Mouse ATF-2 Null Mutants Display Features of a Severe Type of Meconium Aspiration Syndrome

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Toshiro Maekawa‡‡, François Bernier‡, Motohiko Sato§, Shintaro Nomura§§, Mandavi Singh∥, Yoshio Iinoue∥∥, Tomoyuki Tokunaga‡‡, Hiroshi Imai∥∥, Minesuke Yokoyama‡‡, Andreas Reimold‡‡, Laurie H. Glimcher‡‡, and Shunsuke Ishii∥∥∥

From the ¶¶Laboratory of Molecular Genetics, Tsukuba Life Science Center, RIKEN, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan, the Department of Pathology, Osaka University Medical School, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan, the Department of Anatomy, Hokkaido University School of Medicine, Sapporo, Hokkaido 060-0815, Japan, the **National Institute of Animal Industry, 2 Ikenodai, Kukisaki, Inashiki, Ibaraki 305-0901, Japan, the ¶¶Mitsubishi Kagaku Institute of Life Sciences, 11 Minami-Ohya, Machida, Tokyo 194-0031, Japan, the ¶¶Department of Cancer Biology, Harvard School of Public Health, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, and the ¶¶¶CREST (Core Research for Evolutional Science and Technology), JST (Japan Science and Technology Corporation)

Mouse null mutants of transcription factor ATF-2 were generated by the gene targeting method. They died shortly after birth and displayed symptoms of severe respiratory distress with lungs filled with meconium. These features are similar to those of a severe type of human meconium aspiration syndrome. The increased expression of the hypoxia inducible genes suggests that hypoxia occurs in the mutant embryos and that it may lead to strong gasping respiration with consequent aspiration of the amniotic fluid containing meconium. A reduced number of cytotrophoblast cells in the mutant placenta was found and may be responsible for an insufficient supply of oxygen prior to birth. Using the cDNA subtraction and microarray-based expression monitoring method, the expression level of the platelet-derived growth factor receptor α gene, which plays an important role in the proliferation of trophoblasts, was found to be low in the cytotrophoblasts of the mutant placenta. In addition, ATF-2 can trans-activate the PDGF receptor α gene promoter in the co-transfection assay. These results indicate the important role of ATF-2 in the formation of the placenta and the relationship between placental anomalies and neonatal respiratory distress. The ATF-2 null mutants should enhance our understanding of the mechanism of severe neonatal respiratory distress.

Meconium aspiration syndrome (MAS) is a common neonatal problem that results in acute and chronic respiratory morbidity (for review, see Refs. 1 and 2). Unfortunately, our understanding of this entity is incomplete. Aspiration of meconium particles may occur before, during, or after delivery and is associated with deep inspiratory movements because of fetal respiratory depression. Aspiration of meconium may cause mechanical obstruction of the airways, chemical pneumonitis, and surfactant inactivation. Although MAS can be prevented in the majority of infants by appropriate suctioning at birth or by early administration of surfactant, the severe form of MAS is still a neonatal problem that remains to be resolved (3).

A number of transcription factors of the ATF/CREB family, all of which contain a DNA-binding domain consisting of a cluster of basic amino acids and a leucine zipper region (b-zip), have been identified (for review, see Ref. 4). They bind to the cAMP response element (CRE) as homodimers or heterodimers formed through the leucine zipper. Among the numerous transcription factors of the ATF/CREB family, three factors, ATF-2 (also called CRE-BP1), ATF-3, and CRE-BPα form a subgroup (5–9). These factors are capable of forming homodimers or heterodimers with c-Jun (10). A common characteristic of this group of factors is their activation by the stress-activated protein kinases such as the Jun amino-terminal kinase and p38

 EXPERIMENTAL PROCEDURES

Construction of the Targeting Vector—The ATF-2 genomic clones were isolated from a library derived from TT2 cells by the standard plaque hybridization procedure. A 10.0-kb genomic DNA subfragment,
which contains the exon encoding amino acids 327–395, was used to generate the targeting vector. A neomycin cassette driven by the phosphoglycerate kinase gene promoter was inserted into the newly generated BgII site between amino acids 378 and 379 in this exon. To increase the frequency of gene targeting, the diphtheria toxin-poly(A) signal cassette for negative selection was fused to the short arm as described (17).

Generation of ATF-2-deficient Mutant Mice—The embryonic stem (ES) cells used were TT2 cells derived from an F1 embryo resulting from a cross between C57BL/6 and CBA mice (18). The Norl-linearized targeting vector (100 µg) was electroporated into 1.0 × 10⁴ TT2 cells. Targeting vectors were selected after 7–10 days of growth in the presence of G418 (150 µg/ml) and were then expanded in duplicate 24-well plates. The homologous nature of the recombination was confirmed by Southern blot analysis using several restriction enzymes and several probes located either inside or outside the targeting vector. In addition, three different primers, shown in Fig. 1A, were used to amplify a 1100-base pair fragment from the wild type allele or a 1000-base pair fragment from the mutant allele. Chimeras were produced by injecting about 10 ES cells into 40 ICR 8-cell embryos and transplanting the embryos into the uterus of pseudopregnant females. Six- to eight-week-old male progeny with a high degree of chimerism were derived from these clones and were bred with BALB/c, C57BL/6, or ICR females to produce heterozygous mice capable of transmitting the targeted allele through the germ line. The mice were maintained by the Division of Experimental Animal Resources, WPIRIKEN.

Genotyping of ES Cells, Embryos, and Animals—Genomic DNA was isolated from cultured cells, embryos, and tail clippings by digestion overnight at 55 °C in lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% SDS, 0.5 mg/ml proteinase K) followed by RNase A treatment, phenol-chloroform extraction, and ethanol precipitation. For Southern blot analysis, genomic DNA (about 20 µg) was digested with BgII and resolved on 0.8% agarose gels.

Detection of ATF-2 Proteins—The brains of 18.5-days postcoitum (dpc) fetuses were washed in PBS, resuspended in 300 µl of lysis buffer consisting of 45 mM Tris-HCl (pH 7.4), 135 mM NaCl, 2.2 mM EDTA, 0.9% Triton X-100, 0.9% sodium deoxycholate, 0.09% SDS, and 1% Trasylol, and homogenized. After centrifugation, samples of lysates were resolved on a 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a polyvinylidene difluoride membrane, and ATF-2 protein was detected using anti-ATF-2 polyclonal antibodies, which were raised against the full-length recombinant ATF-2 protein and ECL detection reagents (Amersham Pharmacia Biotech).

Histological Analysis, Immunohistochemistry, and in situ Hybridization—Histological analysis was performed essentially as described (20). cDNA Subtraction and Microarray-based Expression Monitoring—cDNA subtraction was done using the PCR-Select kit (CLONTECH) according to the protocol supplied by the supplier. Microarrays containing 18,378 nonredundant mouse cDNA clones chosen from the L-M-A.G.E. collection (Genome Systems, Gene Discovery Array mouse version 1.0) were used to identify the putative target genes of ATF-2 according to the procedure described by the supplier.

Effect of PDGF on Proliferation of Trophoblast-derived Cells—Immortalized choriocarcinoma cells, hTRC-1, were maintained in serum-free medium with 10% fetal bovine serum. PDGF-AA or PDGF-BB (Pepro Tech Inc.) was added to the cells directly at a final concentration of 10 ng/ml. The ability of BrdUr incorporation was examined using the cell proliferation enzyme-linked immunosorbent assay system (Amersham Pharmacia Biotech) according to the protocol from supplier.

The Co-transfection Assay—The luciferase reporter, in which the 2.2-kb human PDGF receptor α promoter (from –2120 to +118) was linked to the luciferase gene, was provided by Dr. S. Mosselman (21). A mixture containing 0.2 µg of the luciferase plasmid pPDGFRE-2120-luc, 0.5 µg of the plasmid to express various forms of ATF-2, and 1 µg of the internal control plasmid pP-β-gal, in which the chicken cytoplasmic signal peptide of β-galactosidase is linked to the β-galactosidase gene, was transfected into Chinese hamster ovary cells, and luciferase assays were performed. The total amount of plasmid DNA was adjusted to 3 µg by adding the control plasmid DNA pact1.

RESULTS

Respiration Defects of the ATF-2-/- Mutant—ATF-2-/- mutant mice were generated by homologous recombination in TT2 ES cells. The gene was disrupted by inserting the neomycin resistance (neo) gene into the exon (amino acids 327–395) that encodes the DNA-binding domain consisting of the basic amino acid region between amino acids 378 and 379. Homologous recombinants were characterized by the appearance of a 2.9-kb BgII fragment with the 3' probe and a 1000-base pair PCR-amplified fragment with the two primers A and C (Fig. 1, A and B). Chimeras were obtained from three independent mutant ES clones and mated with BALB/c females to generate F1 heterozygous mutant mice. Intercrosses between heterozygotes yielded the homozygous mutants. Wild type mice, heterozygotes, and homozygotes were born with the expected 1:2:1 ratio, but the homozygous mutant mice died immediately after birth. After birth, 31 of a total of 326 offspring, 243 breathed regularly, made movements, arched their backs, and turned pink after ventilation of their lungs. Genotype analyses of these mice by Southern blot analysis indicated that 85 were wild type and 158 were heterozygous for the ATF-2 mutated allele. However, none were homozygous for the mutated allele. In contrast, 83 pups exhibited respiratory distress, cyanosis, and subsequently died within 10 min (Fig. 2A). The shape, size, and body weight of these pups were similar to those of the wild type. Genotyping of the dead pups revealed that these pups were all homozygous for the ATF-2 mutated allele. Thus, the ATF-2 mutation results in perinatal recessive lethality. The same result was obtained with homozygous mutants generated from two other independently derived ATF-2-defective ES cell lines, regardless of whether they had been crossed with BALB/c, C57BL/6, or ICR mice. From these results, we conclude that homozygous ATF-2-/- mutant mice are able to develop to term but die shortly after birth as a result of respiratory failure.

This phenotype of the ATF-2-/- homozygotes differed from that we previously reported for the ATF-2-m/m mice. Approximately half of ATF-2-m/m mice survived over one month and exhibited complex anomalies including a defect in endochondral ossification at the epiphyseal plates, an ataxic gait, and reduced numbers of cerebellar Purkinje cells (16). The ATF-2-m/m mutant was generated by inserting the neomycin cassette into the exon encoding amino acids 277–326. However, domain analyses of the ATF-2 protein indicated that the region between amino acids 155 and 338 can be deleted without a loss of trans-activating capacity (22). The difference in the phenotypes of the two independently generated lines of ATF-2 mutant mice suggested that a certain amount of a splice form of ATF-2 protein that we had failed to detect originally may be generated in the ATF-2-m/m mice. Reverse transcriptase-PCR analysis of ATF-2-m/m RNA revealed a transcript that, upon sequencing, contained an in-frame splicing that removed the exon disrupted by the neomycin cassette. The mRNA species expressed in ATF-2-m/m encoded the ATF-2 protein lacking the region between amino acids 277 and 326 (Fig. 1C, lower panel).

To examine whether a mutant protein was made from this transcript, whole cell lysates were prepared from the brains of...
wild type, ATF-2\textsuperscript{m/m}, and ATF-2\textsuperscript{o/o} homozygous mutant fetuses of 18.5 dpc and used for Western blotting (Fig. 1, upper panel). In the wild type lysates, the two forms of ATF-2 protein, consisting of 70- and 62-kDa polypeptides (Fig. 1C, bands 1 and 2), were detected. Although these two forms were not detected in either the ATF-2\textsuperscript{m/m} or ATF-2\textsuperscript{o/o} homozygotes, a novel 66-kDa protein was detected in the ATF-2\textsuperscript{m/m} lysate (Fig. 1C, band 3). These results indicate that the ATF-2\textsuperscript{o/o} mutant described here is a null mutant, whereas the previously described ATF-2\textsuperscript{m/m} mutant contains a mutant protein. It is likely that the use of much larger amounts of extract permitted the detection of this mutant protein, which we\textsuperscript{2} had previously failed to detect. We conclude that small amounts of a mutant ATF-2 protein are sufficient to protect at least some ATF-2\textsuperscript{m/m} mice (but not all, because the viability of the ATF-2\textsuperscript{m/m} mice is only 50%) from death shortly after birth by respiratory failure, and the surviving ATF-2\textsuperscript{m/m} mice allowed an assessment of the role of ATF-2 in other organ systems. Interestingly, when ATF-2\textsuperscript{m/+} and...
ATF-2<sup>−/−</sup> mice were mated, the compound ATF-2<sup>−/−</sup> heterozygotes displayed the same phenotype (neonatal lethality from respiratory failure) as the ATF-2<sup>−/−</sup> homozygotes, demonstrating a gene dosage effect of the ATF-2<sup>−/−</sup> allele. Thus, a 50% reduction in levels of the ATF-2 mutant protein in the compound heterozygote results in the emergence of neonatal lethality, demonstrating exquisite sensitivity of the phenotype to levels of ATF-2.

To understand the mechanism of impaired respiratory function, the lungs prepared from neonates were analyzed. Careful histological analyses of the ATF-2<sup>−/−</sup> mutant lung sections indicated the presence of meconium in the alveoli, showing that prenatal aspiration of the amniotic fluid containing meconium had probably occurred (Fig. 2B). Consistent with this, the wild type lung floated on PBS, but the mutant lung sank, indicating absence of air in the alveoli of the mutant lung at birth (Fig. 2C). Pulmonary histology showed normal architecture of the mutant lung including the presence of branching, alveolization, septation, and alveolar volume. We examined the expression level of the four surfactant proteins, which are important for the mechanical stability of the lung (for review, see Ref. 23).

Although the level of surfactant protein B expression was decreased to half of that of the wild type in the ATF-2<sup>−/−</sup> mutant, the other three surfactant genes were expressed at the same level as that of the wild type (data not shown).

**Hypoxia and a Reduced Number of Cytotrophoblast in Mutant Placentas**—It has been observed that hypoxia in embryos may lead to strong gasping respiration with consequent aspiration of the amniotic fluid containing meconium (1–3, 24). Therefore, we examined whether hypoxia occurs in the mutant embryos. RNA was prepared from various tissues of the wild type and ATF-2<sup>−/−</sup> embryos at E18.5 dpc, and the expression levels of VEGF and tyrosine 3-hydroxylase genes, which are known to be induced by hypoxia (25, 26), were examined by RNase protection (Fig. 3A). The levels of VEGF mRNA in the mutant brain and liver were 4- and 23-fold, respectively, higher than those of the wild type. In addition, the tyrosine 3-hydroxylase mRNA level in the mutant brain was 26-fold higher than that of the wild type. These results suggest that hypoxia occurs in the mutant embryos. In the case of ATF-2<sup>−/−</sup> embryos, a similar induction of VEGF and tyrosine hydroxylase mRNA was observed only in the limited number of embryos (data not shown). This is consistent with the fact that the limited number of ATF-2<sup>−/−</sup> mutant mice also died immediately after birth like ATF-2<sup>−/−</sup>.

To investigate the mechanism of hypoxia in 18.5-dpc embryo, we histologically analyzed the wild type and mutant embryos. No detectable differences in the histological structures of any of the organs could be detected, including the central nervous system, where ATF-2 is expressed at the highest level (15), and the cardiovascular system (data not shown). To examine whether some abnormality in the central nervous system leads to the phenotype of the ATF-2<sup>−/−</sup> mutants, we made transgenic mice expressing ATF-2 from the neurofilament promoter in the central nervous system. However, transgenic ATF-2<sup>−/−</sup> mice were not rescued from postnatal lethality (data not shown).

A histological comparison of E18.5 placentas from wild type mice and homozygous mutants revealed a slight but significant alteration in the labyrinth region of the mutant placentas. The labyrinth region in the normal placenta has numerous fine embryonic vessels surrounded by trophoblast epithelial cells that are bathed in maternal blood. In the labyrinth region of the mutant placenta, the number of cytotrophoblast cells was clearly lower, although the size of the region was almost the same as that of the wild type (Fig. 3B). The average number of cytotrophoblast cells/mm<sup>2</sup> obtained by examining the 18 placentas was 465 ± 52, 483 ± 40, and 216 ± 17 in the wild type, heterozygous, and homozygous placentas, respectively. Furthermore, the network of embryonic vessels and maternal sinuses was poorly developed, and fibrinoid had accumulated around the fetal blood vessels (data not shown). The number of spongiotrophoblast cells of the junctional zone, which produces hormones and growth factors, was approximately the same in the mutant and normal placentas. The expression of ATF-2 in the trophoblast was confirmed by immunostaining using the anti-ATF-2 polyclonal antibody (Fig. 3C), suggesting the idea that ATF-2 is required for the normal proliferation of trophoblasts.

In most of the ATF-2<sup>−/−</sup> mutant placentas, a decrease in number of spongiotrophoblast cells was not evident, and the faint but significant signal of ATF-2 immunostaining was detected (data not shown). However, the placental defect similar to that of ATF-2<sup>−/−</sup> was also detected in the limited number of ATF-2<sup>−/−</sup> mutant placentas (data not shown). This is consistent with the fact that some ATF-2<sup>−/−</sup> pups died immediately after birth like ATP-2<sup>−/−</sup> pups. From approximately E11 onwards, the labyrinth region of the placenta starts to function as a nutrient and oxygen transport unit (27). A decrease in the number of cytrophoblastic cells was not observed at early stages such as E14.5 (Fig. 3B, right panels). This is consistent with the observation that the sizes of the mutant embryos were almost the same as those of the wild type. The reduced number of cytrophoblast cells at the late stage may lead to an insufficient oxygen supply, resulting in gasping respiration and aspiration of the amniotic fluid containing meconium.

**PDGF Receptor α Is a Target of ATF-2**—To identify the gene(s) regulated by ATF-2 in cytrophoblast cells, we employed a cDNA subtraction method. Total RNA was prepared from the placenta of wild type and ATF-2<sup>−/−</sup> homozygous embryos of E18.5 dpc and used for cDNA subtraction analysis. The expression levels of the genes identified by the cDNA subtraction method were further examined by Northern blotting. Five genes were found to be down-regulated in the mutant, and one of them was the PDGF receptor α gene. PDGF receptor α has been reported to be expressed in a subpopulation of cytrophoblasts and to be required for their proliferation (28). On the other hand, six genes up-regulated in the ATF-2<sup>−/−</sup> mutant were identified, and two of them, such as the glucose transporter and cytokeratin genes, are known to be hypoxia inducible (29, 30). Among them, however, there was no obvious candidate that might be expected to block the proliferation of trophoblasts.

In addition to cDNA subtraction analysis, we also used the microarray-based expression monitoring. Microarrays containing 18,378 nonredundant mouse cDNA clones chosen from the I.M.A.G.E. collection, which is thought to be 20% in the mouse genome, were hybridized with the cDNA probes prepared using the wild type and ATF-2<sup>−/−</sup> placenta RNAs. Because the placental tissue prepared from ATF-2<sup>−/−</sup> homozygous embryos should contain a significant amount of ATF-2<sup>−/−</sup> maternal tissue, we speculated that the difference in the expression level of ATF-2 target genes detected by this method is not so high. Therefore, we picked up the genes whose expression level is different more than 2.5-fold between the wild type and ATF-2<sup>−/−</sup> homozygous mutant. Two hundred fifty-three genes were found to be down-regulated in the mutant, and 78 genes among them encoded the known proteins. One of them was the PDGF receptor α gene, and the difference in the PDGF receptor α expression level between wild type and mutant was 2.7-fold. Although some other genes in this group, including the hepatocyte growth factor activator-like protein and the ect2 oncogene, also appear to be involved in the regulation of cellular proliferation, no evidence was reported so far indicating that these genes are
involved in the proliferation control of cytotrophoblast cells. On the other hand, 213 genes up-regulated in the ATF-2/−/− mutant were identified, and 54 genes among them encoded the known proteins. This group contains the hypoxia inducible genes encoding glucose transporter-3, epidermal growth factor-like growth factor, GADD45, and insulin-like growth factor-II. Among them, however, there was no obvious candidate that might be expected to block the proliferation of trophoblasts.

Because the PDGF receptor α gene was identified as a candidate of the target gene of ATF-2 by using two different methods, we examined the expression of the PDGF receptor α gene in wild type and mutant placentas using immunostaining and

FIG. 3. Hypoxia and a reduced number of cytotrophoblast in mutant placentas. A, increased levels of hypoxia inducible mRNAs in the mutant embryos. Total RNA was prepared from various tissues of wild type and ATF-2/−/− homozygous mutants. RNase protection was performed using probes from the VEGF and tyrosine hydroxylase (TH) genes whose expression is induced by hypoxia. The 181- and 192-nucleotide bands were detected with the VEGF probe, whereas the tyrosine hydroxylase probe generated the 161-nucleotide band. The cytoplasmic β-actin probe that gave rise to the 250-nucleotide band was used as a control. The density of the protected bands was normalized with respect to that of the β-actin gene, and the relative amounts are indicated as a bar graph. The shaded bar indicates the data obtained with mutant RNA. B, reduced number of cytotrophoblasts in the 18.5-dpc mutant placentas. Left panels, low power magnification (×5.2) of comparable sections through the wild type (+/+ ) and mutant (−/−) placentas at 18.5 dpc. L, labyrinth region. Middle panels, higher magnification (×200) views of the left panels. The number of cytotrophoblast cells is reduced in the mutant. Right panels, high power magnification (×200) views of sections through the 14.5-dpc placentas. C, expression of ATF-2 in the wild type cytotrophoblast cells. Expression of ATF-2 in cytotrophoblast cells of 18.5-dpc wild type (+/+ ) or mutant (−/−) placentas was examined by immunostaining (×50).
in situ hybridization (Fig. 4A). The expression level of PDGF receptor α was significantly decreased in the mutant, although some PDGF receptor α expression still remained. This partial decrease in PDGF receptor α expression may explain the mild defects of the placenta in ATF-2 o/o homozygotes.

Although PDGF receptor α has been reported to be expressed in a subpopulation of cytotrophoblasts (28), we examined whether PDGF really stimulates the proliferation of cytotrophoblast-derived cells using a human choriocarcinoma cell line, Jar. PDGF-AA and PDGF-BB stimulated BrdUrd incorporation into Jar cells by 40.5 ± 6.7% and 72.0 ± 6.6% (mean ± S.E.), respectively (Fig. 4B). Although this degree of stimulation is not high, it is significant compared with the published data (31).

To further examine whether the promoter activity of the PDGF receptor α gene is directly regulated by ATF-2, we performed co-transfection experiments. A luciferase reporter plasmid, in which the human PDGF receptor α promoter was linked to the luciferase gene, (21) was co-transfected into Chinese hamster ovary cells along with the ATF-2 expression plasmid (Fig. 4C). ATF-2 increased luciferase gene expression about 2.7-fold. In contrast, the ATF-2 mutant (ΔBR), in which the basic region of the b-zip DNA-binding domain was deleted, did not enhance the activity of the PDGF receptor α promoter. Another mutant (C32S), in which the metal finger structure in the NH2-terminal activation domain was disrupted by changing the cysteine residue at amino acid 32 into serine, also did not stimulate the luciferase expression. The modest activation of the PDGF receptor α promoter by wild type ATF-2 is consistent with the partial decrease in PDGF receptor α expression in the ATF-2 o/o homozygotes. Consistent with the phenotype of ATF-2 o/o embryos, the ATF-2 protein lacking the region between amino acids 271 and 338 (Δ 271/338), which is similar to the ATF-2 protein expressed in ATF-2 2m/m mutant,
retained the capacity to enhance the PDGF receptor α promoter activity. These results indicate that ATF-2 activates the PDGF receptor α promoter. Although the PDGF receptor α promoter region contains the sequence suitable for binding to ATF-2, we cannot exclude the possibility that ATF-2 indirectly activates this promoter.

**DISCUSSION**

All of the ATF-2 null mutants examined displayed symptoms of severe respiratory distress with the lungs filled with meconium and had the reduced number of cytotrophoblast cells in the placenta. Although both PDGF receptor α and ATF-2 are expressed in various tissues other than placenta, only the placental defect was evident during embryogenesis. CRE-BPα and ATF-a, which have a striking homology with ATF-2 and form a subgroup in the ATF/CREB gene family (8, 9), may compensate for the lack of ATF-2 in other tissues. Aspiration of the amniotic fluid containing meconium is observed in MAS, which is a common neonatal problem that results in acute and chronic respiratory morbidity (1, 2). Although human MAS has been speculated to be caused by multiple mechanisms, no direct evidence exists to show that a deficiency in any one gene leads to MAS. MAS exhibits a varying severity, but mortality occurs rarely in the human infant and not usually within minutes of birth. In this sense, the phenotype of ATF-2 null mutant is apparently different from a typical type of MAS. However, it should be noted that the human infants can be treated by appropriate suctioning at birth or by early administration of surfactant, whereas the ATF-2 mutant mice could not be treated because of a lack of appropriate systems. In addition, a severe form of human MAS, which cannot be prevented by these treatments, shows features similar to those of ATF-2 null mutants. Interestingly, the placentas of some severe MAS infants showed significant abnormalities (3), suggesting that at least some type of MAS may be a prenatal rather than a postnatal disease. In addition to the presence of fluid, the level of surfactant protein B in the ATF-2 mutant lungs was decreased to half of the level of the wild type. The decrease in the surfactant protein level is associated with respiratory distress syndrome (23). Thus, the ATF-2 null mutants appear to assume the aspects of multiple syndromes of respiration failure. The ATF-2 null mutants described here will be useful to understand the mechanism of severe neonatal respiratory distress and should lead, eventually, to the development of new diagnostic tools and therapies for this type of disease.

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