In Vitro Cell.Dev.Biol.—Animal (2008) 44:10–16
DOI 10.1007/s11626-007-9070-0

Decreased surfactant protein B expression in mice derived completely from embryonic stem cells

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Received: 9 October 2007 / Accepted: 24 October 2007 / Published online: 11 December 2007 / Editor: J. Denry Sato

Abstract ES mice that are derived completely from embryonic stem (ES) cells can be obtained by tetraploid embryo complementation. Many neonate ES mice die because of respiratory distress, but it is not clear what contributes to the phenomenon. Using five microsatellite DNA markers, we confirmed that our ES mice were completely derived from ES cells and contained no tetraploid component. The neonatal ES mice that exhibited respiratory distress were tested for surfactant protein B (SP-B) expression by Western blotting. These mice had no SP-B expression, and even apparently healthy adult ES mice had decreased SP-B levels and aberrant SP-B phenotypes. These data suggest that the expression of SP-B protein is an important factor in the survival of ES mice to term and adulthood.

Keywords ES mice · Respiratory distress · Pulmonary surfactant protein B · Tetraploid embryo complementation

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Introduction

Mouse embryonic stem (ES) cells have the full potential to develop along all lineages of the embryo proper epiblast and show a deficiency in extraembryonic lineages, however, tetraploid embryos rarely contribute to the embryo proper and extensively to the extraembryonic lineage (Nagy et al. 1990; James et al. 1995; Eakin et al. 2005). Therefore, ES cells and tetraploid embryos have a complementary distribution in chimeras made between them. In such chimeras, the embryo proper, the amnion, the yolk sac mesoderm, the allantois, and the chorionic mesoderm-derived part of the placenta are completely ES cell-derived, whereas the yolk sac endoderm and the trophoblast cell lineages are tetraploid embryo-derived (Nagy et al. 1990; James et al. 1995; Eakin et al. 2005). Production of ES cell–tetraploid embryo chimeras, or tetraploid embryo complementation, has proven to be a reliable and simple way of producing completely ES cell-derived mice (ES mice) (Eggan et al. 2001; Li et al. 2005). ES mice can survive to term and adulthood at a higher frequency than nuclear transfer mice (Eggan et al. 2001; Li et al. 2005). In addition, ES mice show fewer developmental abnormalities and the procedure does not require expensive instrumentation and sophisticated manual skills (Eggan et al. 2001; Schwenk et al. 2003; Wang and Jaenisch 2004; Li et al. 2005). ES mice have homologous cytoplasms and karyotypes, whereas nuclear transfer mice have only homologous karyotypes. Tetraploid embryo complementation is a preferable method of generating mice from ES cells.

During the production of ES mice, many abnormalities have been observed, for example, increased placental and birth weights, polyhydramnios, interstitial bleeding, craniofacial abnormalities, axial elongation, polydactyly, swollen edematous skin, poor mandible development, perinatal

death, obesity, abdomen ulcer-like symptoms, elevated hematocrit, and respiratory and circulatory problems (Nagy et al. 1993; Dean et al. 1998; Eggan et al. 2001; Schwenk et al. 2003; Wang and Jaenisch 2004; Li et al. 2005). However, it was obvious that neonatal ES mice died because of respiratory distress with an extremely high penetrance (Nagy et al. 1990; Eggan et al. 2001; Li et al. 2005). This respiratory distress greatly influenced the survival rate of adult ES mice and therefore limited the utility of tetraploid embryo complementation. In our earlier study, we reported that 12 out of 45 ES mice died of respiratory distress at Cesarean section and their life span was less than an hour (Li et al. 2005). The clinical and pathological features of respiratory distress are similar to neonatal respiratory distress syndrome (NRDS) in clinical pediatrics. NRDS results from insufficient levels of pulmonary surfactant. Pulmonary surfactant is essential for the expansion of the alveoli in the lungs, and its deficiency causes the alveoli to collapse, preventing the infants from breathing properly. Symptoms usually appear shortly after birth and slowly become more severe (Goerke 1998). The studies on NRDS led us to investigate the deficiency of pulmonary surfactant in neonatal ES mice with respiratory distress.

Pulmonary surfactant is a lipid–protein complex consisting of 90% lipids and 10% proteins that is synthesized by type II alveolar epithelial cell. Within the protein fraction, four lung-specific surfactant-associated proteins have been isolated and characterized in mice. The larger and more hydrophilic proteins, SP-A and SP-D, are multifunctional lectin-like glycoproteins. The smaller and more hydrophobic SP-B and SP-C, in particular SP-B, have been shown to be critical for normal alveolar function (Goerke 1998). SP-B protein is a 79-amino acid amphipathic polypeptide associated with surfactant phospholipids in the alveolus of the lung. SP-B is encoded by a single gene located on chromosome 2 in humans and at a syntenic site on chromosome 6 in the mouse. The *SFTPB* gene encodes a 40- to 42-kDa glycosylated preproprotein that is proteolytically processed by type II epithelial cells to yield a surface-active mature peptide (6 to 9 kDa) that is secreted into the airspaces (Goerke 1998). Transgenic newborn mice lacking the SP-B gene fail to inflate their lungs and die of respiratory distress shortly after birth (Clark et al. 1995; Melton et al. 2003). Similarly, human infants with mutations leading to SP-B deficiency rapidly develop respiratory distress after birth and invariably die of respiratory failure (Floros et al. 2006). In contrast, targeted disruption of loci encoding the other three surfactant protein genes, SP-A (Korfhagen et al. 1996; Ikegami et al. 1998), SP-C (Glasser et al. 2001, 2003), and SP-D (Fisher et al. 2000; Ikegami et al. 2000), in mice is not associated with neonatal respiratory failure, indicating that SP-B is the only surfactant protein required for the successful transition to air breathing at birth. These data suggest that SP-B deficiency might attribute to respiratory distress in neonatal ES mice. The relationship between the survival of ES mice and SP-B protein level has not been reported in previous documents. In this study, we first used polymerase chain reaction (PCR) in five microsatellite DNA loci to detect the tetraploid embryo contribution in ES mice to determine whether these ES mice are derived completely from ES cells and subsequently assessed mature SP-B protein level in lungs of ES mice by Western blotting.

Materials and Methods

Mice, ES cells, ES–tetraploid embryo. Mice were purchased from Beijing Vitalriver Laboratory Animal, Beijing, China. Hybrid ES cells and (C57BL/6×DBA/2) F2 tetraploid embryos were produced as previously described (Li et al. 2005). Hybrid ES cells was isolated from inbred C57BL/6 females (3- to 4-wk-old) mated to adult inbred 129/Sv males. In brief, blastocysts were collected 3.5 d postcoitum from C57BL/6 females and cultured in ES cell media on a mitomycin C-treated mouse embryonic fibroblasts as previously described (Nagy et al. 1993). At day 5, the outgrowth was dissociated by pipetting in trypsin solution (Gibco, Grand Island, NY), and the cell suspension was replated on a fresh feeder layer. ES cell media was Dulbecco's modified Eagle's medium (DMEM; Gibco) with 20% fetal calf serum (Gibco) containing 1,000 U/ml of leukemia inhibitory factor (Gibco). These plates were screened 3 d later for the presence of ES cell colonies. We started counting passage numbers when we were first able to pass the cells into 35 mm plates (passage 1). The ES cells with early passages (less than 10 passages) were aggregated with tetraploid embryos. We produced 19 ES mice by tetraploid embryo complementation: 7 neonatal ES mice with respiratory distress and 12 adult ES mice.

PCR analysis of genomic DNA. Genomic DNA was extracted from 12 tissues/organs (heart, lung, liver, kidney, stomach, spleen, pancreas, brain, muscle, testis, intestine, bladder) of adult ES mice, from 4 tissues/organs (heart, liver, brain, muscle) of newborn ES mice with respiratory distress, and from ES cells. PCR amplification of five microsatellite DNA loci (http://www.broad.mit.edu/), *D2Mit493*, *D10Mit134*, *D5Mit138*, *D16Mit189*, *D5Mit3468*, was performed using primer pairs synthesized by TaKaRa Biotechnology (Dalian, China; Table 1). Reactions (20 μl) were subjected to 34 cycles of 1 min 95°C, 1 min 55° C, 2 min 72° C, and products were separated on a 4% agarose gel. The PCR results were visualized by direct UV-transillumination of the agarose gel. Because the sequence lengths of the five chosen microsatellite loci are different

Table 1. Sequence lengths and primer pairs of five microsatellite loci

Locus	Sequence lengths (bp)			Primer pairs
	DBA/2	C57BL/6	129/Sv	
D2Mit493	97	111	127	-GTCTCTACCTGAGTTTCCATC -TCCCGAGTTGTCCCTCTATG
D10Mit134	115	95	87	-AATCCTAGAAGATACATGCTGATGC -TTAAGCACCAAAATTGAAATCA
D5Mit138	120	148	142	-AGACAGTTACCTTCTTCCCAAGG -TGTTTCTCCCTTCTGCCATC
D16Mit189	242	270	270	-GAGGGCTAGAACTGCCATGC -CCAGTACAGGAAGTCTTTGCATC
D5Mit346	177	121	141	-TCAAACTCCTCTAATATGG -CTGTCTCATTAATCCATGGATC

among the three mouse strains (DBA/2, C57BL/6, 129/Sv), we can detect occasional tetraploid contributions in ES mice by the phenotype of microsatellite loci in DBA/2.

Histopathological analysis. Lungs were collected from one of the neonatal ES mice with respiratory distress and a normal neonatal mouse and submitted for routine histopathological analysis. The lungs were placed overnight in 10% buffered formalin, embedded in paraffin, and 4-μm-thick sections were stained with hematoxylin–eosin.

Western blots. To assess the SP-B protein level, lung tissue samples were collected from the ES mice and control mice. The tissues were homogenized in 10 mM Tris (hydroxymethyl) aminomethane (Tris, pH 7.5), 0.25 M sucrose, 1 mM EDTA, 5 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of pepstatin A, aprotinin, antipain, leupeptin, and chymostatin. The homogenized liquid was centrifuged at 1,460×*g* (12,000 r/min) for 15 min (4° C). Equal amounts of protein from the supernatant were dissolved in electrophoresis sample buffer and were subjected to 16% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by electrophoretic transfer to nitrocellulose membrane (BioRad). The membrane was blocked with 5% milk in Tris-buffered saline and incubated with rabbit antisheep SP-B polyclonal antibody (CHEMICON International, Catalog No. AB3780, 1:1,500 dilution) or with β-actin antibody (Cell Signaling Technology, #4967, 1:1,000 dilution) for 2 h at room temperature then overnight at 4° C. Subsequently, the membrane was washed with Tris-buffered saline containing 0.05% Tween 20 and 0.05% Nonidet P-40, incubated with a 1:2,000 dilution of alkaline phosphatase (AP)-conjugated goat antirabbit immunoglobulin G (H+L) (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) for 2 h at room temperature, washed again, and developed by conventional method with nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP) (BioRad, Hercules, CA). Band intensity on the membrane was analyzed by densitometric scanning and quantitated using BioRad Quantity One analysis software.

Results

Tetraploid contribution in ES mice. The brown coat color and normal germline transmission of 12 adult ES mice (male) indicated only ES cell contributions. Besides these external characteristics, phenotypes of five microsatellite DNA markers were analyzed to detect occasional tetraploid contribution in the ES mice (12 adult and 7 neonatal). We isolated genomic DNA from 12 organs/tissues of each adult ES mouse, 4 organs/tissues of each neonatal ES mouse with respiratory distress, and the hybrid aggregated ES cells. The samples were analyzed by PCR analysis. At the five microsatellite DNA loci, the sequence length difference between C57BL/6 and 129/Sv is obviously smaller than that of between them and DBA/2, and the PCR bands can be easily determined whether they contain DBA/2 components. The band sizes of *D10Mit134*, *D5Mit138*, and *D16Mit189* were very close in C57BL/6 and 129/Sv; their bands were not separated. The PCR results showed that no DBA/2 phenotypes were found in all the samples, indicating no tetraploid contribution in the ES mice (Table 2). Microsatellite phenotypes of each sample from the ES mice were identical with the aggregated ES cells. Although 15 samples were not determined, this did not influence us to reach a qualitative inference. These data showed that all the ES mice contained only ES cell components and were completely derived from ES cells.

Histopathological characteristics of ES mice that died of respiratory distress. Clinically, the neonatal ES mice with respiratory distress were characterized by dyspnea, profound hypoxemia, decreased lung compliance, and short-

Table 2. Microsatellite DNA analysis of organs/tissues from ES mice

ES mice	Liver					Heart					Brain					Muscle					Lung					Kidney				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
1#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–	–	–	–	–	–	–	–	–	–	–
2#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–	–	–	–	–	–	–	–
5#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7#	–	–	–	–	–	–	–	–	ND	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9#	–	–	–	–	–	ND	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10#	–	ND	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–	–	–
11#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–										
14#	–	–	–	–	–	–	–	–	–	–	–	ND	–	–	–	–	–	–	–	–										
15#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–										
16#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–										
17#	–	–	ND	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–										
18#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–										
19#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–										

Spleen					Pancreas					Stomach					Bladder					Intestine					Testis				
A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	ND	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND
–	–	ND	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND	–	–	–	–	–	–	–	–	–	–	–

A D2Mit493, *B* D10Mit134, *C* D5Mit138, *D* D16Mit189, *E* D5Mit346, *1#–12#* adult ES mice with respiratory distress, *13#–19#* newborn ES mice with respiratory distress, – tissues not containing DBA/2 component, *ND* not determined

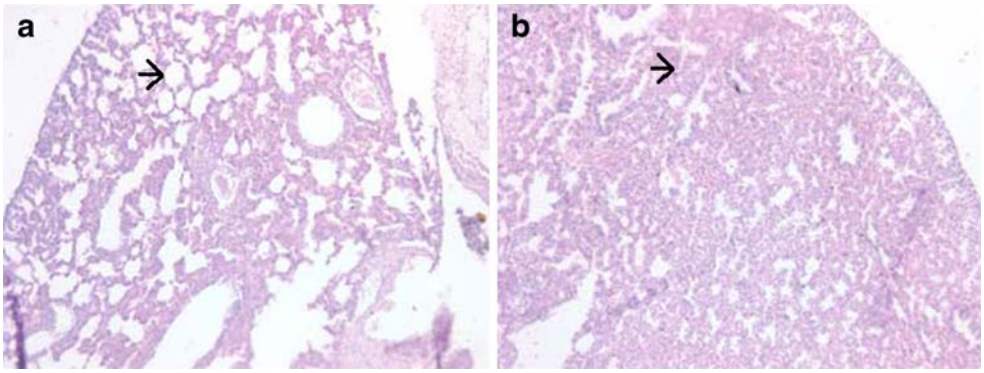

Figure 1. Lung sections from normal mice (*a*) and neonatal ES mice with respiratory distress (*b*). Magnification ×10. *Arrows* indicate the expanded (*a*) and collapsed alveoli (*b*).

ened life span (less than an hour). The frequency of breaths gradually decreased, the intensity become weaker, and the skin color become increasingly darker red after birth. The lungs had the same size as the normal neonatal mice, but were paler in color. Histological examination of the lungs revealed extensive atelectasis, alveoli collapse, and thickened alveolar wall in the ES mice with respiratory distress, whereas the lungs of normal neonatal mice were fully inflated and the alveoli were expanded; as shown in Fig. 1.

Expression of SP-B. We observed weaker SP-B expression in the 12 adult ES mice than in the 12 normal adult mice (C57BL/6×129/Sv; Fig. 2*a*. *A*, *B*). Similarly, we observed no SP-B expression in the six neonatal ES mice with respiratory distress and slight SP-B expression in the four normal neonatal mice (C57BL/6×129/Sv; Fig. 2*a*. *C*, *D*). SP-B expression was lower in the normal neonatal mice than in the normal adult ones, which showed that SP-B expression increased with age (Fig. 2*a*. *A*, *C*). In addition, the phenotypes of SP-B proteins from the 12 adult ES mice were significantly polymorphic in polyacrylamide gel electrophoresis with samples running at different rates (Fig. 2*a*. *B*), whereas the phenotypes of SP-B proteins were absolutely identical in the normal adult mice (Fig. 2*a*. *A*). By using the BioRad Quantity One analysis software, the average relative intensity of SP-B proteins (the rate of SP-B and β-actin) of normal adult mice (*A*), adult ES mice (*B*), normal neonatal mice (*C*), and dead ES mice with respiratory distress (*D*) were 0.23, 0.12, 0.10, and 0, respectively (Fig. 2*b*). The differences between *A* and *B* or *C* was significant, whereas the difference between *B* and *C* was not significant by *t* test.

Discussion

Using five microsatellite DNA markers, we observed that none of the tissue/organ samples from 19 ES mice (12 adult and 7 neonatal) contain a DBA/2 component. Although, theoretically, one-fourth of aggregated tetraploid embryos do not contain a DBA/2 component, these data suggested that tetraploid cells show a stronger tendency to colonize trophectodermal lineages and that the ES mice are entirely derived from the aggregated ES cells. These results were consistent with previous reports (Nagy et al. 1990; Nagy et al. 1993; Schwenk et al. 2003; Li et al. 2005). The glucose phosphate isomerase (GPI) assay was used to determine the chimerism of ES mice in previous publications (Nagy et al. 1990; Nagy et al. 1993; Schwenk et al. 2003). However, polymorphism of GPI is poor. Only three alleles at the GPI locus are found in mouse strains and, thus, the application of the GPI marker is largely limited in ES mice derived from ES cells with complex genetic backgrounds. More than 6,000 microsatellite markers in the mouse genome have been released by the Whitehead Institute/MIT Center for Genome Research (http://www.broad.mit.edu/). An abundant microsatellite marker is more convenient to detect chimerism of ES mice.

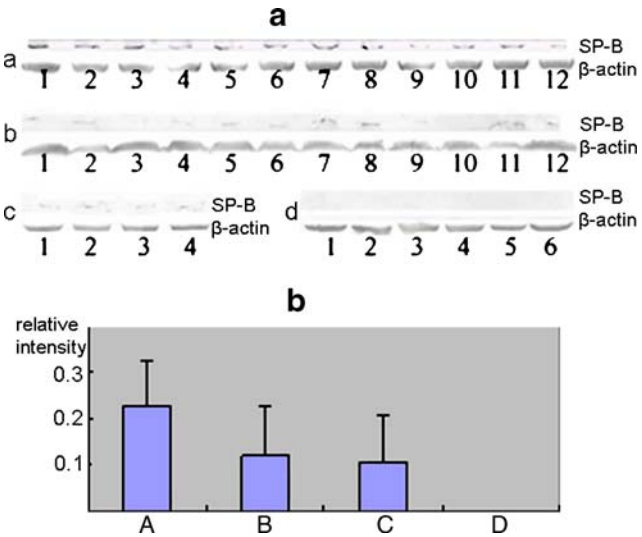

Figure 2. Western blots (*a*) and relative intensity (*b*) of SP-B from lung tissue. Samples containing 100 μg total protein were analyzed by SDS–PAGE followed by Western blotting for SP-B as described in the "Materials and Methods" section. *A* normal adult C57BL/6×129/Sv mice, *B* adult ES mice, *C* normal neonatal C57BL/6×129/Sv mice, *D* neonatal ES mice with respiratory distress.

We observed strong and weak SP-B expression in the normal adult and neonatal mice, respectively. However, no SP-B expression was observed in the lungs of neonatal ES mice that died of respiratory distress. Even the apparently healthy adult ES mice had significantly decreased SP-B expression (Fig. 2*a*). These results suggest that neonatal respiratory distress syndrome in ES mice is caused by SP-B deficiency and that even apparently normal adult ES mice may have decreased or even lost SP-B expression (Fig. 2*a*. *B*), indicating that mammalian development may be rather tolerant to slightly abnormal SP-B expression. All cloned animals (including ES mice) have many abnormally expressed genes, and those that are able to survive to adulthood are no exception, simply with fewer abnormalities in gene expression than those that die early (Humpherys et al. 2001, 2002; Inoue et al. 2002). So we infer that the deficiency and decrease of SP-B in the ES mice may result from abnormal expression of the SP-B gene in this study.

As a critical component of pulmonary surfactant, SP-B plays a key role in promoting adsorption of surface active components to the air–liquid interface as alveolar area increases and is absolutely essential for maintaining film stability during compression and lowering of surface tension (Goerke 1998). Mice with a single mutated SP-B allele (+/−) survived postnatally, whereas homozygous SP-B (−/−) offspring died of respiratory failure immediately after birth. Lungs of SP-B (−/−) mice develop normally but remain atelectatic in spite of postnatal respiratory efforts. SP-B protein and mRNA were undetectable, and tubular myelin figures were lacking in SP-B (−/−) mice. In adult SP-B (+/−) mice, surfactant protein B mRNA and the alveolar lavage SP-B protein were reduced by 50% compared with wild-type littermates, consistent with the inactivation of a single SP-B allele (Tokieda et al. 1997; Sui et al. 1999; Nesslein et al. 2005). In this study, the neonatal ES mice that died of respiratory distress were characterized clinically by dyspnea and hypoxemia, histologically by atelectasis and thickened alveolar wall, and pathologically by deficient SP-B protein. These findings demonstrate that SP-B plays a critical role in perinatal adaptation to air breathing; mice with decreased SP-B level can survive, and the lack of SP-B protein causes respiratory failure at birth.

The adult ES mice had not only decreased and even undetectable SP-B protein expression (samples 3 and 10 in Fig. 2*a*. *B*) but also significantly polymorphic SP-B protein phenotypes by Western blotting (Fig. 2*a*. *B*). In contrast, phenotypes of SP-B proteins were absolutely identical in normal adult mice (Fig. 2*a*). In addition, we also observed that the passage number of ES cells affected the ES mice production, in that ES cells with a higher passage number produced fewer ES mice in our earlier study (Li et al. 2007). We postulate that these differences may result from accumulated aberrant epigenetic modification during ES cells culture *in vitro*. In the mouse, the epigenetic state of the ES cell genome is extremely unstable and *in vitro* culture of ES cells affects their pluripotency and may give rise to fetal abnormalities (Humpherys et al. 2001; Deng et al. 2007). Aberrant gene expression has been observed in ES cells and ES mice (Dean et al. 1998; Humpherys et al. 2001, 2002; Schumacher and Doerfler 2004). The aberrant epigenetic modification consequentially may affect SP-B protein expression procedures such as transcription and translation of the SP-B gene, postprocessing of glycosylated SP-B preproprotein, and mature SP-B peptide level and distribution characteristics in the lungs. This may cause the undetectable SP-B protein in samples 3 and 10 by Western blotting (Fig. 2*a*. *B*).

In the future, it would be useful to compare the level of SP-B of neonatal ES mice with respiratory distress to those of viable ES mice. This preliminary study establishes that the deficiency of surfactant protein SP-B causes neonatal respiratory distress in ES mice. It would be interesting to know if this type of defect also occurs in animals cloned by nuclear transfer or if it is a specific feature of ES mice. This information would be valuable for the field of cloning biology.

Acknowledgements We thank Prof. Zhijie Chang for kindly providing the BCATM Protein Assay Kit (PIERCE) and assisting in the Western blotting. We also thank Dr. Man Hu, Dr. Yuzhong Ma, Mrs. Xianrui Gu, Mrs. Rongyan Zhou, Mr. Jie Tang, and Mr. Lei Ju for the many suggestions and help. In particular, we would like to thanks Mrs. Eileen Rattner for her critical remarks and editing this manuscript. This work was supported by the National Natural Science Foundation of China (No.30571336) and the President Foundation from Agricultural University of Hebei.

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