD+/-^{9th}* (59±6%) and wild-type (63±3%) embryos. Similarly the percentage of immunoreactive lung cells was significantly lower in the mesenchymal

compartment of *mdx:MyoD-/-9th* embryos (34±4%) in comparison to that of *mdx:MyoD+/-9th* (53±9%) and wild-type (49±7%) embryos. The number of proliferating cells in the lung in the epithelial compartment seemed to be higher than that of the mesenchymal compartment in all the embryos. No statistically significant difference was observed in the number of lung proliferating cells between the wild-type and *mdx:MyoD+/-9th* embryos in the epithelial and mesenchymal compartments.

The Number of Apoptotic Cells is Not Different among Wild-type, mdx:MyoD+/- 9th and mdx:MyoD-/- 9th *Embryos*

The number of apoptotic cells was compared in the epithelial and the mesenchymal compartments of wild-type, *mdx:MyoD+/-9th* and *mdx:MyoD-/-9th* embryos. The occurrence of apoptosis appeared to be higher in the lung cells of the mesenchymal compartment in all the embryos. The number of apoptotic cells showed no statistically significant difference either in the epithelial or in the mesenchymal compartment among the genotypes (Fig. 5). The statistical data showed that the average percentage of the TUNEL-positive lung cells in the epithelial compartment of *mdx:MyoD-/-9th* embryos (0.3±0.05%) was similar to that of *mdx:MyoD+/-9th* (0.3±0.08%) and wild-type (0.2±0.05%) embryos. Similarly, the percentage of TUNEL-positive lung cells was not different in the mesenchymal compartment of *mdx:MyoD-/-9th* embryos (0.4±0.05) in comparison to that of *mdx:MyoD+/-9th* (0.3±0.05) and wild-type (0.3±0.05) embryos.

The TTF-1 Expression Gradient is Strikingly Disturbed in mdx:MyoD-/- 9th *Embryos*

Immunohistochemistry was used to evaluate the expression of TTF-1 in the epithelial lung cells of the embryos of all three genotypes (Fig. 6). In wild-type embryos, the pattern of TTF-1 expression seemed to differ between the proximal and the distal ducts of the lung. For instance, almost no positive cell for TTF-1 could be detected among the epithelial columnar cells of the proximal ducts (Fig. 6A), whereas cuboidal cells of the distal ducts were strongly expressing TTF-1. The described gradient of TTF-1 expression observed in wild-type embryos was maintained in

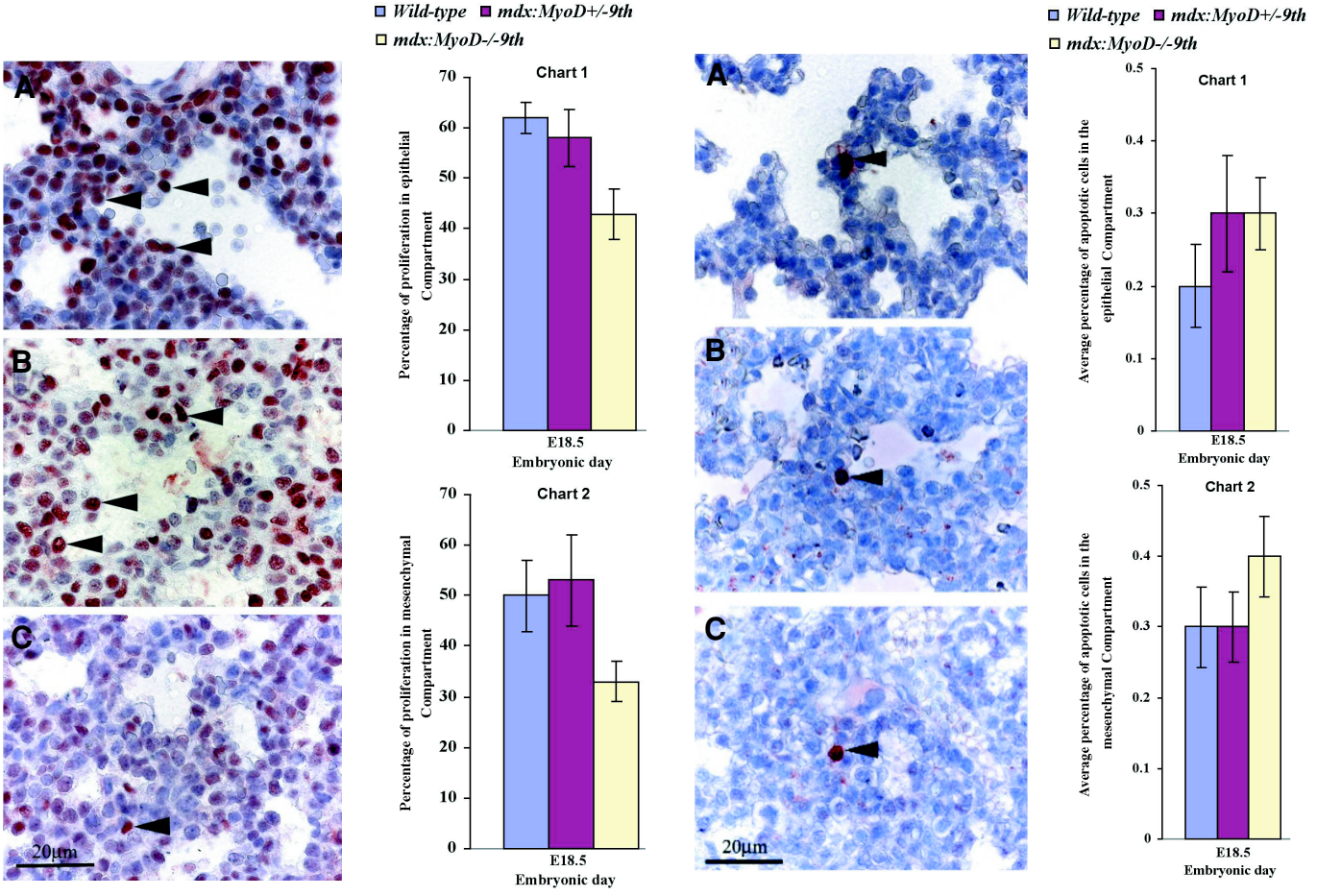

Fig. 4. (Left column) The lung of *mdx:MyoD-/-9th* embryos presents decreased cell proliferation. *The average percentage of proliferating lung cells is lower in the lung of* mdx:MyoD-/-9th *embryos both in the epithelial (chart1) and mesenchymal (chart2) compartments, in comparison to wild-type and* mdx:MyoD+/- 9th *embryos at E18.5. Values are means ± SD.* P *< 0.05 by one-way ANOVA. Similar findings are evident in the micrographs of wild-type* **(A)**, mdx:MyoD+/- 9th **(B)** *and* mdx:MyoD-/- 9th **(C)** *lungs.*

Fig. 5. (Right column) The lung of *mdx:MyoD-/-9th* embryos exhibits a normal rate of programmed cell death (or apoptosis). *The average percentage of apoptotic lung cells is similar in the lung of* mdx:MyoD-/-9th *embryos both in the epithelial (chart1) and mesenchymal (chart2) compartments, in comparison to wild-type and* mdx:MyoD+/- 9th *embryos at E18.5. Values are means ± SD.* P *> 0.05 by one-way ANOVA. Similar findings are evident in the micrographs of wild-type* **(A)**, mdx:MyoD+/- 9th **(B)** *and* mdx:MyoD-/- 9th **(C)** *lungs.*

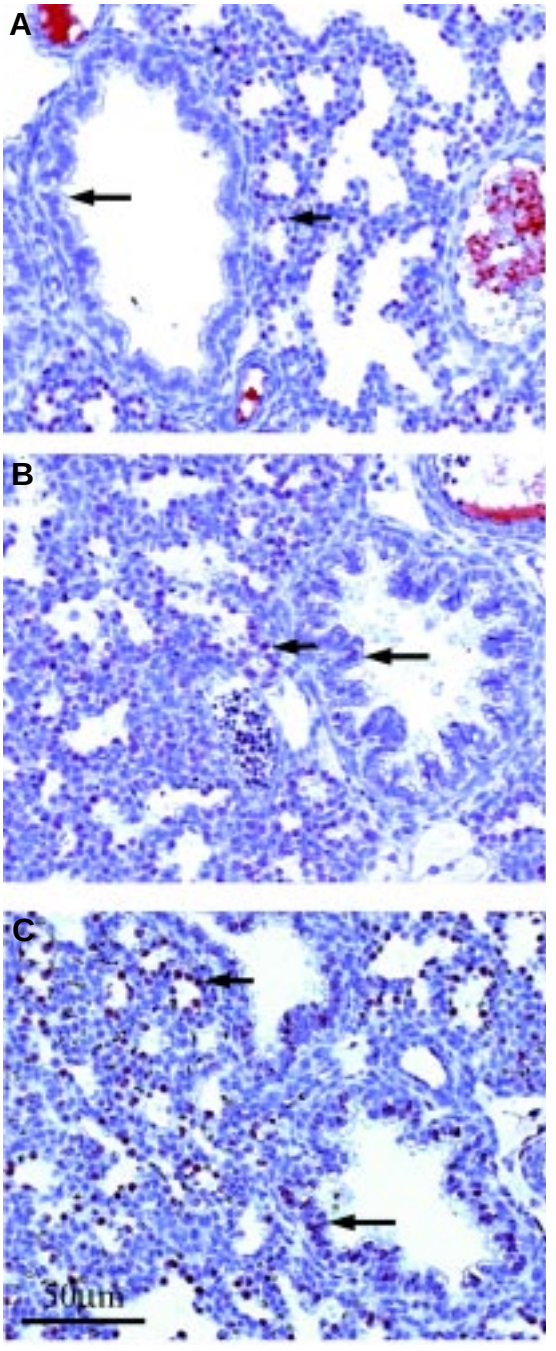

Fig. 6. The gradient of TTF-1 is not maintained in the lung of *mdx:MyoD-/-* ^{9th} embryos. *Paraffin embedded sections of the lung immunostained for TTF-1 show that columnar epithelial cells in the proximal ducts of the lung in wild-type embryos mostly do not express TTF-1; see long arrow in* **(A)**, *in comparison to the adjacent distal ducts (short arrow in A indicates strong bright red nuclear immunohistochemical signal). Similarly, some columnar epithelial cells in the proximal ducts of the lung of* mdx:MyoD+/-^{9th} *embryos express low levels of TTF-1; see long arrow in* **(B)**, *whereas TTF-1 is strongly expressed in the adjacent distal ducts (short arrow in B). In contrast, TTF-1 is strongly expressed in almost all of the columnar epithelial cells in the proximal ducts of the lung of* mdx:MyoD-/-^{9th} *embryos; see long arrow in* **(C)**. *The pattern of TTF-1 expression is indistinguishable between wild-type,* mdx:MyoD+/-^{9th} *and* mdx:MyoD-/-^{9th} *embryos (short arrows in A-C) in the cuboidal cells of the distal lung ducts.*

mdx:MyoD+/-^{9th} embryos (Fig. 6B), but was not maintained in *mdx:MyoD-/-*^{9th} embryos (Fig. 6C). Surprisingly, in *mdx:MyoD-/-*^{9th} embryos, almost all columnar epithelial cells in the proximal ducts were positive for TTF-1 (Fig. 6C). The average percentage of the columnar epithelial cells expressing TTF-1 in the proximal ducts of the lung was 7±2% (wild-type, n=6), 20±3% (*mdx:MyoD+/-*^{9th}, n=4) and 81±2% (*mdx:MyoD-/-*^{9th}, n=3), with statistically significant difference between control and *mdx:MyoD-/-*^{9th} embryos ($P < 0.05$, one-way ANOVA).

Our data revealed no difference in the pattern of TTF-1 expression in the cuboidal epithelial cells of the distal ducts between *mdx:MyoD-/-*^{9th} embryos and their E18.5 embryos. The average percentage of the cuboidal epithelial cells expressing TTF-1 in the distal ducts of the lung was 67±12% (wild-type, n=6), 76±10% (*mdx:MyoD+/-*^{9th}, n=4) and 70±6% (*mdx:MyoD-/-*^{9th}, n=3), with no statistically significant difference among the genotypes ($P > 0.05$, one-way ANOVA).

Together, these data suggest that the absence of an adequate mechanical stimulation (e.g., from the diaphragm) disturbs the proximal-to-distal (or columnar versus cuboidal epithelium) gradient of TTF-1 expression that in turn leads to the development of lung hypoplasia.

Discussion

The aim of this study was to examine *in vivo* the dependence of lung development on the contractile activity of the diaphragm and to provide some insights into the underlying mechanisms that lead to lung hypoplasia in the absence of FBMs. Genetically modified mice and mouse embryos (i.e., *mdx:MyoD-/-*^{9th} embryos) employed in this study provided us with a unique opportunity to investigate the role of FBMs in lung development. Our data suggest that insufficient lung expansion due to the improper development of the diaphragm (and in consequence impairment of FBMs) in *mdx:MyoD-/-*^{9th} embryos leads to lung hypoplasia by decreasing lung cell proliferation and affecting the expression gradient of TTF-1.

Dystrophin and MyoD are clearly lacking in *mdx:MyoD-/-*^{9th} embryos, as reported for *mdx:MyoD-/-*^{1th} mice (Megeny *et al.,* 1996). Importantly, dystrophin protein and MyoD DNA (i.e., the MyoD-*lacZ* transgenes) and protein were not expressed in the lung at any time during development. Moreover, the embryonic lethal phenotype of *mdx:MyoD-/-*^{9th} embryos caused by the impaired diaphragm development and a consequent lung hypoplasia was obtained only after backcrossing mice for nine generations. Furthermore, an absence of gross anatomical and histological abnormalities in the lung of *mdx*, *MyoD-/-* and *mdx:MyoD-/-*^{1st} (1st generation, not backcrossed) mice has previously been reported (Rudnicki *et al.,* 1992; Megeney *et al.,* 1996; Rafael *et al.,* 1999). This is in accordance with our findings that the lack of dystrophin in the diaphragm of *mdx:MyoD+/-*^{9th} mice did not have any effect on lung growth and development. The consequent lung hypoplasia observed in *mdx:MyoD-/-*^{9th} embryos is therefore not due to the absence of the MyoD (Zhang *et al.,* 1999) and dystrophin, but to the decreased availability of muscle fibres in the diaphragm that are insufficient to produce enough mechanical stretch necessary for proper lung development. In support to this statement are the recent findings in *MyoD* null mice, where a significant contractile dysfunction of the diaphragm is reported (Staib *et al.,* 2002).

In addition, our study showed that even though the diaphragm was very thin in *mdx:MyoD-/-^{9th}* embryos, it was always intact across the abdominal wall and no visceral herniation was detected. Consistently, the amount of the amniotic fluid appeared normal in all embryos, suggesting that the cause of lung hypoplasia in *mdx:MyoD-/-:9th* embryos is neither diaphragmatic hernias nor oligohydramnios.

Immunohistochemistry against PCNA revealed that the proliferation index in both the epithelial and the mesenchymal compartments of *mdx:MyoD-/-^{9th}* embryonic lung was significantly reduced in comparison to the wild-type and *mdx:MyoD+/-^{9th}* lung. This pathologic finding is in agreement to what previously reported for *myogenin* null mouse embryos (Tseng *et al.,* 2000). Importantly, the *in vitro* studies show that mechanical forces, especially mechanical stretch, have effects on lung cell proliferation (Liu *et al.,* 1999; Liu and Post, 2000). Moreover, the decreased proliferation index of lung cells is proposed as a probable mechanism for producing lung hypoplasia in the experimentally induced congenital diaphragmatic hernia (Jesudason *et al.,* 2000). Taken together, our data suggest that the abnormal development of the diaphragm (as observed in *mdx:MyoD-/-^{9th}* embryos) and the resulting functional insufficiency of its skeletal muscle compartment consequently generate abnormal FBMs. In term, inadequate FBMs produce an insufficient mechanical stretch that through an unknown mechanochemical signal transduction pathway (Liu *et al.,* 1999; Liu and Post, 2000) causes a decrease in lung cell proliferation and lung hypoplasia.

Our study shows no significant difference in the number of apoptotic cells among the lung tissue (both, the epithelial and the mesenchymal compartment) of wild-type, *mdx:MyoD+/-^{9th}* and *mdx:MyoD-/-^{9th}* embryos. This finding is in agreement with previously reported data (Jesudason *et al.,* 2000; Keijzer *et al.,* 2000) where a decrease in lung cell proliferation, without an increase in lung cell apoptosis, was observed to be the only mechanism leading to lung hypoplasia. By contrast, in *myogenin* null embryos, the number of apoptotic lung cells was higher in comparison to the wild-type embryos (Tseng *et al.,* 2000). The disagreement with our findings may be due to the differences in the experimental models. The diaphragm of *myogenin* null embryos is reported to be thin and fibrous (Tseng *et al.,* 2000), suggesting a total abolishment of functional skeletal muscle (i.e., secondary myogenesis is completely inexistent) and FBMs. By contrast, in *mdx:MyoD-/-^{9th}* embryos, even though the diaphragm is significantly thinner than that of wild-type and *mdx:MyoD+/-^{9th}* embryos, it is not fibrous and contains some striated muscle. It is therefore possible that the available striated muscle fibres in the diaphragm of the *mdx:MyoD-/-^{9th}* embryos produce a sufficient amount of mechanical stretch to prevent activation of a pathway that leads to lung cell apoptosis.

TTF-1 is a homeodomain containing transcription factor expressed in thyroid gland, lung, and brain (Lazzaro *et al.,* 1991). Expression of TTF-1 in the lung is restricted to the epithelium at all stages of development and no expression can be identified in the surrounding mesenchyme (Hackett and Gitlin, 1997). TTF-1 is involved in both morphogenesis of the lung and regulation of the expression of pulmonary surfactant associated proteins (Bohinski *et al.,* 1994; Bruno *et al.,* 1995; Minoo *et al.,* 1995;Yan *et al.,* 1995; Kelly *et al.,* 1996).

Our investigation shows that the pattern of TTF-1 expression in the lung of *mdx:MyoD-/-^{9th}* embryos is dramatically different from what observed in the proximal ducts of wild-type and *mdx:MyoD+/-^{9th}* lung. In wild-type embryos, at the terminal sac stage of lung development (i.e., E18.5), almost none of the columnar epithelial cells in the proximal lung ducts expresses TTF-1. By contrast, TTF-1 is clearly expressed in almost all columnar epithelial cells in the proximal lung ducts of *mdx:MyoD-/-^{9th}* embryos. Similarly, proximal-to-distal gradient of TTF-1 expression is absent in the hypoplastic human lungs (Zhou *et al.,* 2001), whereas it is maintained with the advanced gestational age in the normal human lungs.

Previously, it was reported that in spite of lung hypoplasia in the experimentally induced diaphragmatic hernia in mouse embryos the total amount of TTF-1 mRNA is not decreased (Coleman *et al.,* 1998), suggesting that lung hypoplasia is not necessarily a consequence of a reduction in TTF-1. Indeed, the current study shows no statistically significant difference in the number of distal lung epithelial cells (i.e., cuboidal) expressing TTF-1 among the three examined embryonic genotypes. During mouse lung development, TTF-1 expression starts at E10.5 (Hackett and Gitlin, 1997), while FBMs in mice begin at E14.5 (Abedie *et al.,* 2000). It is therefore clear that the expression of TTF-1 occurs prior to the beginning of FBMs and for this reason FBMs may not have any effect on the early expression of TTF-1. By contrast, it appears that FBMs generated later in development (i.e., by the diaphragm) play a role in the establishment and maintenance of a proper proximal-to-distal expression gradient of TTF-1. Considering the reported importance of TTF-1 in lung development and functional maturation (e.g., TTF-1 is suggested to be involved in surfactant production regulation) (Bohinski *et al.,* 1994; Bruno *et al.,* 1995; Minoo *et al.,* 1995;Yan *et al.,* 1995; Kelly *et al.,* 1996), maintenance of the TTF-1 gradient seems to be an important factor in prevention of pulmonary hypoplasia.

Our next goal is to study the mechanisms employed in generation of pulmonary hypoplasia (i.e., lung growth, epithelial differentiation and lung functional maturation) in the absence of intercostal musculature alone (e.g., *Myf5-/-* knock-out embryos), as well as in the absence of both the diaphragm and the intercostals (e.g., *Myf5-/-:MyoD-/-* compound mutant embryos).

Materials and Methods

Animal Care and Interbreeding

mdx mice (i.e., mice that carry a loss-of-function point mutation in the X-linked *dystrophin* gene and are an animal model for human Duchenne and Becker muscular dystrophy; Sicinski *et al.,* 1989) were bred with *MyoD-/-* mice (Rudnicki *et al.,* 1992) to generate *mdx:MyoD+/-* and *mdx:MyoD-/-* mice. These mice were subsequently backcrossed (i.e., the tenth generation of *mdx:MyoD+/-* mice was bred with the first generation of *mdx:MyoD-/-* mice) until no viable *mdx:MyoD-/-* newborn was detectable. The embryos generated in the described manner were designated as *mdx:MyoD-/-^{9th}* to indicate that they were double-mutant products after nine generations of backcrossing.

The *258/-2.5lacZ* transgenic mice, that carry a construct in which the 258 bp core of the -20 kb *MyoD* enhancer is linked to 2.5 kb of *MyoD* sequence upstream of the transcription start site of the *lacZ* gene (Goldhamer *et al.,* 1995) and the *MD6.0-lacZ* mice, that carry a transgene in which 6.0 kb of *MyoD* sequence upstream from the transcription start site is linked to the bacterial *lacZ* gene (Asakura *et al.,* 1995), were also used.

Embryos and the fetal portion of the placenta were collected by Cesarean section on the required embryonic day (E) and embryos prepared for whole-mount β-galactosidase staining or histology. Ge-

nomic DNA was isolated from the fetal portion of the placenta using the procedure of Laird *et al.* (1991). Embryos were genotyped by Southern analysis (Sambrook *et al.,* 1989) of placental DNA using *lacZ* and *MyoD* specific probes as described previously (Rudnicki *et al.,* 1993). Animal care was in accordance with the institutional guidelines.

β-Galactosidase Staining

β-galactosidase staining was performed as described by Kablar *et al.* (1998). Embryos were fixed 1-2 hours (2% paraformaldehyde, 0.2% glutaraldehyde, 0.1 M phosphate buffer pH 7.4, and 2 mM $MgCl_2$), and washed twice for 30 minutes in solution A (0.1 M phosphate buffer pH 7.4, 2 mM $MgCl_2$, 0.1% sodium deoxycholate, 0.2% Nonidet P-40) and then washed twice, for 30 minutes in solution B (0.1 M phosphate buffer pH 7.4, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.2% Nonidet P-40). β-galactosidase was detected by overnight incubation at 37°C in 1.0 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide. Embryos were washed twice for 1 hour at room temperature in phosphate buffered saline (PBS) and post-fixed in 4% paraformaldehyde overnight (see "Tissue Preparation").

Tissue Preparation

The wild-type (6 embryos), *mdx:MyoD+/-9th* (4 embryos), *mdx:MyoD-/-9th* (3 embryos) and MyoD-*lacZ* embryos (3 embryos per stage and per transgene) were decapitated immediately after Caesarean section recovery. Their lungs were dissected out and fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin (vacuum conditions were routinely used to facilitate the tissue infiltration). Serial 4 μm thick sections were cut with a rotary microtome for immunohistochemistry and hematoxylin-eosin (H&E) staining.

Immunohistochemistry

Cell proliferation was detected by immunolabeling of the sections with mouse monoclonal antibody (Dako) diluted 1:500 against proliferating cell nuclear antigen (PCNA). To detect lung cells expressing TTF-1, mouse monoclonal TTF-1 antibody (Neomarkers) diluted 1:50 was used.

TUNEL In Situ *Detection*

Terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) method was employed to identify the apoptotic cells (R&D Apoptosis Detection Kit). In brief, after protein kinase (15 minutes) and quenching steps, the sections were labelled by biotinylated nucleotides using Terminal deoxynucleotidyl Transferase (TdT) enzyme for 1 hour in a humidity chamber at 37ºC. The incorporated nucleotides were detected using streptavidin solution. The sections were counterstained with hematoxylin.

Morphometry

Lung epithelial and mesenchymal cells with positive reaction against PCNA or TUNEL were counted among 1000 randomly selected cells (i.e., proliferation and apoptotic index respectively) in the epithelial and mesenchymal compartments in serial sections at the magnification of 400x. Nuclei of the cells with positive reaction against TTF-1 were counted in the epithelial lung compartment only.

Statistical Analysis

Data were presented as means ± standard deviation (SD). Statistical significance was considered at $P<0.05$. The statistical analyses were done using SPSS (version 11) program. The values for the three experimental embryos were compared by one-way analysis of variance (ANOVA) with post hoc multiple comparisons using Bonferroni adjustment.

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