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Microarray analysis of *Myf5-/-:MyoD-/-* hypoplastic mouse lungs reveals a profile of genes involved in pneumocyte differentiation

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Summary. Fetal breathing-like movements (FBMs) are important in normal lung growth and pneumocyte differentiation. In amyogenic mouse embryos (designated as *Myf5-/-:MyoD-/-*, entirely lacking skeletal musculature and FBMs), type II pneumocytes fail to differentiate into type I pneumocytes, the cells responsible for gas exchange, and the fetuses die from asphyxia at birth. Using oligonucleotide microarrays, we compared gene expression in the lungs of *Myf5-/-:MyoD-/-* embryos to that in normal lungs at term. Nine genes were found to be up-regulated and 54 down-regulated at least 2-fold in the lungs of double-mutant embryos. Since many down-regulated genes are involved in lymphocyte function, immunohistochemistry was employed to study T- and B-cell maturity in the thymus and spleen. Our findings of normal lymphocyte maturity implied that the down-regulation was specific to the double-mutant lung phenotype and not to its immune system. Immunostaining also revealed altered distribution of transcription and growth factors (SATB1, c-Myb, CTGF) from down-regulated genes whose knockouts are now known to undergo embryonic or neonatal death secondary to respiratory failure. Together, it appears that microarray analysis has identified a profile of genes potentially involved in pneumocyte differentiation and therefore in the mechanisms that may be implicated in the mechanochemical signal transduction pathways underlying FBMs-dependent pulmonary hypoplasia.

Key words: Pneumocyte differentiation, Pulmonary hypoplasia, Mouse embryo, Myf5 and MyoD, cDNA microarray

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Introduction

The development of a functional lung is a process subject to various factors at different stages. The endodermal lung buds evaginate ventrally into the neighboring mesenchyme (Cardoso, 2000) and subsequently undergo four successive developmental stages: the pseudoglandular, canalicular, saccular and alveolar (Ten Have-Opbroek, 1981). During the psuedoglandular stage, the primitive lung appears like a gland made up of acinar tubules (Ten Have-Opbroek, 1981), later developing a definitive blood supply and conductive airways in the canalicular stage (Laudy and Wladimiroff, 2000). The saccular stage depicts the formation of large smooth-walled airspaces or saccules (Laudy and Wladimiroff, 2000), which will give rise to alveoli after birth (Ten Have-Opbroek, 1981). Lung development is composed of growth (increase in size with structural specialization) as well as maturation (cellular and functional differentiation) but, whereas maturation mainly relies on hormonal factors, lung growth appears to depend largely upon physical factors and mechanical forces, which affect cell cycle kinetics and cell differentiation (reviewed by Liu and Post, 2000; Inanlou et al., 2005).

Mechanical forces caused by intermittent respiratory-like movements due to the contractile activity of respiratory muscles in utero, called fetal breathing-like movements (FBMs), appear to have the principal role in this process (Inanlou and Kablar, 2003, 2005a). FBMs are produced with varying frequency and amplitude by rhythmic contractions of the respiratory muscles and are responsible for intermittently reducing intrathoracic pressure and distending the fetal lung. The movements result from neuronal activities of the respiratory center in the brainstem that are transferred to the respiratory muscles (Harding, 1997), and are detectable at embryonic day (E) 14.5 in the mouse (Abadie et al., 2000) and at 10 weeks gestation in human embryos (de Vries et al., 1986). Absence of FBMs

impairs lung growth and leads to pulmonary hypoplasia, which is the most common single autopsy finding in the first week after birth (Nakamura et al., 1992). Hypoplastic lungs appear as small organs with a less than normal wet lung weight to body weight ratio and showing histologic immaturity with fewer and smaller peripheral airspaces, giving the lungs an appearance of being arrested at earlier stages of lung development (Porter, 1998). Importantly, a major feature of the development of pulmonary hypoplasia seems to be the inability of type II pneumocytes to successfully differentiate into type I pneumocytes, the lung cell type responsible for gas exchange, as observed in *Myf5-/-:MyoD-/-* mouse embryos that develop in the complete absence of FBMs (due to the lack of ribs and skeletal muscle) and die from asphyxia at birth (Inanlou and Kablar, 2005b). Indeed, whereas the absence of FBMs has been associated with decreased proliferation and increased apoptosis of pulmonary cells in the hypoplastic lungs (Tseng et al., 2000; Inanlou and Kablar, 2003, 2005a,b), which are in part controlled by mediators including thyroid transcription factor-1 (TTF-1), platelet derived growth factors (PDGFs) and insulin-like growth factors (IGFs), (Pledger et al., 1977; Baxter, 1988; Barres et al., 1992; Harding et al., 1993; Hooper et al., 1993; Liu et al., 1995; Hackett and Gitlin, 1997; Joe et al., 1997; Desai and Gruber, 1999; Zhou et al., 2001; Inanlou and Kablar, 2005a,b), FBMs are, in addition to lung growth, also required for lung maturation, which relies on cell differentiation. The lung is composed of different cell types including Clara cells and pneumocyte types I and II. Biochemical differentiation of lung cells is defined by the expression of their specific markers, and the normal expression of the relevant markers in pulmonary hypoplasia caused by the lack of FBMs indicates that the early embryonic differentiation of these cell types is independent of mechanical forces (Tseng et al., 2000; Inanlou and Kablar, 2005a,b). However, the final differentiation (at term and after) of both type I and II pneumocytes is now largely believed to be dependent on mechanical stimuli, since the accomplishment of morphological differentiation of the two cell types is not possible in the complete absence of respiratory activity (Nagai et al., 1988; Benachi et al., 1999; Inanlou and Kablar, 2005a,b).

Maturation of type II pneumocytes is associated with a decrease in the cytoplasmic glycogen that acts as a substrate for the formation of surfactant-associated proteins and phospholipids as well as a simultaneous increase in the number of lamellar bodies, the intracellular organelles required for assemblage and storage of surfactant (Chi, 1985; Ten Have-Opbroek et al., 1990; Batenburg, 1992). In the lungs of fetuses lacking FBMs, type II cells are unable to utilize glycogen for the synthesis of surfactant, and the number of cytoplasmic lamellar bodies is significantly reduced while the intra-alveolar lamellar bodies are scarce, loose and disorganized (Nagai et al., 1988; Brandsma et al., 1993; Inanlou and Kablar, 2005b). Even the tubular myelins that act as intermediate structures in the formation of a phospholipid monolayer on the alveolar surface are difficult to find in these hypoplastic lungs, and their structure, too, appears loose and disorganized (Inanlou and Kablar, 2005b). These findings suggest that type II cells are unable to complete their morphological differentiation, leading to defects in the synthesis, assemblage and secretion of surfactant. Type I pneumocytes, on the other hand, are morphologically characterized by a flattened nucleus and an extended cytoplasm containing numerous small well-defined vesicles (Williams, 1990). In the absence of FBMs, no cells with these characteristics are found in the hypoplastic lung and, instead, cuboidal cells without well-defined vesicles are observed (Inanlou and Kablar, 2005b), indicating that type I cells are also unable to complete their morphological and functional differentiation. However, the mechanochemical signal transduction pathways that translate mechanical stimuli to meaningful gene instructions for the final cell differentiation have yet to be identified.

Therefore, in this study, we used Affymetrix GeneChip cDNA microarray analysis to compare gene expression in the hypoplastic lungs of *Myf5-/-:MyoD-/-* (double-mutant or amyogenic) embryos to the lungs of normal wild-type control embryos. By this approach, it is possible to perform molecular comparisons (e.g., the type of gene and the amount of that gene expressed) between the double-mutant and wild-type tissues (Schena et al., 1995). One potential pitfall of working with a tissue composed of different cell types, like the lung, is the difficulty of attributing any variation in gene expression to a specific cell type. However, in the case of *Myf5-/-:MyoD-/-* double-mutants, previous analysis has shown that as far as the lung is concerned, the only difference from wild-type embryos is the failure of type I pneumocyte differentiation (Inanlou and Kablar, 2005b). Of course, type II pneumocytes also have some difficulties in finishing their differentiation program. However, unlike type I cells, type II cells are clearly present in the lungs of double-mutants. Therefore, for this study, it can be assumed that most gene expression changes resulted from the absence of differentiated type I pneumocytes, thus providing a profile of genes specific for those missing cells. The identification of candidate molecules responsible for pneumocyte I differentiation (and therefore for functional maturation of the lung) will suggest follow-up studies aimed at increasing our understanding of the molecular processes leading to pulmonary hypoplasia, an important cause of neonatal morbidity and mortality, and the findings from such studies should have an impact on expectations for medical prevention and treatment of pulmonary hypoplasia.

Materials and methods

Animal breeding and fetal collection

Double-mutant (*Myf5-/-:MyoD-/-*) fetuses were obtained by the interbreeding of heterozygous (*Myf5+/-*

:MyoD+/-) parents, as previously described (Rudnicki et al., 1993). All fetuses were collected by Cesarean section at embryonic day (E) 18.5 and genotyped by PCR using *Myf5* and *MyoD* primers (Inanlou and Kablar, 2005b). In addition, the presence or absence of skeletal muscle was confirmed by myosin-fast immunostaining (data not shown). Animal use and care was in accordance with all institutional guidelines.

RNA Isolation, RT-PCR Amplification

Total lung RNA was isolated using the RNeasy^{TM} kit from Qiagen, Mississauga, Ont., Canada, according to the manufacturer's instructions. From each group (wild-type or double-mutant), RNA from two embryos was pooled. Fluorescent labeling of cRNA fragments obtained from the pooled samples and their simultaneous hybridization to MOE340 GeneChip mouse genome arrays was performed at the Ottawa Genome Centre according to standard Affymetrix (Santa Clara, CA) protocols as in Seale et al., 2004. The hybridized chips were then scanned and the results analyzed using the Affymetrix statistical expression algorithms to obtain the expression ratios and fold changes between the wild-type and double-mutant embryo lungs. An examination of gene relationships using PubGene at that time revealed that a number of the differentially expressed genes were known to interact with one another (Fig. 1), suggesting that a coordinated regulation of gene expression and protein function is likely occurring. As is common (Iida and Nishimura, 2002), we used a more traditional gene expression assay as a way of ensuring that the microarray data was reliable. Thus, to confirm the differential mRNA expression and direction of change in expression (up- or down-regulation in double-mutant lungs), total RNA from the lungs of five fetuses in each group was individually reverse-transcribed with M-MLV reverse transcriptase (Promega, Madison, WI) and amplified using the primers and conditions listed in Table 1. All PCR products were obtained within the linear range of the reaction. DNA levels were normalized against the QuantumRNA^{TM} 488 bp 18S ribosomal RT-PCR product (Ambion, Austin, TX) amplified from the same RT reaction, as previously described (Baguma-Nibasheka et al., 2005). These expression data were then compared using a t-test (wild-type versus double-mutant) with differences of $P<0.05$ considered significant. The results (Fig. 2) confirmed the direction of change for the tested genes: *Sdc4*, *Ramp2* and *Krt2-4*, up-regulated, and *Cd3g*, *Rag1 and Tcrb-V13*, down-regulated.

Table 1. Conditions for PCR of transcripts used to verify gene expression differences.

Target	Primer Sequence[a], 5' to 3'	Annealing Temp. (°C)	Product Size (bp)
Sdc4	f: ACC TCC TGG AAG GCA GAT ACT T r: AAC TGG AAG AGA ATG AGG TCA TTC	60	294
Ramp2	f: GCT GTT ACT GCT GCT GTT GC r: GTC TGC CTC GTA CTC CAA GC	63	247
Krt2-4	f: GAA TGC AAG AGT GCT GTG AG r: GGA GTT TCT GCT CTT CAT CC	55	479
Cd3g	f: GAG CAG AGG AAG GGT CTG GCT r: CTT CTT CCT CAG TTG GTT TCC	60	543
Rag1	f: CCA AGC TGC AGA CAT TCT AGC ACT C r: CAA CAT CTG CCT TCA CGT CGA TCC	60	562
Tcrb-V13	f: CAA GGG GCT GGG TGT GGA AT r: GGG AGG GAG GGA GGG AAA GA	58	539

[a] f = forward, r = reverse.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Inanlou and Kablar, 2005a,b) on paraffin-embedded 4 µm sections, with monoclonal mouse anti-CD3, polyclonal rabbit anti-c-Myb and polyclonal goat anti-CTGF, anti-SATB1, and anti-mouse IgM. All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) except anti-mouse IgM (Serotec, Raleigh, NC), and were used at 40 µg/ml, followed by a

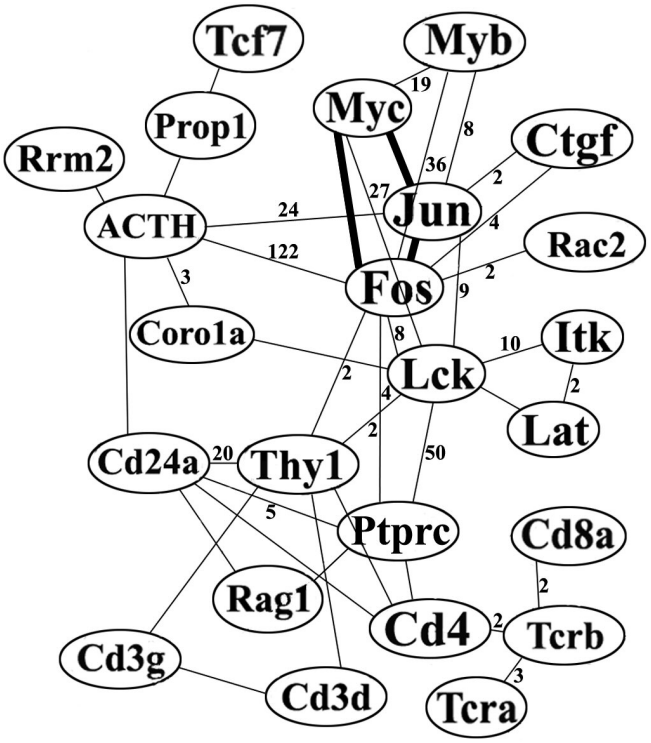

Fig. 1. Relationship of differentially expressed genes. PubGene interaction map, generated from genes with altered expression in the *Myf5-/-:MyoD-/-* double-mutant fetal mouse lung, identified the network of gene interactions pictured. The list of differentially expressed genes was augmented with the following entities to create the cluster: ACTH, Cd24a, Cd4, Fos, Jun, Myc, Prop1, Tcrb. Numbers are the published papers in excess of one linking each two genes (Fos, Jun and Myc were each interlinked by more than 500 publications).

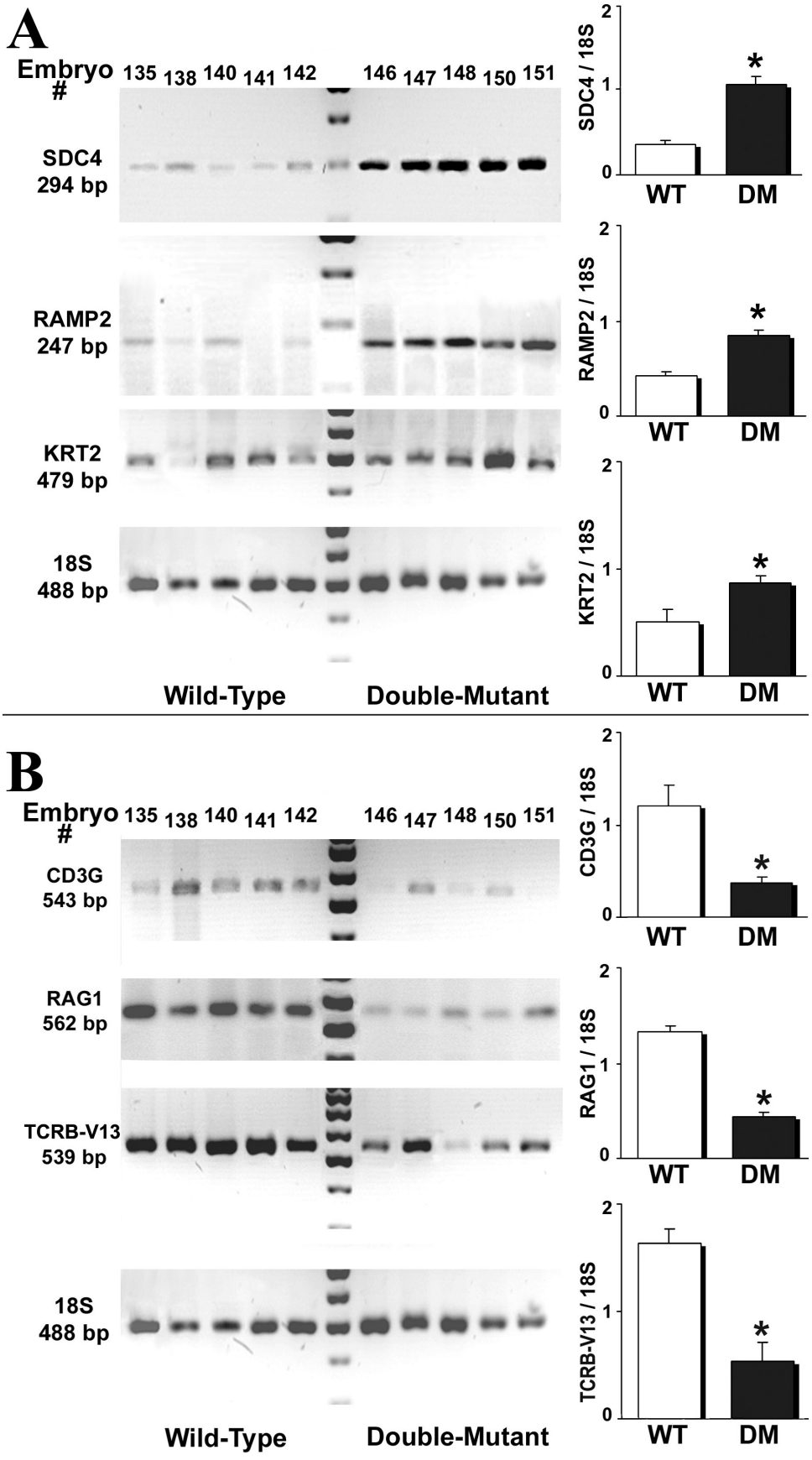

Fig. 2. RT-PCR verification of microarray findings. RT-PCR confirmation of differential gene expression (up-regulated in **A**; down-regulated in **B**) in the lungs of double-mutant (DM) amyogenic embryos. Graphs plot expression relative to 18S rRNA, mean ± SEM, n=5. *: significantly different from expression in wild-type (WT) embryos, $P<0.05$.

hematoxylin counterstain. Control staining to eliminate antibody non-specificity was performed by application of secondary antibodies without prior exposure of the cells to the primaries.

Results

Gene expression is altered in E18.5 Myf5-/-:MyoD-/- lung

As previously mentioned, we compared the lungs of embryos that contain no type I pneumocytes (the lungs of *Myf5-/-:MyoD-/-* embryos, which develop FBMs-dependent pulmonary hypoplasia) to the lungs of normal control embryos, using oligonucleotide microarray analysis. By this approach, it was possible to perform molecular comparisons (e.g., the type of gene and the amount of the gene expressed) between the mutant (i.e., *Myf5-/-:MyoD-/-*) and the control (wild-type) tissues. Molecules that were not present in the mutant lung were assumed to be specific for the lacking type I

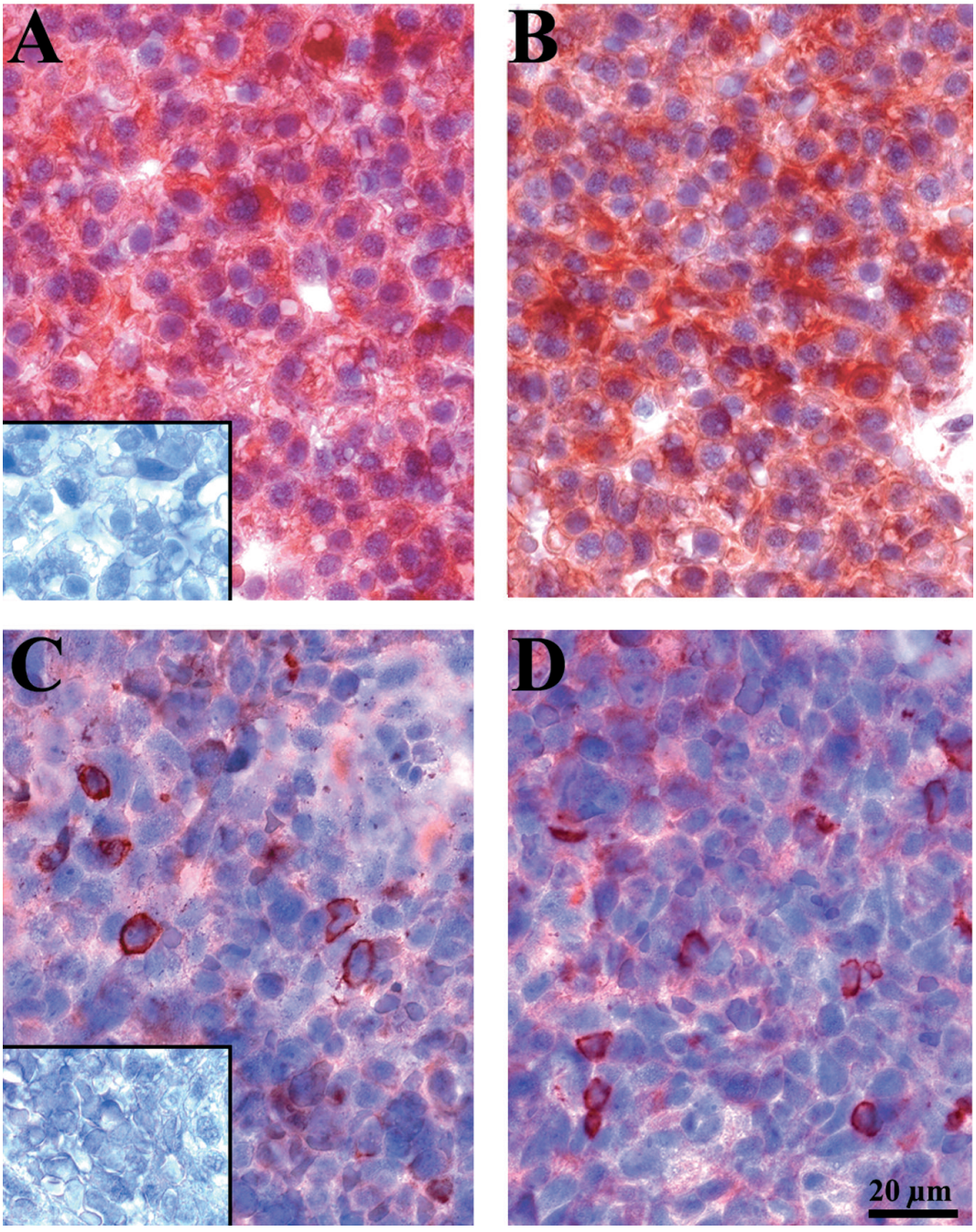

Fig. 3. Immunohistochemical confirmation of T- and B-lymphocyte maturity in double-mutant lungs. The distribution pattern (red-brown staining) of CD3 **(A, B)** and mouse IgM **(C, D)** in the thymus and spleen, respectively, is indistinguishable between wild-type **(A, C)** and double-mutant **(B, D)** embryos. Insets are negative controls (primary antibody omitted). Bar: 20 µm.

Table 2. Genes up-regulated > 2-fold in double-mutants, sorted by function and fold change (FC).

Gene	FC	Gene Title	LE	Molecular Function
Chia	3.63	chitinase, acidic	120	Metabolic (catalytic and transport activity)
Ddx3y	3.44	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	4000	
Ramp2	3.17	receptor (calcitonin) activity modifying protein 2	1300	
Afp	2.62	alpha fetoprotein	NA	
Temt	2.12	thioether S-methyltransferase	75000	
Car3	2.1	carbonic anhydrase 3	500	
Sdc4	3.62	syndecan 4	900	Cytoskeletal organization and biogenesis
Krt2-4	2.61	keratin complex 2, basic, gene 4	NA	
Tnnt2	2.01	Troponin T2, cardiac	500	

LE: mRNA expression (arbitrary units) in the adult mouse lung (Su et al., 2002). NA: data not available.

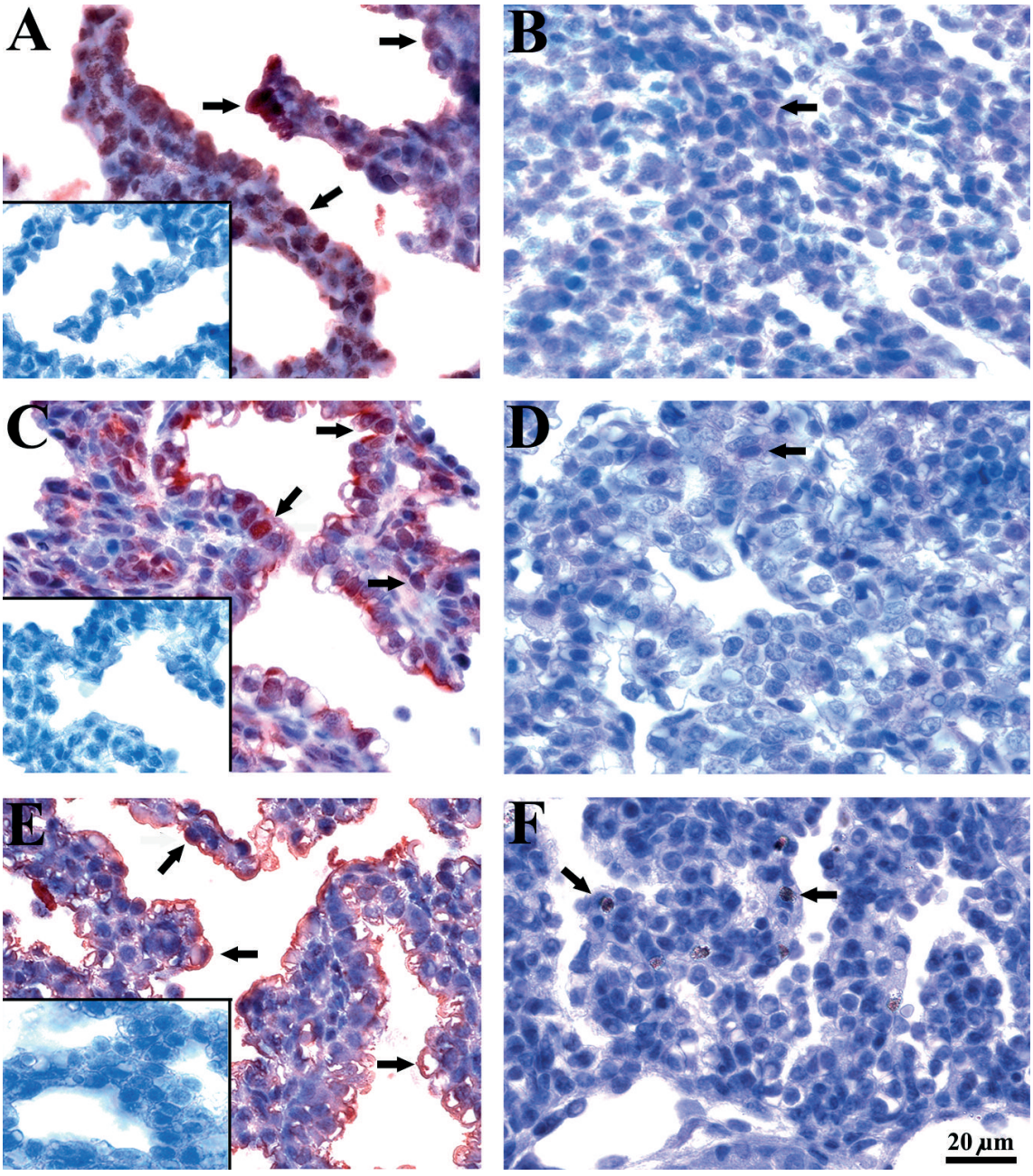

Fig. 4. The distribution of SATB1, c-Myb and CTGF is altered in double-mutant fetal lungs. The distribution of SATB1 **(A, B)**, c-Myb **(C, D)** and CTGF **(E, F)** was analyzed in paraffin sections employing immunohistochemistry. Compared to wild-type (**A, C, E**), double-mutant lungs **(B, D, F)** show a completely different distribution pattern of all three proteins. A reduction in the number of positive cells (arrows in **A-F**) as well as decreased staining intensity is observed throughout the double-mutant lung alveolar epithelium and the adjacent mesenchyme. Insets are negative controls (primary antibody omitted). Note that, whereas CTGF staining is predominantly cytoplasmic in wild-type lungs **(E)**, it is only nuclear in the double-mutants **(F)**. The lungs of double-mutants depict histopathological features typical of pulmonary hypoplasia, evidenced by the absence of expanded saccules **(B, D, F)**, as previously described (Inanlou and Kablar, 2005a). Bar: 20 µm.

Table 3. Genes down-regulated ≥ 2-fold in double-mutants, sorted by function and fold change (FC).

Gene	FC	Gene Title	LE	Molecular Function
Tcrb-V13	-12.5	T-cell receptor beta, variable 13	10	
Satb1	-7.69	special AT-rich sequence binding protein 1	100	
Tcf7	-5.56	transcription factor 7, T-cell specific	250	
Spatial	-4.76	RIKEN cDNA 1700021K02 gene, spatial	50	
Gtf2h4	-2.78	general transcription factor II H, polypeptide 4	200	Transcription factors
Myb	-2.44	myeloblastosis oncogene	50	
Zfpn1a1 (Ikaros)	-2.27	zinc finger protein, subfamily 1A, 1	100	
Uhrf1	-2.13	ubiquitin-like, containing PHD and RING finger domains, 1	200	
Mcm5	-2.08	minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)	125	
Ankrd1	-2.04	ankyrin repeat domain 1 (cardiac muscle)	100	
Coro1a	-4.55	actin binding protein 1A, coronin	500	
BC032204	-2.86	cDNA sequence BC032204	300	
Myl1	-2.56	myosin, light polypeptide 1	150	Cytoskeletal organization
Def6	-2.38	differentially expressed in FDCP 6	120	and cell adhesion
Rac2	-2.33	RAS-related C3 botulinum substrate 2	500	
Ctgf	-2.00	connective tissue growth factor	725	
Ptprc (Cd45)	-6.25	protein tyrosine phosphatase, receptor type, C	50	
Lck	-6.25	lymphocyte protein tyrosine kinase	160	Tyrosine kinase
Itk	-2.22	IL2-inducible T-cell kinase	150	pathway activity
Lat	-2.04	linker for activation of T cells	410	
Pcna	-2.08	proliferating cell nuclear antigen	600	
Ccna2	-2.08	cyclin A2	240	Cell cycle regulation
Rrm2	-2.00	ribonucleotide reductase M2	150	
Rag1	-11.1	recombination activating gene 1	245	
Thy1	-5.88	thymus cell antigen 1, theta	100	
Cd3g	-5.56	CD3 antigen, gamma polypeptide	225	
Cd3d	-4.55	CD3 antigen, delta polypeptide	28	
Prss16	-4.35	protease, serine, 16 (thymus)	NA	Lymphocyte differentiation,
Tcrg-V4	-4.35	T-cell receptor gamma, variable 4	56	maturation and function;
Cd8b1	-3.85	CD8 antigen, beta chain 1	75	histocompatibility and antigen-
H2-Aa	-3.03	histocompatibility 2, class II antigen A, alpha	2300	antibody activity
Ii	-3.03	Ia-associated invariant chain	4000	
Scap1	-3.03	src family associated phosphoprotein 1	NA	
H2-Ea	-2.86	histocompatibility 2, class II antigen E alpha	18	
Ccr9	-2.38	chemokine (C-C motif) receptor 9	80	
Cd8a	-2.38	CD8 antigen, alpha chain	50	
Ccl25	-2.22	chemokine (C-C motif) ligand 25	200	
Igh-6	-2.00	immunoglobulin heavy chain 6 (heavy chain of IgM)	70	
Phgdh	-2.86	3-phosphoglycerate dehydrogenase	150	
Rasgrp1	-2.78	RAS guanyl releasing protein 1	100	
Dck	-2.08	deoxycytidine kinase	12	Metabolic and housekeeping
Mscp	-2.08	Slc25a37, mitochondrial solute carrier protein	600	
Psmb8	-2.04	proteosome (prosome, macropain) subunit, beta type 8 (Large Multifunctional Protease 7)	750	
Eif2s3x	-2.00	eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked	800	
Reg3g	-3.45	regenerating islet-derived 3 gamma	50	
Xist	-3.12	inactive X specific transcripts	180	
Gzma	-2.94	granzyme A	225	Other*
Scgb3a1	-2.27	secretoglobin, family 3A, member 1	2300	
Deadc1	-2.00	deaminase domain containing 1	160	
9130430L19Rik	-3.23	RIKEN cDNA 9130430L19 gene	248	
E430003D02Rik	-2.56	RIKEN cDNA E430003D02 gene	NA	
1700097N02Rik	-2.38	RIKEN cDNA 1700097N02 gene	200	Not yet specified
AI662270	-2.04	expressed sequence AI662270	260	
BC068171	-2.04	CDNA sequence BC068171	225	

Bold: predominant immune response activity; *: "Other" functions include inflammation, apoptosis, X-inactivation. LE: mRNA expression (arbitrary units) in the adult mouse lung (Su et al., 2002). NA: data not available.

pneumocytes. Microarray analysis identified a large number of genes that were differentially expressed between the control and the mutant lung tissues, and an arbitrary cut-off value of 2-fold (i.e. ≥ 2-fold or ≤ -2-fold) was chosen as a means of determining the up- and down-regulated probesets, respectively. A total of 94 probesets met this criterion (19 probesets were up-regulated and 75 down-regulated in the lungs of double-mutant embryos). There was a total of 19 redundant probesets leaving 75 unique transcripts. Of the latter, 12 were expressed sequence tags (ESTs, i.e., nucleotide sequences clustering near known genes, but themselves not yet annotated to code for a characterized gene), leaving 63 unique named genes. Nine named genes were up-regulated more than 2-fold in the lungs of double-mutant embryos, (Table 2) whereas a total of 54 were down-regulated (Table 3). Tables 2 and 3 also show that over 90% (49 out of 54) of the named genes are measurably expressed in the lungs of adult mice (Su et al., 2002).

Lymphocyte maturation is not affected in Myf5-/-:MyoD-/- term fetuses

Remarkably, about half of the down-regulated genes were previously found to be involved in the immune response, with particular relation to thymocyte function (Table 3). Because of this, we thought it was necessary to analyze the status of lymphocyte maturity in double-mutant term fetuses and find out whether they were in some way immune-compromised. However, immunohistochemical staining with antibodies against CD3 and mouse IgM showed that the maturity of T- and B-lymphocytes in the thymus and spleen, respectively, was equivalent to that in the wild-type embryos (Fig. 3). Therefore, the lung down-regulation of genes is unlikely a consequence of the presence of the relatively scarce

Table 4. Genes down-regulated ≥ 2-fold in double-mutants and leading to death of the null mutants before or soon after birth when knocked out.

Gene	Comments on Deletion Mutants
Tcrb-V13	Have compromised vascular integrity and die at E12.5-14.5 from severe hemorrhage (1).
Satb1	Have reduced T-cell development, fail to thrive, and die at 2-3 weeks after birth (2).
Myl1	Fail to form mesoderm, and are resorbed by E8.5 (3).
Myb	Die at about E15 from severe anemia and hepatic hematopoietic failure (4).
Ccna2	Die at E5.5 due to failure of cell division (5).
Ctgf	Skeletal defects cause death at birth due to respiratory failure (6).

1. Kuo et al., 1997; 2. Alvarez et al, 2000; 3. Jiang et al., 2002; 4. Mucenski et al., 1991; 5. Murphy et al., 1997; 6. Ivkovic et al., 2003.

Table 5. Genes down-regulated ≥ 2-fold in double-mutants and with viable knockout (null mutant) mouse models.

Gene	Comments on Deletion Mutants
Rag1	Healthy and fertile to at least 21 weeks, though lacking mature lymphocytes (1).
Ptprc	Survive beyond 5 weeks despite impaired T-cell maturation (2).
Lck	Lack mature T-cells but survive beyond 8 weeks (3).
Thy1	Fertile and overtly normal, but with impaired hippocampal formation (4).
Cd3g	Survive beyond 6 weeks despite severely inhibited T-cell development (5).
Tcf7	Healthy and fertile with normal lifespan, but lack T-cell immunocompetence (6).
Cd3d	Viable and fertile in specific pathogen-free conditions; disturbed T-cell selection (7).
Prss16	Phenotypically normal and fertile; susceptible to autoimmunity (8).
Tcrg-V4	T-cell selection is perturbed, but appear normal and survive beyond 5 weeks (9).
Cd8b1	Healthy and fertile with reduced thymic maturation (10).
Xist	Half the females die in early embryogenesis, all other mutants healthy and fertile (11).
H2-Aa	Severe immune system perturbations, but viable and fertile (12).
Ii	Viable but have poor antigen presentation (13).
Gzma	Normal and fertile; deficient in cytotoxic lymphocyte activity (14).
H2-Ea	Severe immune system perturbations, but viable and fertile (12).
Rasgrp1	40% of expected mutants born; appear normal and fertile, but with immature T-cells (15).
Ccr9	Apparently normal and fertile despite deficient thymocyte responses (16).
Cd8a	Very susceptible to viral infection and alloantigens (17).
Rac2	Impaired neutrophil function; increased vulnerability to fungal infection (18).
Zfpn1a1	Viable and fertile but with severe anemia and megakaryocytic and lymphoid defects (19).
Itk	Viable and fertile; severe T-cell dysfunction and impaired response to allergic asthma (20).
Lat	Appear healthy though lacking mature T-cells. (21).
Psmb8	Poor cytotoxic T-cell activity; can survive and reproduce in pathogen-free conditions (22).
Igh-6	Appear healthy though lacking mature B-cells (23).

1. Mombaerts et al., 1992; 2. Byth et al, 1996; 3. Dal Porto et al., 2004; 4. Barlow et al., 2002; 5. Haks et al., 1998; 6. Verbeek et al., 1995; 7. Dave et al., 1997; 8. Cheunsuk et al, 2005; 9. Sunaga et al., 1997; 10. Fung-Leung et al., 1994; 11. Marahrens et al., 1997; 12. Madsen et al., 1999; 13. Viville et al., 1993; 14. Shresta et al., 1997; 15. Dower et al., 2000; 16. Wurbel et al., 2001; 17. Fung-Leung et al., 1991; 18. Roberts et al., 1999; 19. Lopez et al., 2002; 20. Mueller and August, 2003; 21. Zhang et al., 1999; 22. Basler et al., 2004; 23. Kitamura et al., 1991.

resident and/or migratory lymphocyte population in the double-mutant lung. Instead, it more likely reflects the real possibility that the identified genes have a role in lung alveolar epithelial cell differentiation as well.

The distribution pattern of several examined proteins was found to be altered in double-mutant lungs at term

For subsequent study, down-regulated genes for which knockout mouse models have, according to Mouse Genome Informatics (www.informatics.jax.org), already been generated were subdivided into those whose knockout is lethal or sublethal, i.e., their null mutants die before or soon after birth (Table 4), and those whereby the null mutants are viable and have a fairly normal lifespan (Table 5). The rationale behind this strategy was that the genes whose deletion is lethal could be directly involved in pulmonary development and therefore deserve a closer look in an investigation of the mechanisms leading to pulmonary hypoplasia. Based on this information, the lung distribution of SATB1 (special AT-rich binding protein 1), c-Myb (myeloblastosis oncogene) and CTGF (connective tissue growth factor), a few of the proteins coded for by genes whose deletion mutants are known to undergo embryonic or neonatal death, was immunohistochemically tested.

The results (Fig. 4) indicated a drastic change in the distribution pattern of all three proteins in the double-mutant lungs. Whereas in wild-type lung SATB1 and c-Myb (both transcription factors) were clearly and abundantly contained within the nuclei of lung cells (mostly in the alveolar epithelium and in some mesechymal cells), the double-mutant lung had an obviously decreased number of cells positive for these proteins and very reduced staining intensity. In addition, it was notable that while CTGF immunostaining was largely cytoplasmic in the lungs of wild-type fetuses (Fig. 4E), it was restricted to the nucleus in a few double-mutant lung cells (Fig. 4F). Taken together, these results show that differences in gene expression detected by the microarray analysis do indeed correlate with altered protein distribution patterns in the lungs of our double-mutant embryos, further suggesting a role for SATB1, c-Myb and CTGF in lung development and possibly in the differentiation of the alveolar epithelium (considering their distribution patterns).

Discussion

Our microarray analysis, as typically happens, identified a large number of genes that are differentially expressed between the control and the *Myf5-/-:MyoD-/-* lung tissues. Using a cut-off fold change value of 2 (i.e. ≥ 2-fold or ≤ -2-fold), we found that 9 unique named genes were up-regulated, and 54 down-regulated, in the lungs of double-mutant fetuses (Tables 2 and 3). Over 90% of these named genes are well expressed in the lungs of adult mice (Su et al., 2002), indicating some level of functional necessity. Also, a PubGene cluster analysis of gene associations at that time revealed several literature co-occurrence networks (one is illustrated in Fig. 1), indicating that the identified genes were already known to be related by expression pattern and/or molecular function. The differential expression between normal and mutant lungs was further examined by RT-PCR (Table 1 and Fig. 2), which verified the direction of change for the six genes tested. (N.B., The RT-PCR levels of up- and down-regulation differ from those reported from the microarray analysis, for which we only showed the highest fold change from different probesets or transcripts for each gene.)

With varying degrees of gene ontology information available on the identified genes, developing a protocol to prioritize candidate genes was an essential aspect of the analysis. Candidates for subsequent analysis were ordered by the criterion of whether they represent unique regulatory genes (transcription factors, signaling intermediates, cytoskeleton organizers, cell cycle modulators, etc.) rather than widely expressed genes for metabolic and housekeeping proteins. Despite considerable overlap, many of these genes could be grouped into a number of molecular functional categories with priority given to the functions related to the focus of our pneumocyte differentiation research project. Among the down-regulated genes, for instance, nine are transcription factors, which may regulate the expression of downstream genes in different developmental pathways. Others are involved in cytoskeletal organization and may therefore affect the ability of type II pneumocytes to give rise to the flattened type I cells. Genes in the tyrosine kinase pathway were considered important because that pathway mediates the signal transduction of mechanical stretch into cellular proliferation (Liu and Post, 2000). Similarly, genes regulating the cell cycle would have an important role in the development of all organs, including the lung.

Because of the finding that a large number of the down-regulated genes may be involved in the immune response (Table 3), we found it necessary to study the immune competence of the double-mutant fetuses. However, immunostaining with antibodies against CD3 in the thymus and mouse IgM in the spleen revealed the presence of both proteins at a level similar to that in the corresponding wild-type organs (Fig. 3). This points to normal lymphocyte development and function in the double-mutants, implying that the microarray down-regulation of these particular genes may be specific for the double-mutant lung phenotype, as opposed to the immune system. It could also reflect the very real possibility that the affected genes may play some specific role, as yet unidentified, in pulmonary cell differentiation and/or maturation and lung function in addition to their effect on lymphocytes.

Also, the down-regulated genes were grouped into those whose deletion or knockout is known to be lethal or sub-lethal to the null mutants (Table 4) and those

whose knockout allows a fairly normal lifespan (Table 5), as per Mouse Genome Informatics (www.informatics.jax.org), on the basis that the genes whose knockout is lethal could be directly involved in the growth and differentiation of the lungs and therefore need further examination in the study of molecular mechanisms leading to pulmonary hypoplasia. From that information, we therefore chose to use immunohistochemistry and compare the expression and distribution of the protein products of several genes whose deletion mutants are known to undergo embryonic or neonatal death. Specifically, these were: i) SATB1, a nuclear protein with transcription factor activity, is predominantly expressed in T-cells (Alvarez et al., 2000) and was down-regulated 7.69-fold in double-mutant lungs. Knockout models exhibit up to 100-fold reduction in thymocyte number as well as profound histological and size deficiencies in the thymus, spleen and lymph nodes, fail to thrive, and die at 2-3 weeks due to lack of an essential function in nonthymic tissues including the brain (Alvarez et al., 2000). Importantly, they are also known to have difficulty breathing (Dr. T. Kohwi-Shigematsu, personal communication), while our own immunohistochemical results show that SATB1 staining is almost abolished in double-mutant lungs (Fig. 4B).

ii) c-Myb, a transcription factor promoting cell proliferation, was down-regulated 2.44-fold in double-mutant lungs. As reviewed by Oh and Reddy (1999), c-Myb is closely involved with the transactivation of many cell cycle regulatory genes including *cdc2* and *c-myc*, and is therefore implicated in cell division and differentiation. Its deletion mutants die at about E15 from anemia and hematopoietic failure (Mucenski et al., 1991), and we have preliminary evidence of significant lung hypoplasia in these knockouts (Baguma-Nibasheka, Frampton and Kablar, unpublished observations on tissues kindly provided by Dr. J. Frampton). Our present study also shows a distinct reduction in c-Myb immunostaining in the lungs of double-mutant amyogenic fetuses (Fig. 4D).

iii) CTGF, an integrin-binding mitogen involved in cell growth, proliferation, differentiation and migration (Lau and Lam, 1999), was down-regulated 2-fold in double-mutant fetal lungs. CTGF is thought to exert its effects in part as a mediator of transforming growth factors ß and bone morphogenetic protein, and null mutants for the gene die at birth due to respiratory failure related to skeletal defects (Ivkovic et al., 2003). Although studying CTGF in relation to its osteogenic, chondrogenic and angiogenic effects, Ivkovic and colleagues did note the inability of *Ctgf-/-* newborns to expand their lungs (Dr. K.M. Lyons, personal communication), resulting in lung hypoplasia (Baguma-Nibasheka, Lyons and Kablar, unpublished observations on *Ctgf-/-* lung tissue kindly provided by Dr. Lyons). This is not entirely unexpected since pulmonary hypoplasia, probably related to thoracic restriction, has been noted in chondrodystrophic mice (Hepworth et al., 1990). The current investigation revealed a reduction in both the number of positively stained cells and the staining intensity for CTGF in the lungs of double-mutants (Fig. 4F). Surprisingly, there was also redistribution of CTGF into the nuclear rather than the cytoplasmic compartment (Fig. 4F). Although CTGF is described as a secreted and cytoplasmic protein characterized by an N-terminal secretory signal peptide (Lau and Lam, 1999), its export may depend on interacting factors absent from the *Myf5-/-:MyoD-/-* double-mutant lung, an intriguing feature that calls for further investigation.

Since these three genes are transcription and growth factors, it is probable that the absence of FBMs may be disrupting a number of developmental pathways. Interestingly, both *c-Myb* and *Ctgf* appear in the Fig. 3 gene cluster, indicating some possible biological association. Other revealed genes that may be of interest in pneumocyte differentiation include Tcrb-V13, also known as lung Kruppel-like factor (LKLF), a transcription factor found to be 12.5 times down-regulated in the mutant lung tissue. Although initially described as lung-specific and highly expressed in the embryonic mouse lung at a time coinciding with the onset of FBMs (Anderson et al., 1995), LKLF is now known to be important in vasculogenesis and T-cell activation as well (Kuo et al., 1997; Conkwright et al., 2001). However, because homozygous LKLF deletion mutants die at E12.5-14.5 (Kuo et al., 1997), studies on its involvement in lung development have had to use $LKLF^{-/-}$ embryonic stem (ES) cells in chimeric animals (Wani et al., 1999). Their results show that the mutant ES cells do not contribute to lung tissue in chimeric mice that survive birth and that the chimeric mice that die at birth have their lung development curtailed at the canalicular stage, suggesting that LKLF expression is essential for normal lung development (Wani et al., 1999). We have recently obtained ES cells from that same group (kindly provided by Dr. J.B. Lingrel) and will be using selective pneumocyte markers and transmission electron microscopy (TEM) in follow-up studies on cell differentiation in the resulting E18.5 chimeras.

In summary, further studies on these lead candidate molecules (SATB1, c-Myb, CTGF and LKLF) will be performed. They will be conducted in a similar fashion to that described in our earlier publications (Inanlou and Kablar, 2005a,b), with special attention to the TEM analysis to precisely determine what steps of pneumocytes type I and II differentiation might be affected in each of these knock-outs, hence attributing a precise function in lung development to each of the four molecules of interest and thereby introducing new molecular players in the field of lung developmental biology.

Finally, it will also be important to investigate the expression and function, in the lungs, of the genes whose deletion allows the null mutants to live a fairly normal life. Even though such genes may not be considered

strictly essential for the differentiation of a functional pulmonary epithelium, it could be possible that, when they are absent, some other genes take over their function. Indeed, such "genetic redundancy" has been implicated in murine skeletal myogenesis where one allele of *MyoD* appears capable of rescuing myogenesis in *Myf5*-deficient embryos whereas two functional copies of *Myf5* are required to rescue *MyoD-/-* mice, even though the latter exhibit a 3.5-fold increase in Myf5 mRNA (Rudnicki et al., 1993). Similar alternative mechanisms for overcoming deficient gene function have been suggested for the survival of spinal cord motor neurons in mice lacking brain-derived neurotrophic factor and neurotrophic factor -4/5 (Kablar and Belliveau, 2005). Follow-up experiments on such modifying loci would call for double-knockouts of the candidate gene and its suspect alternate and, for instance, likely couples might include *Coro1a* and *Sdc4*. These are both involved in cytoskeletal organization and cell locomotion (de Hostos, 1999; Zimmermann and David, 1999) and one is down-regulated 4.55-fold while the other is up-regulated 3.62-fold in the lungs of our double-mutants. Another example is Rag1-/-. Considering that Rag1 was found the second most down-regulated in double-mutant lungs (after Tcrb-V13), identification of an up-regulated molecule (with a viable knockout) in Rag1-/- lungs (kindly provided by Dr. C. Nerlov) would provide a clue for the generation of a double-mutant between Rag1 and the up-regulated molecule. Furthermore, TEM analysis of lung tissue from some Table 4 mutants might detect defects in pneumocyte differentiation that are not necessarily incompatible with life but could still be helpful in attributing a precise lung development function to the molecules of interest, even though such non-lethal differentiation failures would more likely be detectable in type II rather than type I pneumocytes.

In conclusion, the results from these studies will provide valuable insights into the molecular regulation of pneumocyte differentiation as a preliminary step in the prevention, early diagnosis and medical treatment of pulmonary hypoplasia.

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