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THE INFLUENCE OF GENETIC BACKGROUND ON THE MORPHOLOGY OF THE CARDIOPULMONARY SYSTEM IN TRANSGENIC MICE

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ABSTRACT

Assessment of the influence of genetic background on the phenotype of genetically modified mice is important in evaluating mouse models of human disease. In the present study, we examined the morphological and pathophysiological aspects of the cardiopulmonary system of newborn C57BL/6J and CBA mice transgenic for the *Msx1* gene. We investigated the possible influence of genetic background on the phenotype of these animals. For this, the expression of extracellular matrix components (fibronectin and collagen I) and smooth muscle ∝-actin was studied. Transgenic newborn heterozygous C57BL/6J or CBA mice showed characteristic early (neonatal/perinatal) death and shared common alterations in the cardiopulmonary system. Hematoxylin-eosin stained sections of transgenic newborn C57BL/6J mice showed myocardial infarction and lungs with congestion and hemorrhage. One CBA-derived transgenic newborn showed a few areas of cardiac mineralization while in the remaining mice of this group showed no significant cardiac alterations**.** All CBA-derived transgenic mice showed varying degrees of pulmonary congestion. Similar patterns of extracellular matrix and smooth muscle ∝-actin expression were observed in transgenic newborn mice derived from both strains based on light microscopy of sections stained with Gomori's trichrome, reticulin and picrosirius plus polarization microscopy, as well as with immunohistochemical reactions for fibronectin, type I collagen and smooth muscle ∝-actin. These results indicate that the genetic background influenced the structure of the cardiopulmonary system but not the expression of extracellular matrix components or smooth muscle ∝-actin in C57BL/6J and CBA mice transgenic for *Msx1*.

Key words: Cardiopulmonary, genetic background, morphology, newborn mice, transgenic mouse strain

INTRODUCTION

The genetic background of mice plays an important role in their susceptibility to various disorders [7], and this also applies to genetically engineered strains [6,9,17,23,32- 34]. Transgenic mouse strains have served as useful models for studying gene regulation and developmental and homeostatic functions [29]. Genes may be introduced into inbred strains to search for extratransgenic enhancers or suppressors of disease or for cooperation between gene products [2]. The variability in mutant phenotypes affords an excellent opportunity for identifying unlinked modifiers that affect the phenotype of interest. This is extremely important if the mice are a model for a human disease and the unlinked modifiers suppress the disease phenotype. By mapping and cloning these unlinked modifier genes, it may be possible to identify ways of suppressing the corresponding human disease [2].

In this study, we examined some morphological and pathophysiological aspects of the cardiopulmonary system of neonatal mice derived from a transgenic *Msx1* strain with C57BL/6J and CBA backgrounds. We investigated the possible influence of genetic background on the phenotype of these animals since neonates with C57BL/6J or CBA backgrounds had common, characteristic alterations in their cardiopulmonary system and a similar neonatal or perinatal mortality. The expression of extracellular matrix (ECM) components (fibronectin and collagen I) and smooth muscle ∝-actin was also examined since these proteins contribute to the structural

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integrity and functions of the cardiovascular and pulmonary systems [12,15,31].

MATERIAL AND METHODS

Animals

Transgenic mice obtained by microinjection of DNA into the pro-nuclei of fertilized C57BL/6J eggs were used in this study. The transgene consisted of 4 kb of the *Msx1* promoter sequence driving the bacterial lacZ gene [1]. Seven transgenic lineages were obtained by this approach, with one of them consistently showing a 30% neonatal mortality when backcrossed to wild type C57BL/6J or CBA animals. Six C57BL/6J and seven CBA newborns transgenic mice for *Msx1*, all obtained from different litters were studied in detail. Six healthy wild type newborn mice (three C57BL/6J and three CBA) were used as controls. All of the control mice were killed with CO_2.

Screening by PCR

The mice were genetically screened using PCR and DNA obtained from tail tissue by a standard procedure [11]. The LacZ gene was amplified using two primers: 5Lac (sense), GCA TCG AGC TGG GTA ATA AGC GTT GGC AAT (2303-2332 nt) and 3Lac (antisense), GAC ACC AGA CCA ACT GGT AAT GGT AAT GGT AGC GAC (3095-3124 nt). The *Msx1* gene was amplified with the primers *Msx1*- D730 (sense), GCG GAA TTC TCC AGC TGC CTC AGC CTC ACC (730-759 nt) and *Msx1* – R1345 (antisense), TTP GGC CTC TGG TCT CCT TCA GCC TCT QQT (1345-1374 nt). The amplification conditions consisted of 40 cycles of 94ºC for 1 min, 65ºC for 1 min and 72ºC for 2 min.

Histological analysis

Tissue samples for light microscopy were collected from mice that characteristically experienced neonatal or perinatal death after cardio respiratory dysfunction. Histological analysis of the cardiac and pulmonary systems of transgenic newborn mice was done in formalin-fixed, paraffin-embedded (FFPE) sections stained with hematoxylin/eosin (HE), Gomori's trichrome (G), reticulin (R) and picrosirius plus polarization microscopy (PP). The stains were based on standard protocols [13,27].

Immunohistochemistry

Immunohistochemistry for fibronectin (FN), type I collagen (CI) and smooth muscle ∝-actin (SMA) was done using the streptavidin-biotin-peroxidase method [5] in formalin-fixed, paraffin-embedded tissue sections (4 µm thick), mounted on poly-L-lysine- coated microscope slides. The sections were deparaffinized and rehydrated through xylene and graded alcohols. A diluent with background reducing components (Code S 3022, Dako Corp., Carpinteria, CA, USA) was used for all the antibodies. The tissue sections were incubated in a humidified chamber.

FN was analysed using a rabbit anti-human FN polyclonal antibody (Code A 0245, Dako Corp.). Endogenous peroxidase was blocked with 10% H_2O_2 diluted in 70% methanol. Antigen retrieval by proteolytic digestion was achieved by incubating the slides for 10-15 min at 37ºC in 1% trypsin (T-81200, Sigma Chemical Co., St. Louis, MO) diluted in 0.05 M Tris-HCl, pH 7.6. The slides were incubated overnight (4-8ºC) with a freshly prepared solution of 1% non-fat powder milk containing 1% bovine serum albumin (A-2153, Sigma Chemical Co.) diluted in 0.5 M Tris-HCl, pH 7.6 to block endogenous immunoglobulins (nonspecific binding). The milk solution was discarded and the slides then incubated (without washing) with the primary antibody (diluted 1:1500) at room temperature (RT) for 35 min. Incubation with the secondary biotinylated goat anti-rabbit antibody (Code E 0432 Dako Corp.; diluted 1:200) was also for 35 min (RT) after washing the slides twice by immersion in 0.5 M Tris-HCl, pH 7.6 for 10 min. The slides were subsequently washed twice in 0.5 M Tris-HCl, pH 7.6 for 10 min prior to incubation with an avidin-biotin-horseradish peroxidase conjugate (Strept ABC complex / HRP Code K 0377, Dako Corp.) for 35 min. FN was visualized with the chromogen 3,3'-diaminobenzidine (DAB, Code K 3465, Dako Corp.). Negative control sections were processed simultaneously as described above but with the first antibody being replaced by 0.5 M Tris-HCl, pH 7.6. None of the negative controls showed immunoreactivy to FN. The sections were counterstained with Mayer's hematoxylin, dehydrated in a graded alcohol series, cleared in xylene and coverslipped.

CI immunodetection was done essentially as for FN, but with the following modifications: 1) antigen retrieval was achieved by immersing the sections in a target retrieval solution (Code S 1699, Dako Corp.) at 95ºC in a water bath for 30 min, 2) endogenous immunoglobulins (nonspecific binding) were blocked by incubation for 3-4 h instead of overnight, and 3) the primary antibody used a rabbit anti-type I collagen polyclonal antibody (Cat nº 2150-0020, Biogenesis Inc., Kingston, USA; diluted 1:50) with overnight incubation.

For immunodetection of smooth muscle ∝-actin (SMA), antigen retrieval was done by incubating with target retrieval solution as described for CI. An Animal Research Kit (ARK, code K 3954, Dako Corp.) was used in conjuction with a diluted (1:100) mouse anti-human SMA monoclonal antibody (Clone 1 A4, code M 0851 Dako Corp.) for the detection of SMA in mice tissue.

RESULTS

Mice with neonatal or perinatal death were heterozygous for the transgene and showed a high number of in "tandem insertions" and low levels of *LacZ* expression (data not shown). Neonates with a C57BL/6J or CBA background had symptoms of cardiorespiratory dysfunction before death. Mice with a C57BL/6J background died 1-2 h after birth whereas those with a CBA background died within 24 h of birth.

Morphological aspects of neonates with a C57BL/6J background

Gross examination of all heterozygous neonates derived from the C57BL/6J strain showed abundant

bleeding in the thoracic region (Fig. 1A). HE-stained sections showed severe alterations in the heart, including areas of hemorrhage near the apex in the left ventricle. The right ventricle was more severely compromised in the outer wall, with diffuse hemorrhage, a region of coagulation necrosis and a spreading infiltration of mononuclear inflammatory cells (Fig. 1B). The adjacent adipose tissue also showed hemorrhage. No alterations were observed in the right and left atria in HE sections.

Histological analysis of the pulmonary system of these mice revealed diffuse hemorrhage and congestion (Fig. 1C). Erythrocytes were seen in the trachea, alveolar sacs and bronchioli. The blood vessels appeared congested.

Morphological aspects of neonates with a CBA background

In heterozygous mice with a CBA background the alterations were most evident in pulmonary system. Six out of seven of these neonates showed no significant cardiac alterations based on examination of HE-stained sections (Fig. 1D). One neonate had a few areas of cardiac mineralization represented by basophilic granular material in degenerated myofibers and an infiltrate of mononuclear inflammatory cells. All of these mice showed varying degrees of pulmonary congestion (Fig. 2A). Erythrocytes were observed within alveolar sacs in only three of these CBA-derived neonates.

Expression of ECM components and immunohistochemistry

Despite the alterations observed in HE sections of C57BL/6J- and CBA-derived mice, the ECM components were generally well-defined in the heart and lungs of these newborn mice, as seen in sections stained with Gomori's trichrome, reticulin, and picrosirius plus polarization microscopy (Fig. 2A-F).

Histological sections stained with Gomori's trichrome (Fig. 2A,B) showed a well-defined conjunctive tissue stained in green/blue and muscle and endothelial layers stained in red, with a similar distribution among mice of both backgrounds. The interstitial distribution of ECM components in the heart and lungs of mice of both backgrounds was similar to that of wild type neonatal mice (not shown). The ECM was expressed in the pericardium and epicardium of the atria and ventricles and in the atrioventricular valves (Fig. 2B), but was more discrete in the interventricular septum and among cardiac muscle fibers. The conjunctive tissue of coronary vessels of ventricular walls was also seen. In arteries, the conjunctive tissue stained in green/blue was delimited by red rings and anchored the intimal, medial and adventitial layers. In veins, the conjunctive tissue was expressed in the adventitial layer. The basement membrane of the blood vessels was well- defined in green.

As with the cardiac system, the pulmonary system (Fig. 2A) also showed a similar pattern of ECM expression in mice of both backgrounds as visualized by Gomori's trichrome stain, despite the alterations seen in HE sections. This pattern of staining was also similar to that of wild type newborn mice (not shown). Histological sections of the pulmonary system of all mice showed a thin layer of conjunctive tissue (green) anchoring the internal mucosal layer of the trachea surrounding the chondrocytes (stained in red). ECM was also expressed in the basement membranes of tracheal rings and blood vessels, in delimiting bronchioles, and discretely in pleural membranes, bronchi and alveolar septa.

Sections stained for reticulin showed black brownish stained reticular fibers in the pericardium, around cardiomyocytes and in the adventitial layer of blood vessels from the cardiovascular system of mice from both backgrounds. In the respiratory system, reticular fibers surrounded the tracheal rings, perichondrium, pleural membrane, basement membrane of the bronchioli, and the walls of the alveoli. Figure 2C shows the reticular fibers in a lung section from a CBA-derived mouse.

In the hearts of all mice, the birefringency of the collagen fibers (in red and green) in PP revealed the presence of these fibers in the pericardium and atrioventricular valves, but not in the endocardium. The labeling of collagen fibers was discrete, except for the regions of hemorrhage and necrosis. The atrioventricular valves contained collagen in fibrillar form. Collagen fibrils were seen in the adventitia of large blood vessels but not in all layers of small blood vessels of the cardiopulmonary system, as well as in the basement membrane of the tracheal cartilage and in chondrocytes, stroma and pericondrium of all mice.

Immunohistochemical staining for FN was seen in small and large blood vessels of the heart (Fig. 2D) and lungs of mice of both backgrounds. FN was present in the basement membranes of blood vessels, as well as in the intimal layer of veins, and intimal and medium layers of arteries. Cardiac FN was evi-

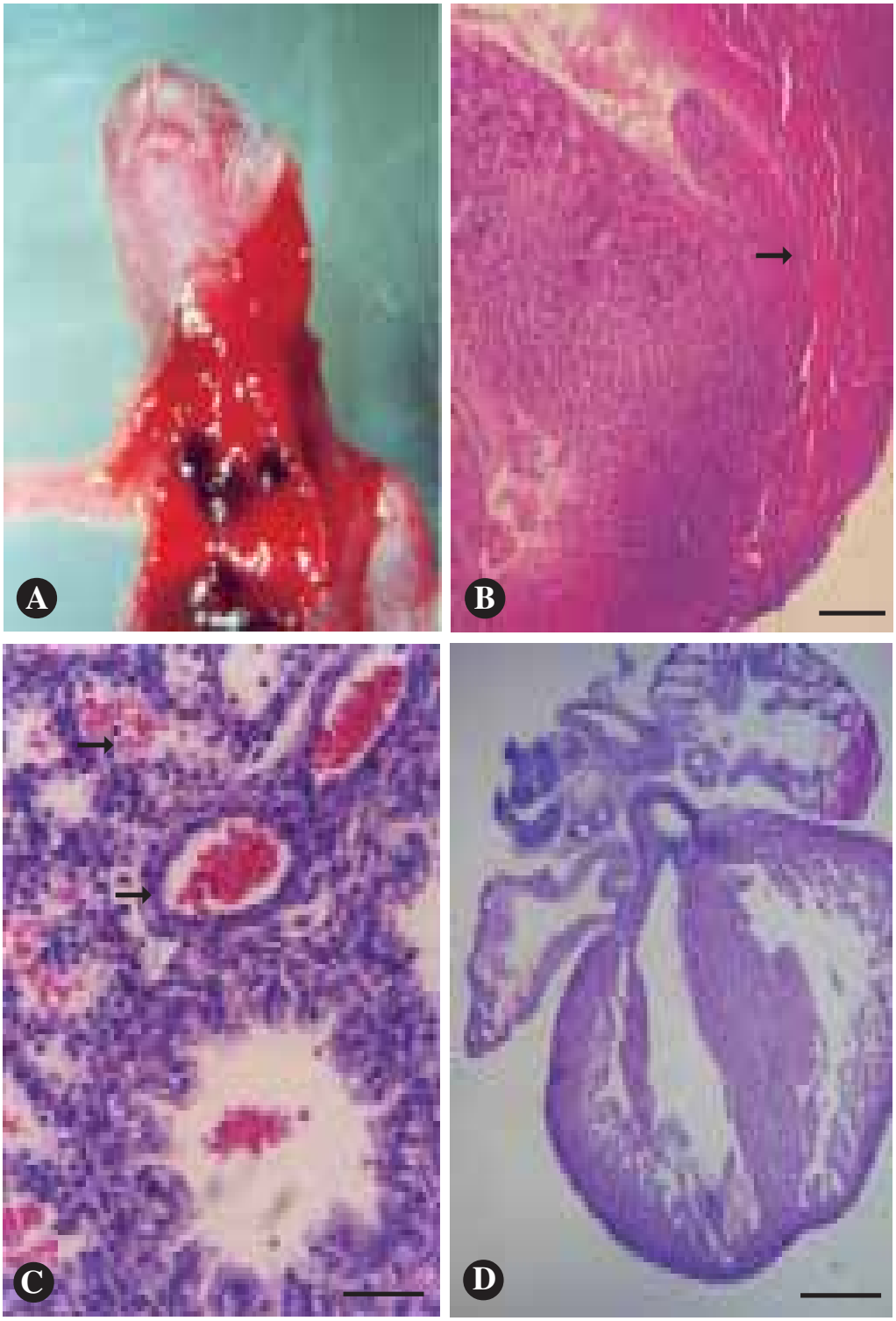

Figure 1 A-D. Macroscopic appearance and histology of heart and lung sections of newborn C57BL/6J and CBA mice transgenic for *Msx1*. **(A)** Macroscopic appearance of a transgenic C57BL/6J mouse showing a ventral view of the thoracic region with profuse hemorrhage. **(B)** Heart section from mouse in Figure 1A showing profuse hemorrhage in the ventricule with regions of coagulation necrosis (arrow). (Bar = 100 μm). **(C)** Lung section from the mouse in Figure 1A showing congestion and hemorrhage (arrows) (Bar = 30 μm). **(D)** Heart section from a transgenic newborn CBA-derived mouse. (Bar = 300 μm).

dent at the atrioventricular valves and discrete in the pericardium, endocardium and interstitium. FN was expressed in blood vessels, bronchi, alveolar septum and pleura in the lungs.

CI was expressed in the cardiac septum, pericardium, and atrioventricular valves (Fig. 2E), as well as in the walls of the bronchioli and pleura. CI was not observed in capillaries or in the basement membrane of blood vessels, but was detected in the medial and adventitial layers of large blood vessels .

SMA was well expressed in the medial layer of small and large blood vessels of the heart and lung (Fig. 2F), and in the muscle layer of bronchioli from mice of both genetic backgrounds.

The smooth muscle cells were as well preserved as in healthy wild type newborn mice (not shown).

DISCUSSION

The effects of genetic background on phenotypic variation

An influence of genetic background on phenotype has been reported for knockout [9,32,34] and transgenic [23,33] mice and has been documented in studies of tumor phenotype [23], embryonic lethality [17,34], epithelial defects [32], carcinogenesis [9,33], and developmental defects [7].

In our study, the variability in phenotype between C57BL/6J- and CBA-derived mice could reflect the influence of genetic background, with the former mice having a more aggressive phenotype pattern than the latter. Neonates derived from the C57BL/6J strain died within 1-2 h of life and had much more severe cardio respiratory dysfunctions than CBA-derived mice which died within 24 h of birth. Interestingly, Msx1 deficient mice also died more rapidly (within a few hours after birth) when backcrossed with C57BL/6J mice compared with the homozygotes from a mixed genetic background [11].

As shown here, the heart and lung lesions in newborn mice derived from the C57BL/6J strain were more severe than in the mice derived from CBA background. The lesions in the latter occurred predominantly in the respiratory system, except for one mouse which had pulmonary congestion and cardiac mineralization. Mineralization in the heart of mice has generally been considered to be indicative of dystrophic calcification within the myocardium or epicardium and is a genetically determined trait with marked variability between mouse strains [21]. Since inbred mouse strains with a high incidence of cardiac calcification include DBA/2, C, C3H, Balb/c A, CHI, and CBA [35], it seems most likely that the cardiac mineralization seen in this one newborn mouse reflected its genetic background.

Pathophysiological and histopathological aspects

Neonatal and perinatal death often result from congenital defects such as cardiovascular and lung malformations. Cardiovascular anomalies may occur singly or in combination and, although compatible with intrauterine life because of the fetal circulation [3], they tend to be fatal for life outside the uterus when heart function is compromised [36].

Cardiac and/or pulmonary alterations could have contributed to the death of present newborn mice. Pulmonary congestion process can be consequence of varied degrees of cardiovascular alterations [3,10,14]. However no heart dysfunctions which could have contributed to the congested lungs in CBA-derived mice were observed by light microscopy. In addition, no vascular anomalies, such as stenosis, fibrosis, vasculitis, and hypertrophy, which can alter the expression of ECM and SMA in blood vessels, were detected.

In pathophysiological states such as myocardial infarction, interruption of the blood supply leads to the rapid death of cardiomyocytes in the affected part of the cardiac wall. In adult mammals, cardiomyocytes are terminally differentiated cells that have lost the ability to divide [4]. As a result, wound healing and the replacement of necrotic cardio-myocytes occurs via the deposition of ECM (fibronectin, collagen I) in the wound area. The granulation tissue formed in the infarcted area eventually matures into a scar [4]. In neonatal mice, the cardiomyocyte retain some ability to divide. In most mammals terminal differentiation of cardiac myocytes occurs at, or shortly after, birth and is characterized by a transition from hyperplastic growth (cell division) to hypertrophic growth (an increase in cell size) [18]. The ability of cardiomyocyte to divide may persist for 3-4 days after birth in wild type newborn mice [26]. The age at which our newborn mice suffered myocardial infarction could explain the absence of extracellular matrix deposition in the wound area (Fig. 2B).

ECM and smooth muscle ∝-actin (SMA)

Several studies have shown the significance of cellular interactions with the ECM in physiological and pathological processes [8,12,15,22,28,30]. The

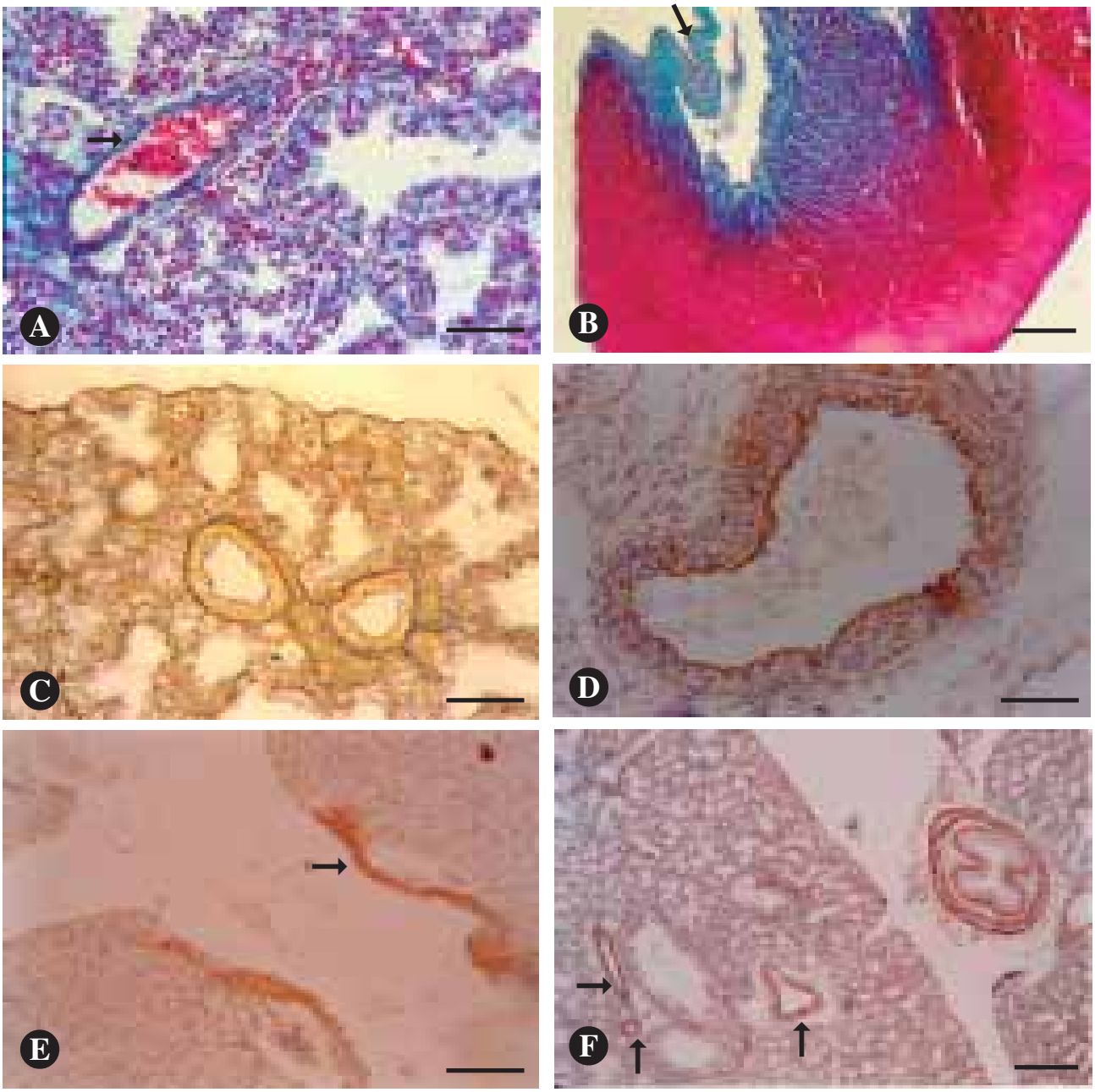

Figure 2 A-F. Expression of ECM components and SMA in heart and lung sections of newborn C57BL/6J and CBA mice transgenic for *Msx1*. **(A)** Lung section from a transgenic, newborn CBA mouse showing congested blood vessel (arrow) and the distribution of ECM components stained in green/blue (Gomori's trichrome; Bar = 30 μm). **(B)** Heart section of transgenic, newborn C57BL/6J mouse showing the distribution of ECM components stained in green/blue. Arrow shows the atrioventricular valve (Gomori's trichrome; Bar = 100 μm). **(C)** Lung section from a transgenic, newborn CBA mouse showing the distribution of reticular fibers (black) in the alveolar walls, (Reticulin stain Bar = 30 μm). **(D)** Large heart blood vessel (transgenic, newborn CBA mouse) immunostained with anti-fibronectin antibody (Mayer's hematoxylin counterstain; Bar = 30 μm). **(E)** Heart section (transgenic, newborn CBA mouse) showing atrioventricular valve (arrows) immunostained with anti-type I collagen antibody (Mayer's hematoxylin counterstain Bar = 30 μm). **(F)** Lung section (transgenic, newborn CBA mouse) immunostained with anti-SMA antibody (arrows), (Mayer's hematoxylin counterstain Bar = 100 μm).

ECM provides proteins for structural support and also facilitates the exchange of information among cells, thereby modulating processes such as development, cell migration, attachment, and repair. The extracellular matrix plays a crucial role in wound healing through its chemotactic, opsonic, and attachment properties [30]. As reviewed by Ronguish *et al.* [28], the matrix provides extracellular signaling molecules which regulate intracellular events, such as growth and differentiation, through interactions between their receptors, the cytoskeleton, and intracellular signalling molecules. Matrix macromolecules can regulate key functions of cells, and the composition of the cardiovascular ECM is under strict control. The control mechanisms involved produce long-term changes in tissue structure, but can also be very rapid, and cause almost immediate changes in cell behavior [19].

FN expression was similar in mice of both genetic backgrounds and similar to that of wild type mice [24,25]. A role for FN in heart and blood vessel morphogenesis has been proposed [8]. As in neonate wild type rat hearts [28], no CI was observed in capillaries or the basement membrane of blood vessels in both groups of transgenic mice. The expression of CI in our mice was identical to previous reports of its presence mainly in the adventitia of large diameter vessels compared to capillaries, and its occurrence in septa and epicardium. The pattern of ECM revealed by the special staining methods used was similar in mice of both genetic backgrounds.

Actin is a multifunctional protein that plays a fundamental role in a wide variety of cellular processes, including contractility, maintenance of the cytoskeleton, cell division, cell motility, and muscle contraction [16]**.** Smooth muscle ∝-actin is an important cytoskeletal filament that has a central role in regulating vascular contractility and blood pressure homeostasis [22,31]. Smooth muscle cells (SMC) of the vascular wall, bladder, myometrium, gastrointestinal and respiratory tracts retain the ability to proliferate postnatally, which enables adaptive responses to injury, hormonal, or mechanical stimulation [20]. As with ECM, the expression of SMA was similar in mice of both backgrounds. Despite the ability of SMA to undergo remodeling, the SMC of the transgenic mice were as well preserved as in healthy, wild type neonate mice (not shown).

The results of this study revealed phenotypic differences in the cardiopulmonary system among newborn C57BL/6J and CBA mice transgenic for *Msx1*. However, the pattern of ECM and SMA expression was similar in the groups of transgenic and wild type mice. The lesions in CBA-derived mice involved predominantly the pulmonary system and were less aggressive than in C57BL/6J-derived mice in which all animals had myocardial infarction and congestion and hemorrhage of the pulmonary system. These observations indicate that genetic background influenced the cardiopulmonary system but had no effect on the expression of ECM and SMA.

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