9-15-2003

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Developmental Biology 261 (2003) 313–323

DEVELOPMENTAL BIOLOGY

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A null mutation for *Tissue Inhibitor of Metalloproteinases-3* (*Timp-3*) impairs murine bronchiole branching morphogenesis

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Received for publication 17 December 2002, revised 15 May 2003, accepted 16 May 2003

Abstract

Tissue inhibitors of metalloproteinases (TIMPs) regulate extracellular matrix (ECM) degradation by matrix metalloproteinases (MMPs). We have examined the role of TIMP-3 on ECM homeostasis and bronchiole branching morphogenesis during murine embryogenesis. Employing an in vitro organ culture system, we found decreased bronchiolar branching in null lungs when compared with wild type (WT) counterparts after 2 days in culture. When a synthetic inhibitor of MMPs at low dose was added to the culture system, branching was augmented regardless of genotype. Gelatin and in situ zymography revealed that null lungs exhibited enhanced activation of MMPs throughout lung development. We analysed the impact of increased MMP activity on a number of ECM molecules by Western blot analysis, but found that only fibronectin abundance was consistently reduced in the null lungs throughout development. To confirm that our observed defect in culture was not simply a developmental delay in the null lung, we examined null and WT lungs from newborn pups. Here, we found not only a reduced number of bronchioles in the null, but also that the bronchiole tubes were dilated compared with controls and that alveologenesis was attenuated. We propose that the deletion of TIMP-3 disrupts the exquisite TIMP/MMP balance required for proper focal ECM proteolysis, which leads to correct bronchiole branching morphogenesis in the developing mouse lung.

Keywords: TIMP; MMP; ECM; Lung development; Epithelial–mesenchyme interactions

Introduction

The mouse lung originates on embryonic day 9.5 (Ed9.5) as paired endodermal buds from the primitive foregut invade into surrounding mesoderm. The epithelium develops into the airways, while the mesoderm becomes the lung stroma. Between Ed9.5 and Ed15.5 there is rapid growth and branching of the primitive lung epithelium to form the bronchiole pattern of the lung (Ten Have-Opbroek, 1991). This branching morphogenesis results from a repetitive series of epithelial cleft and bud formation, and is dependent on the surrounding mesenchyme and the basement membrane (BM) that separates the two compartments. Elegant grafting experiments demonstrated that distal mesenchyme, when grafted next to denuded tracheal epithelium, could induce multiple branches from a supernumerary tracheal bud. Thus, the mesenchyme has an inductive action upon an epithelium which would not normally bud and branch (Alescio and Cassini, 1962).

Several lines of evidence suggest that remodeling of the BM has an active role in the induction of branching. For example, the BM separating epithelial and mesenchymal cells at the tips of actively budding regions becomes relatively thin with reduced amounts of nidogen, collagen IV, fibronectin, and laminin-1 (Mollard and Dziadek, 1998). Specific domains of the laminin-1 molecule may be involved in eliciting a branch outgrowth by facilitating cell attachment and consequently cell proliferation since neutralizing anti-laminin antibodies inhibit branching in lung organ culture (Schuger et al., 1991). Fibronectin, an extracellular matrix (ECM) molecule found predominantly in the

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doi:10.1016/S0012-1606(03)00318-X

clefts of the developing lung (Roman et al., 1997), is possibly involved in the regulation of bronchial branching morphogenesis as reagents that inhibit fibronectin matrix assembly also caused decreased branching (Roman et al., 1991). Culture of embryonic lung single cell suspensions in Matrigel, a synthetic BM, can lead to organotypic rearrangement of the cells into structures that closely resemble those found in vivo, which reinforces the concept that BM is a major regulator of organogenesis in the lung (Schuger et al., 1990). These data imply a fundamental role for epithelial–BM–mesenchymal interactions during lung organogenesis and airway branching.

Controlled remodeling of the ECM is required for tissue morphogenesis during development (Vu and Werb, 2000), and this remodeling is achieved, in part, by the activities of matrix metalloproteinases (MMPs). Developmental expression profiles during the second half of mouse gestation have been documented for only a few MMPs. For example, the *Mmp-2* mRNA is expressed in mesenchymal cells in 14–15 day lung (Reponen et al., 1992) and *Mmp-11* is expressed in lung on Ed14.5 through Ed16.5 in mesenchymal cells lining the BM (Lefebvre et al., 1995). The *Mmp-9* mRNA is expressed in the primordial alveoli at Ed15 and in the bronchiole tree on Ed13 and Ed15 (Canete-Soler et al., 1995), placing the MMP-9 protein in a prime position to mediate BM thinning associated with bud outgrowth of the bronchiolar tree. Recent work with mice deficient for the epidermal growth factor receptor (EGFR) has implicated MMP-2 along with MMP-14 (MT1-MMP) in the process of lung branching morphogenesis (Kheradmand et al., 2002). EGFR null mice demonstrated decreased abundance of MMP-14 (MT1-MMP) leading to decreased activation of MMP-2, which, in turn, contributed to impaired branching observed in the null lungs.

One of the functions of tissue inhibitors of metalloproteinases (TIMPs) is to regulate ECM degradation by MMPs. The expression profiles for *Timps-1*, *-2*, and *-3* in developing lung have been previously documented. The *Timp-1* mRNA is expressed in lung on Ed15 and Ed17 and increases by Ed18 (Nomura et al., 1989), while *Timp-2* is expressed by mesenchymal tissues surrounding developing epithelia from Ed10.5 through Ed18.5 (Blavier et al., 1997). Alternatively, abundant *Timp-3* expression is seen in the developing bronchiole tree at Ed12.5 and Ed14.5, but not in the mesenchyme. In the newborn mouse, *Timp-3* expression is predominantly in cells of the pulmonary stroma (Apte et al., 1994). This unique expression pattern in the developing lung, and the fact that TIMP-3 is the only TIMP family member known to bind to the ECM (Leco et al., 1994; Yu et al., 2000), places the TIMP-3 protein in an ideal location to control the degradation of bronchiole ECM and therefore possibly contribute to the control of airway branching.

Recently, we developed a mouse line that is deficient for TIMP-3 (Leco et al., 2001). The TIMP-3 null animals spontaneously develop progressive alveolar air space enlargement similar to that seen in human emphysema, with an impaired capacity for gas exchange and premature morbidity and mortality. The air space enlargement was observed as early as 2 weeks of age, which implies a congenital abnormality in lung development in the TIMP-3 null animals. We also observed a significant difference between null and control animals in the distribution of terminal bronchiole branches relative to the lung pleura, which further indicates a role for TIMP-3 in murine lung morphogenesis (Leco et al., 2001).

In the present study, we report that the deletion of TIMP-3 results in decreased bronchiole branching in null animals beginning as early as Ed12.5 of gestation. The results indicate that this defect stems from enhanced MMP activation in the absence of TIMP-3 throughout murine lung development. The role of MMPs in the defect was corroborated by the inclusion of a synthetic inhibitor of metalloproteinases during 48 h of culture of Ed11.5 lung rudiments. At low dose of inhibitor, branching was augmented in lung cultures, indicating that substitution of TIMP-3 by a synthetic inhibitor could rescue the defect. Of the BM molecules examined by Western blot analysis, only fibronectin demonstrated a reduced abundance in the null lungs during development in vivo. Immunohistochemistry with fibronectin antiserum revealed this reduction of fibronectin was localised to the BM surrounding bronchioles and in the stromal ECM and was presumably due to uninhibited MMP activity. These findings imply an essential role for TIMP-3 in the protection of the ECM from degradation by MMPs during lung branching morphogenesis and provide further support of an indispensable function for the ECM in directing pattern formation throughout lung development.

Materials and methods

Mice

A full description of the generation of the TIMP-3 null mice used in this study was reported by Leco and coworkers (2001). For these experiments, the mutant allele was backcrossed seven generations onto the C57/Black6 strain of mice for clone seven, and back-crossed six times onto the C57/Black6 strain of mice for the independently targeted clone eight. In the last backcross for each line, *Timp-3* homozygous null male animals were crossed with wild type (WT) female C57/Black6 to produce *Timp-3* heterozygous offspring. Non-brother/sister matings then produced both WT and null animals which were crossed in like genotype, non-brother/sister matings to produce the WT and null animals used in these studies. In some experiments, where appropriate, heterozygotic matings produced timed litters containing all genotypes. The TIMP-3 null line(s) used for each experiment are identified in legends to figures.

Timed pregnancies

WT crossed with WT, *Timp-3* heterozygote crossed with heterozygote, and *Timp-3* null crossed to null mice were employed for the generation of timed pregnancies with the day of discovery of a vaginal plug being considered embryonic day 0.5 (Ed0.5). Tail biopsies were performed on all litters to provide DNA for PCR genotyping and establishment of embryonic genotype (not shown). Pregnant dams were sacrificed at desired time points (Ed11.5 to Ed17.5), and the embryos were removed and placed into phosphate-buffered saline (PBS; Life Technologies Inc., Burlington, ON, Canada). Newborn animals were sacrificed at post partum day 1 (PP1). Lungs were removed from the embryos or pups and then rinsed in fresh PBS. Lungs were either placed into organ culture, snap frozen on dry ice for protein analysis, placed in embedding compound (Stephens Scientific, Riverdale, NJ, USA) and submersed in isopentane cooled on dry ice for fresh frozen sectioning, or fixed with 4% PBS-buffered paraformaldehyde for 3 h at 4°C, rinsed in PBS, dehydrated through an alcohol series, and embedded in paraffin wax for sectioning.

Embryonic lung organ culture

Lung buds were removed from WT, heterozygote, and null mouse embryos on Ed11.5 of gestation and placed into serum-free, BGJ_b medium supplemented with antibiotic and antimycotic (Life Technologies Inc.). The lungs were placed onto culture inserts with 8-μm pores using a glass pipette and cultured at the air-medium interface for 48 h at 37°C and under a 5% CO_2 in air atmosphere. Images of lungs were captured at 0, 24, and 48 h by using a SMZ1500 dissecting microscope, a DXM1200 digital camera, and ACT-1 software (Nikon Canada, Mississauga, ON, Canada). For experiments using a synthetic MMP inhibitor, GM6001 (Chemicon International, Temecula, CA, USA), lungs were cultured under four conditions: 2.5 μM GM6001 in BGJ_b medium, 20 μM GM6001 in BGJ_b medium or an equivalent volume of dimethylsulfoxide (DMSO, vehicle for GM6001) for each of the GM6001 concentrations in BGJ_b medium. Use of either volume of DMSO did not significantly influence lung branching compared to media alone, and thus DMSO data were pooled.

Quantification of lung bronchiole branching

Extent of peripheral terminal bronchiole branching in vitro was expressed according to the following equation (adapted from Schuger et al., 1991):

$$\frac{\text{number of terminal bronchiole buds in null lung at 48 h} - \text{number of buds in null lung at 0 h}}{\text{number of terminal bronchiole buds in WT lung at 48 h} - \text{number of buds in WT lung at 0 h}} \times 100.$$

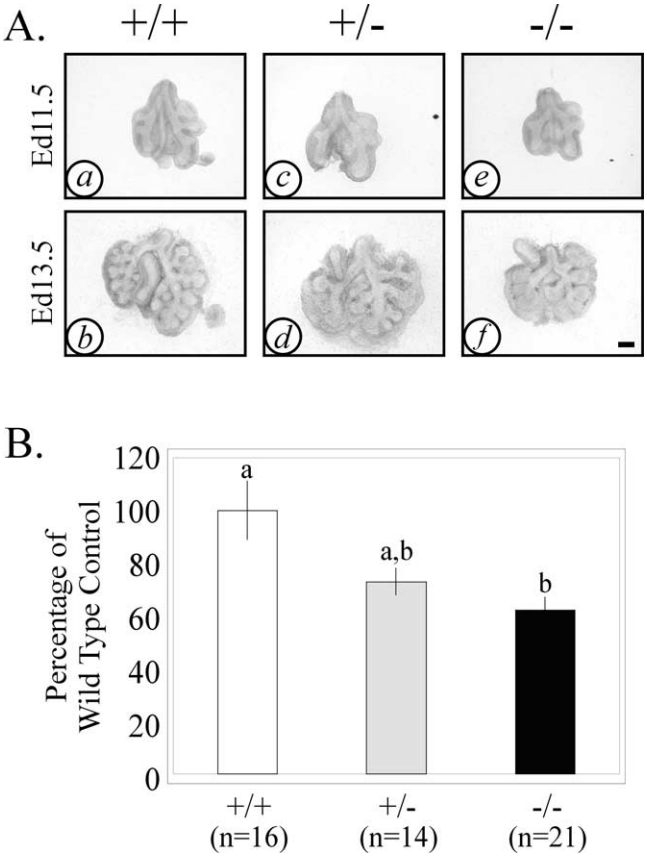

Fig. 1. Impaired bronchiole branching in TIMP-3 null lungs in vitro. (A) Embryonic day 11.5 (Ed11.5) lungs at time zero of culture (a,c,e); lungs after 48 h in culture (b,d,f). TIMP-3 null lungs demonstrated impaired bronchiole branching and dilated terminal buds (f) when compared with wild type counterparts (b), while heterozyote lungs (d) had an intermediate number of branches. Scale bar, 200 μm. (B) Quantification of the number of peripheral terminal bronchiole buds after 48 h in culture, expressed as a percentage of the wild type control (+/− SEM). Both heterozygote and null lungs showed decreased branching when compared with wild type lungs; however, only the null lungs showed a significant decrease (one-way ANOVA; $P < 0.05$). Data for this experiment were collected from both independently derived clones (clone 7 and clone 8).

Images of cultured lungs were digitally captured and the peripheral terminal buds were counted by two individuals who were blinded as to the genotype of the lung. A one-way ANOVA with a Tukey's post-hoc test was used to determine the statistical significance between genotypes (Fig. 1), and a two-way ANOVA with a Tukey's test was used to determine the statistical significance in the GM6001 experiments (Fig. 2).

Gelatin zymography

Lung homogenates were obtained from a developmental series of murine WT and null lungs (Ed13.5–Ed17.5 and PP1) by grinding frozen lungs in extraction buffer (50 mM Tris, 150 mM NaCl, 1% SDS plus one EDTA-free proteinase inhibitor cocktail pill per 10 ml buffer; Roche Diagnos-

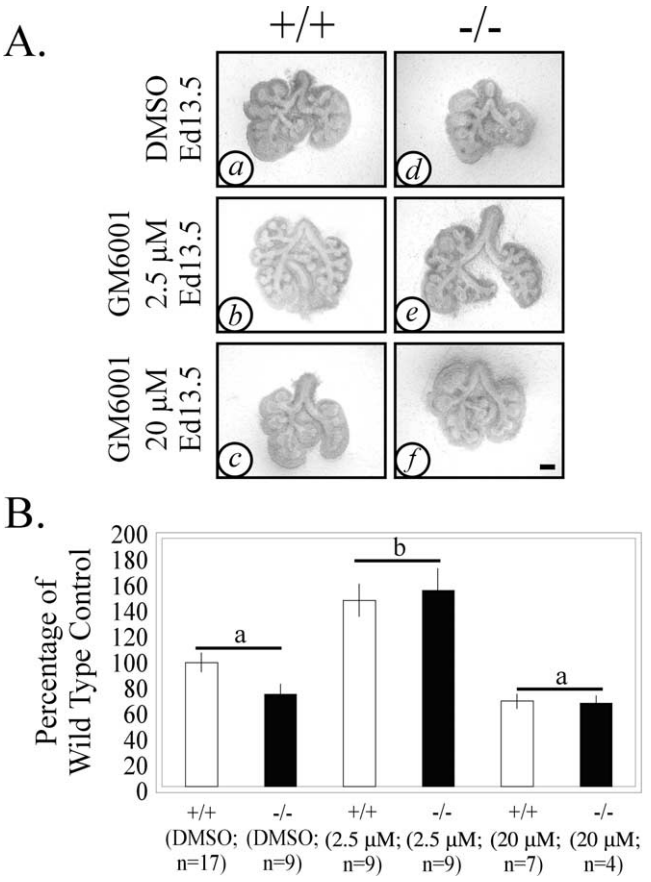

Fig. 2. Inhibition of MMPs effects lung branching morphogenesis in vitro. (A) Addition of DMSO (vehicle for GM6001) had no effect on lung cultures, with impaired branching observed in null lungs after 48 h of culture, as in Fig. 1; wild type (a) versus null (d). Low concentration of GM6001 (2.5 μM) led to an enhanced branching in both wild type (b) and null lungs (e), while high concentration of GM6001 (20 μM) led to impaired bud formation in both genotypes; wild type (c), null (f). Scale bar, 200 μm. (B) Quantification of peripheral terminal lung buds after 48 h in culture expressed as a percentage of the wild type control (+/− SEM). Treatment of lungs with low concentration of GM6001 led to significantly increased bronchiole branching (two-way ANOVA; $P < 0.05$) regardless of genotype. Data for this experiment were collected from both independently derived clones (clone 7 and clone 8).

tics, Mannheim, Germany). Protein content of the homogenate was determined by the method of Bradford (BioRad Laboratories Inc., Hercules, CA, USA). Equal amounts of protein (50 μg each) were loaded on a 12% polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, gels were washed twice for 45 min in 2.5% Triton X-100, rinsed in water, and incubated in substrate buffer (50 mM Tris, 5 mM $CaCl_2$, 5 μM $ZnCl_2$) for 18 h at 37°C. Gels were stained in Coomassie R350 (BioRad Laboratories Inc.) for 1 h, destained for 20 min, and then allowed to equilibrate in water overnight.

Gelatin in situ zymography

Gelatin in situ zymography was performed on fresh frozen sections (cut at 7μm) as previously described (Leco et al., 2001). In order to demonstrate that proteolysis was due to metalloproteinase activity, WT and null tissue sections were overlayed with substrate containing 5 mM EDTA as a negative control. Images of experimental sections were captured after first reducing the exposure time to yield a dark image with the negative control (EDTA) sections. Once set, light intensity, exposure time, and the filter used (FITC; 460–500 nm) were identical for all samples. Areas of fluorescence are indicative of gelatinolytic activity.

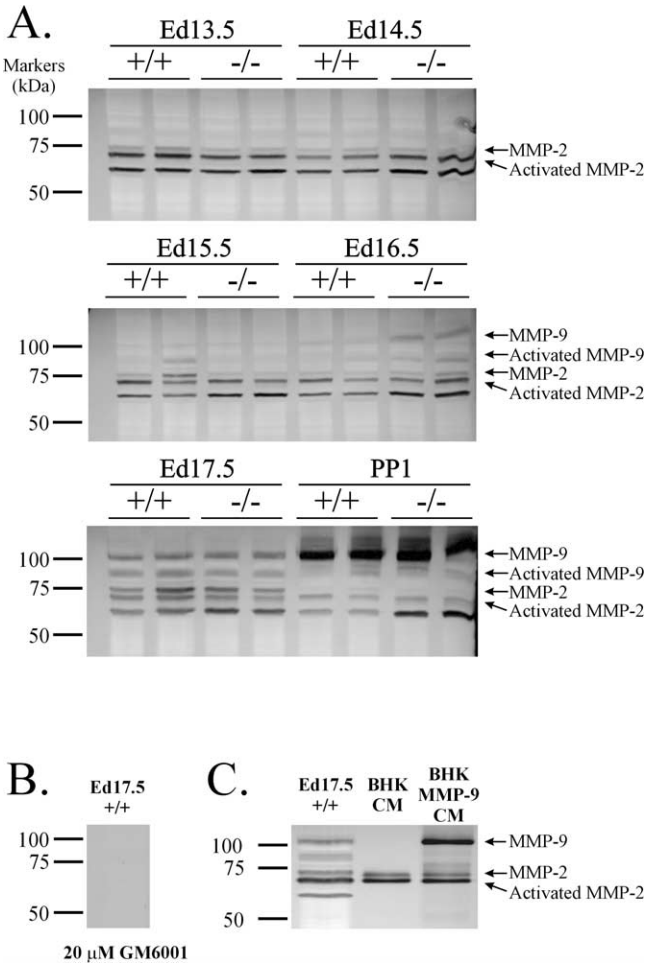

Fig. 3. Zymographic analysis of wild type and null lung tissue homogenate from Ed13.5 through postpartum day 1 (PP1), inverse images shown. (A) Increased abundance of a gelatin degrading activity (approximately 55 kDa) was apparent in null lungs throughout development, becoming more prevalent in later stages (Ed16.5 to PP1). Abundance of latent MMP-9 (105 kDa) was also elevated in null lungs at PP1. (B) Incubation of a sample lane with GM6001 confirmed that gelatin digestion in all of the bands was due to metalloproteinase activity. (C) Control conditioned media from baby hamster kidney cells (BHK CM; source of MMP-2) or conditioned media from BHK cells transfected with an MMP-9 cDNA (BHK MMP-9 CM) were compared on a gelatin zymograph to wild type Ed17.5 lung extracts to distinguish which bands represented MMP-2 and MMP-9. The identity of the lowest molecular weight band has not been confirmed. Lungs from clone 8 animals were used in these experiments.

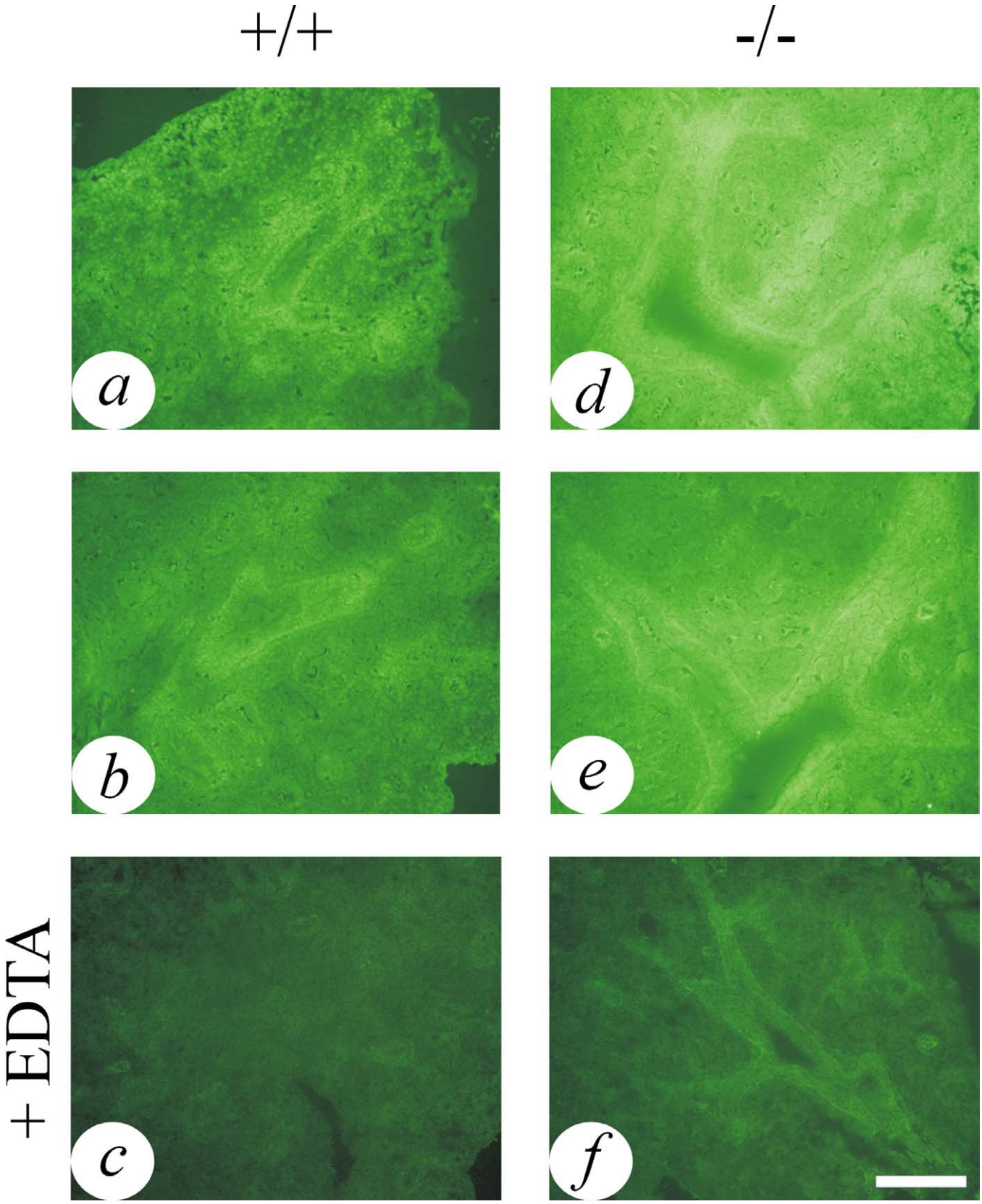

Fig. 4. Enhanced lung metalloproteinase activity in the absence of TIMP-3. Increased gelatinolytic activity was observed at Ed15.5 by in situ gelatin zymography in both the mesenchyme and the epithelium surrounding developing bronchioles in TIMP-3 null lungs (d, e; $n = 10$) when compared with wild type lungs (a, b; $n = 10$). Fluorescence indicates gelatinolytic activity. Incubation of wild type and null lungs with EDTA reduced the fluorescence to negligible levels confirming that the gelatinolytic activity was due to metalloproteinases (c, f). Scale bar, 50 μm. Lungs from clone 8 animals were used in these experiments.

Western blot analysis

Western blot analysis was performed on lung homogenates from WT and null lungs (Ed13.5–Ed17.5 and PP1). Lungs were homogenised in extraction buffer (50 mM Tris, 150 mM NaCl, 1% SDS, 0.1 M β-mercaptoethanol plus one EDTA-free proteinase inhibitor cocktail pill per 10 ml buffer; Roche Diagnostics), boiled for 3 min, protein content determined, and 50 μg of total protein from each sample loaded per lane. The samples were electrophoresed on 4–20% Tris–HCl gradient SDS-PAGE gels (BioRad Laboratories Inc.). Protein was transferred to nitrocellulose membranes (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) and filters were blocked with 5% skim milk in Tris-buffered saline (TBS). Individual blots were incubated with antibodies directed against fibronectin (rabbit anti-mouse polyclonal; 1:5,000 dilution; DAKO Diagnostics Canada Inc., Mississauga, ON, Canada), collagen type-I (rabbit anti-mouse polyclonal; 1:1000 dilution; Chemicon International), laminin (rabbit anti-mouse polyclonal; 1:1000 dilution; DAKO), elastin (rabbit anti-mouse polyclonal; 1:1000 dilution; Elastin Products Inc, Owensville, MO, USA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; mouse monoclonal; 1:10,000 dilution; Cedarlane Laboratories Limited, Hornby, ON, Canada) diluted in TBS-T (TBS and 0.1% Tween 20) plus 0.5% skim milk. After washing in TBS-T, blots were incubated with anti-mouse or anti-rabbit secondary antibodies conjugated with horseradish peroxidase (1:5,000 or 1:10,000 dilution for mouse and rabbit, respectively; Sigma-Aldrich, St. Louis, MO, USA) diluted in TBS-T plus 0.5% skim milk. After washing in TBS-T, bands of interest were visualised with ECL chemiluminescence reagent (Amersham Pharmacia Biotech) using X-AR film (Eastman Kodak, New Haven, CT, USA).

Immunohistochemistry

Paraffin-embedded sections from WT and null embryonic lungs were cut at 7 μm, dewaxed in toluene, and rehydrated through an alcohol series. After blocking endogenous peroxidase activity with 3% H_2O_2 and antigen retrieval by pepsin digestion, sections were incubated with DAKO blocking solution. Sections were then incubated with fibronectin antiserum (1:100 dilution in DAKO blocking solution) or blocking solution alone (negative control). After washing in TBS-T, all sections were incubated with anti-rabbit secondary antibodies conjugated with horseradish peroxidase (1:200 dilution in DAKO blocking solution). After washing in TBS-T, sections were incubated with AEC substrate (ICN Biomedicals Inc., Aurora, OH, USA), counterstained with Mayer's hematoxylin (Sigma), and mounted under Crystal/Mount (Biomeda Corp., Foster City, CA, USA). Images were captured by using a Nikon E1000 microscope as described above.

Results

Impaired bronchiole branching in TIMP-3 null mice

The effect of TIMP-3 deletion on murine bronchiole branching morphogenesis was first examined in vitro. Embryos were removed from timed pregnant dams on Ed11.5 and lungs placed in organ culture. After 48 h in culture, bronchiole branching was significantly impaired in the null lungs ($P < 0.05$), which had 62% of the peripheral terminal buds when compared with WT lungs. Lungs from heterozygotic pups demonstrated an intermediate number of buds (Fig. 1A and B). The null terminal buds also had dilated ends when compared with their WT counterparts (Fig. 1A).

In an attempt to rescue the bronchiole branching defect seen in the null lungs, a synthetic inhibitor of metalloproteinases, GM6001, was added to the organ cultures. Addition of the vehicle for GM6001 (DMSO) to organ culture medium had no effect on bronchiole branching in WT or null lungs (Fig. 2). At the lower concentration of GM6001 (2.5 μM), there was significantly enhanced branching ($P < 0.05$) observed in both the WT and null lungs (150 and 158% of WT controls, respectively; Fig. 2B). When a higher concentration of GM6001 (20 μM) was used, we observed a decrease in bronchiole branching in the WT lungs (69% of WT controls; Fig. 2B); however, this result was not deemed significant.

Enhanced activation of MMPs in the absence of TIMP-3

To examine whether abundance and/or degree of activation of gelatinolytic MMPs in the developing lung had been affected by the deletion of TIMP-3, we employed gelatin zymography. Confirmation of the bands representing MMP-2 and -9 was achieved by comigration of bands with those in conditioned media samples from BHK cell cultures (MMP-2) or BHK cell cultures engineered to overexpress MMP-9 (Fig. 3C). Confirmation that all bands observed in the zymographs were metalloproteinases was achieved by incubation of a lane in the presence of 20 μM GM6001 (Fig. 3B).

No consistent difference in levels of latent or active MMP-2 or -9 was observed between genotypes at any of the time points examined, with the exception of elevated latent MMP-9 in newborn null lungs (PP1, Fig. 3A). However, the null lungs did show increased activity of an unidentified MMP running at an apparent molecular weight of approximately 55 kDa compared with their WT counterparts throughout development, with this difference becoming more apparent in the later stages (Ed16.5 to PP1; Fig. 3A). Two independently derived lungs for each time point are represented, and a negative image of the zymography gels is shown. This experiment was repeated two times with independently isolated embryos with similar results (not shown).

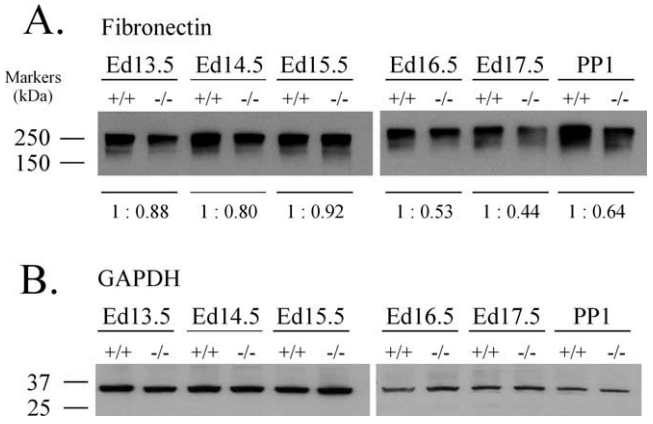

Fig. 5. Reduced abundance of fibronectin in the lungs of TIMP-3 null animals. (A) Western blot analysis of lung tissue homogenate from wild type or TIMP-3 null lungs demonstrated that null lungs had a decreased abundance of fibronectin compared with controls throughout development. Numbers below the fibronectin lanes represent the ratio of wild type lung fibronectin (set to one for each time point) compared with null lung fibronectin abundance after normalizing to the signal obtained for the loading control, GAPDH (B). Lungs from clone 8 animals were used in these experiments.

We next explored how presence or absence of TIMP-3 would impact MMP activity within intact lung tissue using gelatin in situ zymography (Fig. 4). We observed enhanced gelatinolytic activity associated with the epithelium of developing bronchi and throughout the mesenchyme of TIMP-3 null lungs. Two independently derived Ed15.5 lungs for each genotype are shown. Addition of a metal chelator (EDTA) to the assay reduced fluorescence to negligible levels confirming that degradation was due to metalloproteinase activity (Fig. 4c and f).

Decreased fibronectin in lungs of TIMP-3 mutant mice

Western blot analysis using antibodies directed against various ECM components was used to examine whether there was enhanced ECM degradation resulting from the deletion of TIMP-3. We examined lung protein extracts from a developmental series (Ed13.5 to PP1) and found that there were decreased amounts of fibronectin in the TIMP-3 null lungs throughout development (Fig. 5A). This experiment was repeated three times with independently isolated embryos with similar results (not shown). Blots were reprobed for GAPDH as a loading control (Fig. 5B). We also examined collagen type-1, collagen type-IV, laminin, and elastin, but were unable to detect differences between TIMP-3 WT and null lungs at the protein level (not shown).

In order to identify where fibronectin protein abundance was reduced in vivo, we performed immunohistochemistry with fibronectin antiserum. Using sections from Ed15.5 and Ed16.5, we observed reduced fibronectin staining associated with the terminal ends of bronchiole buds, as well as in cleft regions of the bronchiole tree in the TIMP-3 null sections (Fig. 6). Further, overall intensity of fibronectin staining was reduced throughout the lung mesenchyme (Fig. 6). A difference was observed at both time points; however, the reduction was more apparent in the null lung at Ed16.5, consistent with Western blot analysis.

Defects in bronchiole branching in vivo

We next investigated whether the organ culture phenotype resulted from a developmental delay by direct examination of PP1 null and WT lungs. Branching morphogenesis normally terminates between the pseudoglandular and canilicular phases of lung development, at around Ed16.5 (Ten Have-Opbroek, 1991). Therefore, we reasoned that examination of PP1 lungs would allow 2 extra days for the TIMP-3 null lungs to fully develop if there was a developmental delay. In whole-mount lung preparations, we observed not only fewer bronchioles in equivalent lobes of the lung, but also that the termini of these bronchioles were dilated in the null animals (Fig. 7A). Images of eight lungs for both genotypes were captured and examined, and in all cases, a consistent observation was made. Examination of PP1 lungs at the histological level, by hematoxylin and eosin staining of newborn lung sections, confirmed the whole-mount observations (Fig. 7B). Further, the degree of alveolarization was diminished in the null lungs compared to WT controls. Thus, the defects in branching morphogenesis that we observed in the lung organ culture experiments are not simply due to the culture environment, but are recapitulated in vivo.

Discussion

The role of the ECM in bronchiole branching morphogenesis is well established; however, the mechanisms regulating its involvement must still be elucidated. One current model of lung development proposes that a deposition of ECM components forms clefts which create a mechanical obstruction restricting the growing lung bud. The lung bud is then forced to expand around the cleft where there are no restrictions, thereby forming branches (Ganser et al., 1991; Mollard and Dziadek, 1998). This model is supported by evidence showing a thickening of the BM in cleft regions and a thinning of the BM at the tips of the growing buds. Further, epithelial cells in the regions of active budding demonstrate an increased proliferation when compared with regions of cleft formation (Mollard and Dziadek, 1998).

Remodeling of the ECM is believed to result from a shift in the balance between activated MMPs and TIMPs (Baker et al., 2002), and controlled remodeling of the ECM is required for tissue morphogenesis during development (Vu and Werb, 2000). As well, proteolytic activity of MMPs has been implicated in lung branching morphogenesis. For example, disruption of a functional EGF receptor impaired bronchiole branching (Miettinen et al., 1997) by a mechanism subsequently shown to involve the regulation of

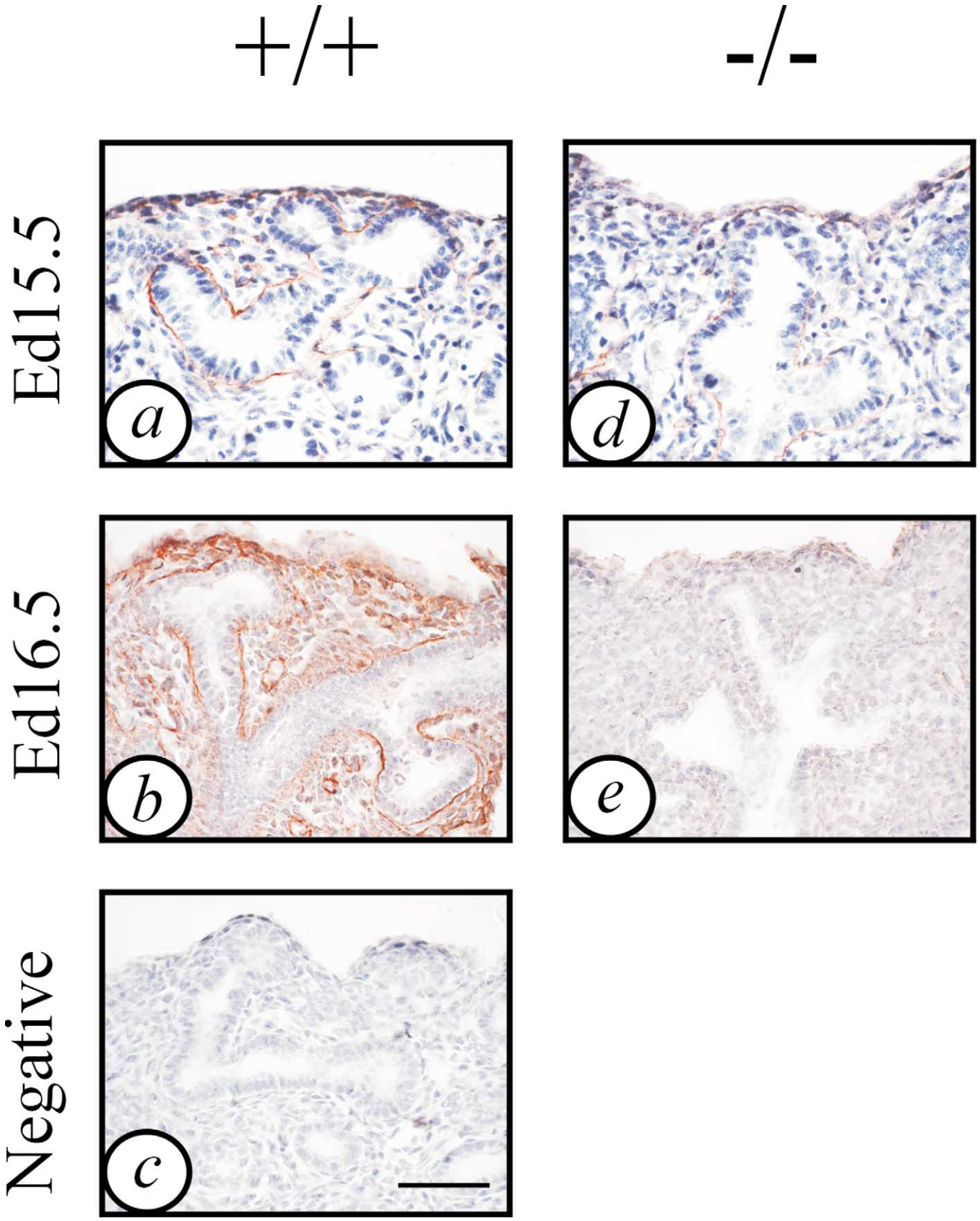

Fig. 6. Fibronectin abundance is decreased in both the basement membrane surrounding bronchiole epithelium and the mesenchymal matrix in the absence of TIMP-3. Immunohistochemical analysis performed at Ed15.5 demonstrated reduced fibronectin in the basement membranes surrounding the terminal ends and within the cleft regions of developing bronchioles in the null lungs (d; $n = 4$) when compared with wild type lungs (a; $n = 4$). At Ed16.5, decreased fibronectin was observed in the bronchiole basement membrane and within the mesenchymal ECM of the null lung (compare b and e; $n = 4$). The negative control was a wild type Ed16.5 sample incubated without primary antibody (c). Scale bar, 50 μm. Lungs from clone 7 animals were used in these experiments.

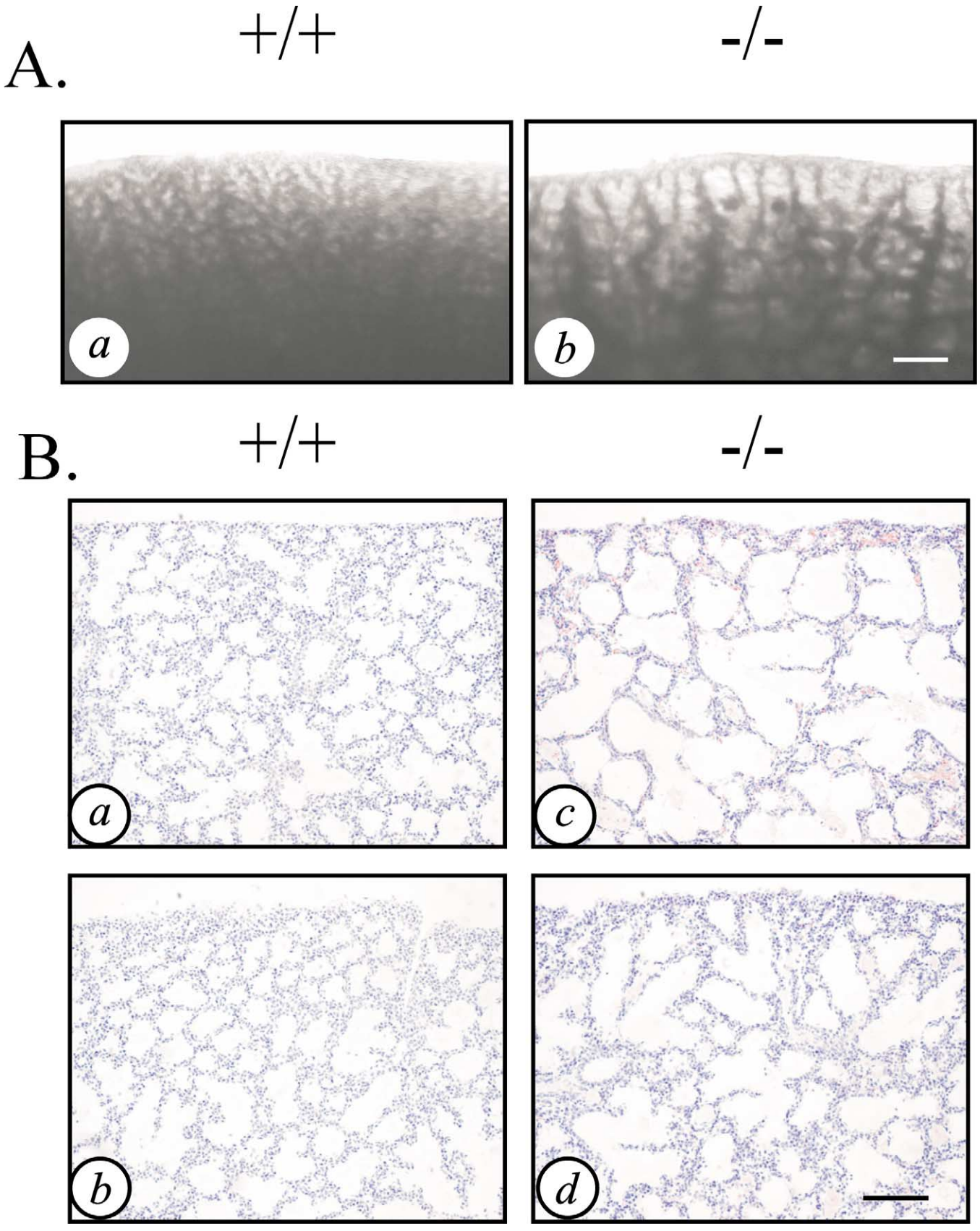

Fig. 7. Bronchiole branching in the TIMP-3 null lung is reduced in vivo. (A) Whole-mount photomicrographs show that lungs from PP1 null animals (b; $n = 8$) have impaired branching and dilated terminal buds when compared with lungs from wild type animals (a; $n = 8$). Scale bar, 200 μm. (B) Hematoxylin and eosin staining of PP1 lungs confirm dilation of bronchioles, as well as deficient alveolarization in null lungs. Compare wild type (a, b) with null (c, d); scale bar, 50 μm. Lungs from clone 7 animals were used in these experiments.

MMP-14-mediated activation of MMP-2 (Kheradmand et al., 2002). Additionally, lungs deficient for MMP-2 phenocopied those of the EGFR null (Kheradmand et al., 2002).

In the present study, we have provided evidence that the deletion of TIMP-3 results in impaired bronchiole branching, both in vitro and in vivo, during murine embryogenesis. In the TIMP-3 null lung, we have shown by gelatin and in situ zymography that there is enhanced MMP activity, which may result in inappropriate degradation of fibronectin in the BM and stroma surrounding the developing bronchiole epithelium. Consistent with this observation, we previously demonstrated that an unscheduled activation of MMP-2 correlated with earlier fragmentation of fibronectin in the involuting mammary gland of TIMP-3-deficient mice (Fata et al., 2001). Thus, this mechanism of MMP activation and fibronectin degradation appears to be of importance in BM breakdown in two distinct branched tissues in the absence of TIMP-3. We propose that enhanced MMP-mediated degradation of fibronectin in the absence of TIMP-3 leads to decreased cleft formation and thinning of the BM at the tips of buds, which together results in the decreased bronchiole branching observed in TIMP-3 null lungs.

The role of MMPs in lung development has also been explored by using the synthetic metalloproteinase inhibitor, GM6001. Total inhibition of MMP activity during lung development in vivo using high concentration of GM6001 resulted in impaired branching (Kheradmand et al., 2002). In our study, at a low concentration of inhibitor (2.5 μM), we observed a rescue of the TIMP-3 null branching defect and an augmentation of WT bronchiole branching in vitro. Taken together, these results imply that total inhibition of MMP activity results in less branching, possibly due to lack of BM thinning at the tips of elongating buds, while moderate inhibition of MMP activity could enhance branching due to a stabilization of clefts. We observed, both in vitro and in vivo, that heightened MMP activity in the TIMP-3 null lung led to dilation of the terminal buds. These data are consistent with the work of Ganser and colleagues (1991), who found that addition of mammalian collagenase to lung cultures led to impaired branching and enlarged terminal buds. Thus, MMP activity and subsequent ECM remodeling during embryonic morphogenetic processes, such as bronchiole branching, requires exquisite regulation.

Growth factors and growth factor receptors have been implicated in bronchiole branching. For example, hepatocyte growth factor (HGF) and the HGF receptor (c-Met) are expressed by the mesenchyme and epithelium of the developing lung, respectively, and addition of HGF to organ culture of fetal lung stimulates branching (Ohmichi et al., 1998). Further, c-Met can be shed from the cell surface by an as yet unidentified TIMP-3-sensitive metalloproteinase (Nath et al., 2001). We are currently exploring the hypothesis that, in addition to the loss of fibronectin from the BM surrounding the developing bronchiole tree, the reduced branching we see in the null lungs may result from a disruption in the HGF/c-Met signaling pathway due to shedding of the c-Met receptor in the epithelial compartment by the activity of a TIMP-3 sensitive metalloproteinase.

A candidate sheddase for cleavage of c-Met may come from the class of metalloproteinases known as the ADAMs (a disintegrin and metalloprotease domain) which are involved in shedding cell surface receptors, a process known as ectodomain shedding (Black and White, 1998). A recent study has shown that some ectodomain shedding is required for normal branching morphogenesis. Lungs deficient for ADAM-17/TNF-α converting enzyme (TACE) have impaired branching both in vivo and in vitro (Zhao et al., 2001). Since TIMP-3 is able to inhibit TACE activity (Amour et al., 1998), this leads to the expectation that TACE activity is likely increased in the TIMP-3 null lungs. Despite this apparent contradiction, subtle modifications of TACE activity in the TIMP-3 null mice may have pleiotropic effects on ectodomain shedding and therefore tissue organization during development. Further, TIMP-3 is able to inhibit many other ADAMs (Amour et al., 2000; Hashimoto et al., 2001; Kashiwagi et al., 2001), leaving open the possibility of enhanced ADAM mediated ectodomain shedding in the TIMP-3 null lungs.

Here, we have provided additional evidence that the ECM plays an integral role in murine lung branching morphogenesis, involving the dynamic processing of the ECM, which must not be altered either temporally or spatially. We have also demonstrated that TIMP-3 has a critical function in the control of the finely orchestrated ECM degradation that occurs during lung development. Future experiments will focus on further defining the precise mechanism(s) by which TIMP-3 regulates bronchiole branching morphogenesis.

Acknowledgments

This work was supported by grants from the Canadian Institutes of Health Research (37927) and the Canada Foundation for Innovation (2860). Salary support for S.E.G. was provided by the Ontario Graduate Scholarship Program. A.J.W. and K.J.L. are recipients of the Ontario Premier's Research Excellence Award.

Note added in proof. While this manuscript was being prepared for publication, Sakai and coworkers reported that appropriate fibronectin expression is absolutely required for branching morphogenesis of the salivary gland (Sakai, T., Larsen, M., Yamada, M., 2003. Fibronectin requirement in branching morphogenesis. Nature 423, 876–881). Our results are consistent with this report, in that we have also identified fibronectin as a key regulator of branching morphogenesis in the developing lung.

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