Tissue inhibitor of metalloproteinases 3 regulates extracellular matrix—Cell signaling during bronchiole branching morphogenesis

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Abstract

Tissue inhibitors of metalloproteinases (TIMPs) regulate extracellular matrix (ECM) degradation by matrix metalloproteinases (MMPs) throughout embryogenesis. We examined lungs from TIMP3 null mice and found decreased bronchiole branching, enhanced activity of MMPs and enhanced fibronectin degradation throughout lung development compared to controls. Activation of focal adhesion kinase (FAK) was also reduced from embryonic days 12.5 through 14.5 in TIMP3 null lungs. Treatment with a synthetic MMP inhibitor, GM6001, in utero enhanced the branching pattern in both wild type and null lungs accompanied by a restoration of fibronectin localization, signaling through FAK and epithelial cell proliferation in null lungs. Direct down-regulation of FAK abundance in WT lung organ culture by siRNA targeting resulted in reduced bronchiole branching, phenocopying the TIMP3 defect. We propose that enhanced MMP activity in the absence of TIMP3 interferes with focal ECM proteolysis, perturbing the intracellular signaling necessary for correct pattern formation of the bronchiole tree during bronchiole branching morphogenesis. Thus, TIMP3 can indirectly regulate epithelial cell proliferation via MMP inhibitory activity. While others have demonstrated this function for MMPs, and there is in vitro evidence that TIMP3 controls proliferation, to our knowledge this is the first evidence of TIMP3 regulating proliferation in vivo.

Keywords: TIMP; MMP; E-cadherin; ECM; FAK; Fibronectin; Bronchiole branching morphogenesis; Lung development; Epithelial–mesenchymal interactions

Introduction

Murine lung development begins on embryonic day 9.5 (Ed9.5) as paired endodermal buds from the primitive foregut invade into the surrounding mesoderm (Ten Have-Oppreok, 1991). Beginning at Ed11.5, these buds enter into a period of rapid growth and begin a series of cleft and bud formations in a process termed bronchiole branching morphogenesis, resulting in the formation of the prospective bronchiole tree (Ten Have-Oppreok, 1991). Bronchiole branching morphogenesis depends on interactions between the prospective bronchiole epithelium, the surrounding mesenchyme and the specialized extracellular matrix (ECM) structure, the basement membrane, which serves as an interface between the two compartments (Alescio and Cassini, 1962; Schuger, 1997). Specific ECM molecules, including the multi-adhesive basement membrane protein fibronectin (FN), are required for bronchiole branching morphogenesis (Mollard and Dziadek, 1998; Roman, 1997; Sakai et al., 2003). During lung bud outgrowth, there is a thinning of the basement membrane and reduced amounts of FN at the tips of the growing buds (Mollard and Dziadek, 1998; Roman and McDonald, 1992). Further, accumulation of FN in presumptive cleft forming regions is thought to regulate the formation of two daughter branches from one lung bud (Affolter et al., 2003). Loss of FN, or the inability to form a FN matrix in vitro, leads to impaired bronchiole branching (Gill et al., 2003; Roman et al., 1991; Sakai et al., 2003) indicating an essential function for FN during murine lung morphogenesis.

Remodeling of the ECM is believed to result from a shift in the balance between active matrix metalloproteinases (MMPs) and their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs; Baker et al., 2002; Vu and Werb, 2000). Multiple MMPs and TIMPs are present in the developing lung (Kheradmand et al., 2002; Nuttall et al., 2004) and the MMP/TIMP balance is tightly regulated during bronchiole branching

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morphogenesis (Gill et al., 2003; Kheradmand et al., 2002). Either decreased levels of active MMPs (Kheradmand et al., 2002) or decreased levels of TIMPs resulting in increased MMP activity (Gill et al., 2003), lead to impaired bronchiole branching morphogenesis both in vitro and in vivo. While MMPs have the potential to exert many effects through cleavage of bioactive molecules or shedding of receptors from the cell surface (Mott and Werb, 2004; Parks et al., 2004), their classical role in ECM degradation presumably has an essential function during lung development. Impaired branching observed in developing lungs in the absence of TIMP3 is accompanied by reduced FN abundance (Gill et al., 2003), which could reflect enhanced degradation mediated by increased MMP activity. While the importance of FN in lung morphogenesis is certain, the specific role of the MMP/TIMP balance in the regulation of FN distribution and abundance remains to be determined.

Cells bind FN via integrins, predominantly integrin α5β1 (Pankov and Yamada, 2002), which serve to transduce signals from the ECM into the cell. Binding of FN to integrins results in the localization of focal adhesion kinase (FAK) to developing focal adhesions followed by activation of FAK through autophosphorylation of tyrosine 397 (Carragher and Frame, 2004; Schaller and Parsons, 1994). FAK then interacts with multiple downstream signaling pathways, including the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, to regulate cell functions such as cell migration, proliferation and differentiation (Danen and Yamada, 2001; Parsons, 2003; Schaller and Parsons, 1994).

Epithelial cells of the prospective respiratory system undergo tightly regulated proliferation as they invade into the surrounding mesenchyme during bronchiole branching morphogenesis (Nogawa et al., 1998). The epithelial cells at the tips of invading buds may have a higher rate of proliferation than epithelial cells in the cleft forming regions (Mollard and Dziadek, 1998). This difference in proliferation rates, however, is not evident until after the bud has begun to form (Nogawa et al., 1998). Enhanced proliferation allows the epithelial sheet to expand as the prospective bronchiole tubes elongate and is regulated through a host of cellular signals. Growth factors, such as members of the fibroblast growth factor (FGF) family, have been implicated in the interaction between the invading epithelium and the surrounding mesenchyme and are capable of regulating proliferation in the developing lung (Minoo, 2000). This regulation occurs through interaction with multiple signaling pathways including the PI3K and MAPK pathways (Wang et al., 2005b). Proliferation may also be regulated in the developing lung through the deposition and degradation of ECM proteins like FN, which are capable of enhancing proliferation in vitro (Han et al., 2005; Nguyen et al., 2005). In addition, interactions between the basement membrane and growth factors regulate the localization of growth factor gradients and thereby the localization of proliferation (Sannes and Wang, 1997). Thus, proliferation in the developing lung is a complex process that requires multiple levels of regulation and the coordination of many signaling pathways.

In light of the above information, we hypothesized that the reduction in the abundance of FN previously detected in TIMP3 null lungs (Gill et al., 2003) could result in defective signaling through FAK and thus contribute to the reduced branching observed. In the current study, we demonstrate that enhanced degradation of FN in the developing lungs of TIMP3 null mice leads to impaired ECM-cell signaling through FAK. This is accompanied by a reduction in epithelial cell proliferation. Addition of a low concentration of a synthetic inhibitor of metalloproteinases (GM6001) in utero rescues bronchiole branching morphogenesis as well as FN abundance, FAK signaling and epithelial cell proliferation. Furthermore, we provide evidence that FAK signaling is required for bronchiole branching morphogenesis and that down-regulation of FAK signaling ultimately leads to the impaired branching observed in TIMP3 null lungs. These results highlight the necessity for tight regulation of the MMP/TIMP balance during development and also provide evidence in support of a novel role for TIMP3 in regulation of ECM-cell signaling through FN and FAK and the subsequent regulation of epithelial proliferation during bronchiole branching morphogenesis. While many papers have demonstrated a role for MMPs in the regulation of cellular proliferation via ECM modulation or growth factor activation (reviewed by McCawley and Matrisian, 2001; Mott and Werb, 2004; Vu and Werb, 2000), and there is in vitro evidence that TIMP3 controls proliferation (Baker et al., 1998; Yang and Hawkes, 1992) to our knowledge, ours is the first evidence of TIMP3 indirectly regulating proliferation in vivo.

Materials and methods

Mice

A full description of the generation of the TIMP3 null mice used in this study was reported by Leco et al. (2001). Animals were cared for in accordance with protocols approved by the UWO Animal Care Committee, following the guidelines of the Canadian Council on Animal Care. For these experiments, the mutant allele was back-crossed eight generations onto the C57/Black6 strain of mice for the independently targeted clone 8. Mice were maintained and bred as previously described (Gill et al., 2003) and the TIMP3 null line(s) used in each experiment are identified in legends to figures. Pregnant dams were sacrificed in order to retrieve embryonic lungs at the desired time points (Ed11.5 to 14.5), with the day of discovery of a vaginal plug being considered Ed0.5. Lungs were either placed into organ culture, snap frozen on dry ice for RNA or protein analysis or fixed with PBS-buffered 4% paraformaldehyde for 3 h at 4°C, dehydrated through an alcohol series and embedded in paraffin wax for sectioning.

GM6001 injections

Pregnant wild type (WT) and null dams were injected intraperitoneally with carboxymethyl cellulose (CMC; Sigma-Aldrich) or 20 mg/kg GM6001 (3.33 mg/ml in CMC; Chemicon International) on both Ed11.5 and Ed12.5 (n=12 for each group). Dams were sacrificed on Ed13.5, the embryonic lungs removed and either snap frozen on dry ice for protein analysis or fixed with PBS-buffered 4% paraformaldehyde for 3 h at 4°C, photographed, then dehydrated through an alcohol series and embedded in paraffin wax for sectioning.

Quantification of lung bronchiole branching plus/minus GM6001 in utero

Images of fixed lungs were digitally captured using a SMZ1500 dissecting microscope equipped with a DXM1200 digital camera and accompanying ACT-1 software (Nikon Canada). The number of terminal buds around the periphery...
of the left lobe from each Ed13.5 embryo±GM6001 was counted to be among two independent investigators who were blind as to the genotype and treatment. Extent of peripheral terminal bronchiole branching in vivo at Ed13.5 was expressed according to the following equation adapted from Schuger et al. (1991): (TB experimental/TB WT CMC control) × 100, where TB experimental is the number of terminal buds in each experimental group. Images of the left lobe were also digitally outlined and the area of the lobe calculated using SimplePCI software (C*Imaging Systems, Compix, Inc.). A two-way ANOVA with a Tukey’s post hoc test was used to determine statistical significance.

Western blot analysis

Western blot analysis was performed on lung homogenates from WT and null lungs (Ed12.5 to Ed14.5) or Ed13.5 lungs treated in utero with either GM6001 or CMC essentially as described (Gill et al., 2003) with the exception that the protein extraction buffer contained 1× phosphate buffer (Roche Diagnostics). Antibodies used in these studies were directed against FN (1:10,000 dilution; DAKO Diagnostics; Catalogue Number A0245), focal adhesion kinase (FAK; 1:500 dilution; Oncogene Research Products; Catalogue Number P314), FAK phosphorylated at tyrosine 397 (pFAK; 1:1000 dilution; Sigma-Aldrich; Catalogue Number F7926), E-cadherin (1:2500 dilution; BD Biosciences; Catalogue Number 610182), proliferating cell nuclear antigen (PCNA; 1:1000 dilution; Novocastra; Catalogue Number NCLPCNA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000 dilution; Helena Biosciences; Catalogue Number ASM020). Secondary antisera were either anti-rabbit or anti-mouse conjugated to horseradish peroxidase (1:5000; Sigma-Aldrich). Optical density of autoradiographic bands was determined using SimplePCI software on a minimum of three independent blots. The control (WT sample for each time point in a developmental series) was set to one and arc sine transformation was performed on the data. A two-tailed, independent Student’s t test was used to determine significance between genotypes at each gestational age.

Northern blot analysis

Northern blot analysis was performed as previously described (Leco et al., 1994) on homogenates from WT and null lungs (Ed12.5, 13.5 and 14.5). Total RNA (5 μg) was separated by electrophoresis, transferred to a nylon membrane and hybridized sequentially with 32P-labelled E-cadherin, Fn and Gapdh cDNA probes. Bands of interest were visualized by exposure to X-AR film and the optical density of each band was determined as described above.

Protein degradation assay

Lungs from three Ed13.5 WT or null embryos were homogenized in extraction buffer (50 mM Tris–Cl (pH 7.5), 150 mM NaCl and 1% Triton X-100 plus one EDTA-free protease inhibitor cocktail pill (Roche Diagnostics) per 10 ml buffer), sonicated for 2 s and protein concentration determined. Samples (50 μg) were diluted in substrate buffer [50 mM Tris–Cl (pH 7.5), 5 mM CaCl2 and 5 μM ZnCl2] incubated at 37°C for 3 h in the presence or absence of 10 mM EDTA, then analyzed by Western blot for FN, E-cadherin and GAPDH as described above. This experiment was repeated three times using embryos from different litters.

Immunohistochemistry

Paraffin-embedded lung tissue sections (7 μm) were prepared for immunohistochemistry as previously described (Gill et al., 2003). Sections were incubated with either FN, pFAK or PCNA antisera or DAKO blocking solution alone (negative control) overnight at 4°C. Signal on sections incubated with FN antisera (1:100) were visualized with secondary antibodies conjugated to horseradish peroxidase (1:200) and AEC substrate (ICN Biomedical), counterstained with Mayer’s hematoxylin and mounted under Crystal Mount (Biomeda Corp). Sections incubated with pFAK (1:100) or PCNA (1:100) were visualized using secondary antibodies conjugated to rhodamine or FITC (1:200, Jackson Laboratories) and counterstained with Hoechst nucleic acid stain (2.5 μg/ml; Molecular Probes), or propidium iodide (0.5 μg/ml; Sigma-Aldrich). Sections were mounted with fluoroguard (Biorad) and a coverslip. Images were captured with the E1000 upright microscope and the digital camera and software were described above. Once set for a minimal signal with the negative control, exposure times were kept constant for all subsequent images.

Primary lung cell co-culture

Lungs were removed from WT mouse embryos on Ed13.5 and placed into serum-free BGJb medium (Invitrogen). Lungs (5–8 per experiment) were rinsed in PBS, pooled and treated with trypsin twice for 15 min at 37°C, then pipetted gently to generate a single cell suspension. After centrifugation at 400×g, cells were resuspended in BGJb medium with 10% fetal calf serum (FCS; Invitrogen), counted and plated in 6-well plates at 5×103 cells per well. Primary co-cultures consisted of both epithelial and mesenchymal cells. After 18 h, the cells were given fresh BGJb medium with 10% FCS 1 h prior to transfection with siRNA.

The Fak siRNA sequence (sense: 5′-AACCCACCCGCGCCAGAUAAUU-3′; antisense: 5′-AUUUACUGGCCACGGUUGG-3′) and the scrambled siRNA sequence (sense: 5′-AAUUCUCCAGGUGUCAGCUU-3′; antisense: 5′-ACGUGACAGUCUGGAGAAA-3′) were based on a previous study (Duxbury et al., 2003) and were purchased from Qiagen Inc. Double stranded RNA duplexes (16 nM) were introduced into primary co-cultures using lipofectamine 2000 (Invitrogen) according to the manufacturer’s guidelines. Cells were incubated 24 h, then either lysed with extraction buffer for Western blot analysis or fixed with ice-cold methanol for immunohistochemistry. These experiments were repeated 4 times with WT embryonic lung cells from different litters.

Primary cell co-cultures were prepared as above and after 18 h, transfected with 1 μg of either an expression plasmid containing Fak cDNA or an empty vector (gift from Dr. David Schlaepfer, Scripps Research Institute, La Jolla, CA) using lipofectamine 2000. Cells were cultured in BGJb medium with 10% FCS for 24 h and then lysed with extraction buffer or fixed with ice-cold methanol. These experiments were repeated 3 times with lungs from different litters.

Primary cultures were also cultured in 6-well plates in the presence of either BSA or FN (1.3 μg/ml; Sigma-Aldrich). Cells were cultured in BGJb medium with 10% FCS for 24 h and then lysed with extraction buffer as described above. This experiment was repeated 3 times with lungs from different litters.

Immunohistochemical analysis on primary cell co-cultures

Antibodies used were pan-cytokeratin antisera (1:250; DAKO-Z622), PCNA antisera (1:100) or 0.5% normal lamb’s serum alone (negative control). Detection was achieved with secondary antibodies conjugated to horseradish peroxidase (1:200) and AEC substrate. Ten random fields of view were captured for each well using a TS100 tissue culture microscope equipped with the camera and software described above (Nikon Canada) and immunostained cells were counted by individuals blind to the treatment group. A two-tailed independent Student’s t test was used to determine significance.

Embryonic lung organ culture

Lung rudiments were removed from WT and null mouse embryos on Ed11.5 and cultured as described (Gill et al., 2003). WT lungs from three separate litters were transfected with either scrambled siRNA (16 nM; n=7) or Fak siRNA (16 nM; n=5) using lipofectamine 2000. Peripheral terminal buds from the entire lung were quantified at 72 h as described (Gill et al., 2003) and the lungs fixed in ice-cold methanol.

Whole-mount immunofluorescence

Lungs from organ culture were incubated with pFAK antisera (1:100) followed by anti-rabbit secondary antibody conjugated to rhodamine (1:200), or E-cadherin antisera (1:200) followed by anti-mouse secondary antibody conjugated to rhodamine (1:200), placed on a glass slide and covered with fluoroguard (Biorad) and a coverslip. Images were captured using a Zeiss LSM 410 laser-scanning microscope and the accompanying Zeiss LSM software package. Once set for a minimal signal with the negative control (secondary antibody only), exposure times were kept constant for all subsequent images.
Results

**GM6001 rescues bronchiole branching of TIMP3 null lungs in vivo**

We previously demonstrated that the loss of TIMP3 results in impaired bronchiole branching morphogenesis, which can be rescued in vitro by the addition of a low concentration of GM6001, a synthetic inhibitor of metalloproteinases (Gill et al., 2003). In the present study, we questioned whether GM6001 treatment would also rescue lung development in TIMP3 null mice in utero. Pregnant dams were injected with carboxymethyl cellulose (CMC, vehicle for GM6001) or GM6001 (20 mg/kg) at Ed11.5 and Ed12.5, and embryonic lungs were harvested at Ed13.5 for analysis. The number of peripheral terminal buds in the left lung lobe from control TIMP3 null animals was reduced significantly when compared to the left lung lobe from control WT counterparts after injection of pregnant dams with CMC (80% of WT, Fig. 1A and B). Injection of pregnant dams with GM6001 led to a significantly enhanced branching pattern in the embryonic lungs regardless of genotype. The number of terminal buds present in the GM6001 treated TIMP3 null left lung lobe at Ed13.5 was similar to those observed in WT controls (104% of WT, CMC control) and the number of buds present in the WT animals treated with GM6001 was increased above those observed in their vehicle control treated counterparts (112% of WT, CMC control; Figs. 1A and B). Images of left lobes were also digitally outlined and the area of the tissue calculated. The area of the left lung lobe from control TIMP3 null animals was reduced significantly when compared to the left lung lobe from control WT counterparts (82% of WT, Fig. 1C). Injection of GM6001 tended to reduce the area of WT lungs, and conversely increased the area of null lungs; however, these results did not attain statistical significance (Fig. 1C).

**Enhanced degradation of FN in lungs of TIMP3 null mice**

In our earlier studies, FN was the only ECM protein examined that was markedly decreased in embryonic TIMP3 null lungs compared to WT (Gill et al., 2003). Here, we extended this analysis with lungs from WT and null animals at Ed12.5, 13.5 and 14.5 (Fig. 2A). FN protein abundance was reduced at each
time point in the TIMP3 null lungs when compared to WT counterparts with a statistically significant reduction at Ed12.5 and 13.5 (Fig. 2B).

To investigate whether this decrease in FN protein abundance was a result of impaired synthesis or enhanced degradation, we first employed Northern blot analysis. Fig. 2C demonstrates that Fn mRNA abundance is actually higher in TIMP3 null lungs compared to WT at both Ed12.5 and Ed13.5. Therefore, the reduced FN protein abundance (Fig. 2A) is not due to reduced Fn mRNA abundance.

We questioned whether MMPs present in the developing lung were capable of degrading endogenous FN protein. For this analysis, Ed13.5 lungs from WT and TIMP3 null embryos were homogenized and incubated for 3 h at 37°C in the presence or absence of 10 mM EDTA (a metalloproteinase inhibitor) before FN protein abundance was examined by Western blot. After 3 h incubation, FN protein levels were significantly decreased in both WT and null lung homogenate when compared to WT or null samples incubated in the presence of EDTA indicative of FN digestion by endogenous MMPs (Fig. 2D). Taken together, the mRNA and protein degradation experiments demonstrate that reduced FN protein abundance in the developing TIMP3 null lung is mediated by metalloproteinase degradation of FN protein, and not reduced Fn mRNA abundance in null lungs.

**FN abundance is restored in embryonic TIMP3 null lungs by GM6001**

To determine if GM6001 could restore FN protein abundance, we employed Western blot analysis of Ed13.5 WT and null lung tissue from embryos treated in utero on Ed11.5 and Ed12.5 with either GM6001 or vehicle (CMC). Treatment of null embryonic lung with GM6001 did indeed restore FN protein levels to that seen in the WT lung. We next examined the immunolocalization of lung FN at Ed13.5 after treatment with GM6001 in utero. Examination of the tips of the developing bronchioles at the lung periphery revealed a reduction in the accumulation of FN in the absence of TIMP3 (Fig. 3B). In the WT lung, FN immunoreactivity was present predominantly in the basement membrane, in both cleft forming regions and to a lesser extent at the tips of growing buds (Fig. 3B, panel a). FN was also present at low levels throughout the WT lung mesenchyme. Control, CMC injected TIMP3 null lungs had far less immunostaining for FN (Fig. 3B, panel b). Treatment with GM6001 increased the amount of FN in null lungs to levels comparable to those present in the WT control, with a similar distribution pattern (Fig. 3B, panel d vs. a). Therefore, a rescue of FN accumulation in TIMP3 null lungs treated with GM6001 was clearly revealed by immunohistochemistry.

**Impaired intracellular signaling downstream of FN in the absence of TIMP3**

To explore the effect of reduced FN abundance in the TIMP3 null lungs, we examined the abundance of phosphorylated focal adhesion kinase (pFAK), which is a downstream target of FN-integrin interactions. In the absence of TIMP3, pFAK abundance was reduced during the initial stages of bronchiole branching morphogenesis when compared to lungs from WT animals (Fig. 4A). Densitometry confirmed that the quantity of pFAK was
reduced in the null lungs, with a statistically significant reduction at Ed12.5 and 13.5 compared to WT lungs (Fig. 4B).

Administration GM6001 in utero enhances FAK activation in TIMP3 null lungs

The next question to address was whether enhanced FN abundance in TIMP3 null lungs after treatment with GM6001 led to a rescue of FAK activation. Similar to FN, the abundance of pFAK was rescued in null lungs treated with GM6001 as demonstrated by Western blot analysis (Fig. 5A). pFAK immunoreactivity was localized to the basal aspect of the bronchiole epithelial cells of WT lungs, but was virtually absent from similar regions in null lungs (Fig. 5B, panel a vs. b). pFAK was also diffusely localized throughout the WT lung mesenchyme, consistent with the pattern we observed for FN (Fig. 5B, panel a vs. Fig. 3B, panel a, respectively). Treatment with GM6001 in utero led to increased accumulation of pFAK in epithelial cells of branching bronchioles and throughout the mesenchymal compartment of TIMP3 null lungs, restoring pFAK immunoreactivity to a pattern consistent with WT lungs (Fig. 5B, panel d vs. a). Furthermore, the distribution of pFAK in epithelial cells after rescue was coincident with the distribution of FN in the basement membrane after rescue in the null lungs (Fig. 5B, panel d vs. Fig. 3B, panel d).

E-cadherin abundance is reduced in the lungs of TIMP-3 null mice

Others have shown that FN may regulate cleft formation in the lung by altering E-cadherin cell–cell adhesions (Sakai et al., 2003). We therefore hypothesized that in the TIMP3 null lungs, the loss of FN and subsequent loss of epithelial cell FAK signaling could result in a reduction of E-cadherin expression in potential cleft forming regions with fewer branch points formed in developing bronchioles. To explore this potential downstream
effect of reduced FAK signaling, we examined TIMP-3 null lungs by Western blot analysis with E-cadherin antisera. In a pattern similar to both FN and pFAK abundance, E-cadherin abundance was reduced throughout the early stages of branching morphogenesis with a statistically significant reduction observed at Ed12.5 (Figs. 6A and B). Northern blot analysis demonstrated a reduction of E-cadherin mRNA abundance at Ed12.5, Ed13.5 and Ed14.5 demonstrating reduced abundance of E-cadherin protein in TIMP3 null lungs compared to WT lungs at the same gestational age. (B) Densitometric ratio of pFAK protein abundance normalized for GAPDH signal where WT is set to 100% for each time point (*=significance; two-tailed, independent Student’s t test between genotypes at each time point; P<0.02; n=3 for each time point; densitometry was performed on a lesser exposure for all replicates).

FAK is required for bronchiole branching morphogenesis

To this point, there were correlative data supporting the hypothesis that a reduction in FAK signaling may be responsible for the reduced branching observed in the TIMP3 null lung. We tested this hypothesis directly utilizing siRNA down-regulation of FAK expression in whole lung organ culture. Lungs from Ed11.5 WT mice were cultured in the presence of either scrambled siRNA or Fak siRNA for 72 h. Treatment of lungs with Fak siRNA led to a significant 30% reduction in the number of peripheral terminal bronchiole buds (Figs. 7A and B) and an abnormal branching pattern compared to control lungs treated with scrambled siRNA (Fig. 7A). Analysis by whole-mount immunofluorescence demonstrated that the abundance of pFAK was reduced in lungs treated with Fak siRNA (Fig. 7C, panel c) when compared to control lungs (Fig. 7C, panel a). Contrary to our expectation that direct down-regulation of pFAK signaling would reduce E-cadherin protein levels, no difference in E-cadherin abundance between the two treatment groups was observed by immunofluorescence (Fig. 7C, panels b and d).

FAK signaling regulates epithelial cell proliferation

We next asked if reduced FAK activation could result in decreased epithelial cell proliferation, which may contribute to the reduced branching in the null lung. Here we utilized an epithelial and mesenchymal primary cell co-culture from Ed13.5 WT lungs incubated in the presence or absence of Fak siRNA. Down-regulation of FAK expression in monolayer cultures led to a significant decrease in the number of cytokeratin immunopositive cells (epithelial cells) in the cultures (30% reduction in Fak siRNA treated cultures compared to scrambled siRNA; Figs. 8A and C). To investigate whether the reduced epithelial cell number could be attributed to decreased proliferation, siRNA treated cells were immunostained for proliferating cell nuclear antigen (PCNA). Quantification revealed a significant reduction of 50% in PCNA positive cells observed in cultures treated with Fak siRNA compared to cultures exposed to scrambled siRNA (Figs. 8A and C). Treatment of cells with Fak siRNA led to a significant reduction in FAK protein abundance compared to cells treated with scrambled siRNA as determined by Western blot analysis (Fig. 8B). Consistent with the reduction in epithelial cell number in the cultures, Western blot analysis revealed a significant reduction in E-cadherin protein abundance in extracts from cells treated with Fak siRNA compared to control (Fig. 8B).

We also performed complementary experiments, up-regulating FAK signaling using two approaches. To determine whether FN was involved in regulation of cell proliferation through FAK activation, primary epithelial and mesenchymal cells from Ed13.5 WT lungs were cultured on either BSA or FN. Cells cultured on BSA or FN had similar levels of FAK protein; however, the abundance of pFAK and PCNA was significantly increased in cells cultured on FN vs. BSA (Fig. 8D). To confirm that these effects were a direct result of increased FAK activation, primary co-cultures were transfected with either empty vector or a Fak-expression vector. Co-cultures transfected with the Fak-containing vector had significantly increased abundance of FAK and pFAK, indicating that FAK was being over-expressed in these cells (Fig. 8D). As a result of enhanced signaling through pFAK, PCNA...
abundance was significantly increased as determined by Western blot analysis (Fig. 8D). Immunohistochemical analysis confirmed that there were significantly more cytokeratin immuno-positive cells, and therefore a significantly enhanced number of epithelial cells after over-expression of FAK (Fig. 8E). Consistent with the increased epithelial cells in cultures over-expressing FAK, the amount of E-cadherin protein was also significantly increased over cultures transfected with empty vector (Fig. 8D).

**Proliferation is reduced in the absence of TIMP3 in vivo**

In monolayer culture, we demonstrated that loss or gain of FAK expression led to reduced or enhanced proliferation of epithelial cells. Since FAK activation is reduced in embryonic TIMP3 null lungs, we examined if cell proliferation was also affected by the loss of TIMP3. Immunostaining for PCNA revealed that the number of proliferating cells was reduced in TIMP3 null lung (b) and restored to a pattern similar to WT in a null lung after treatment with GM6001 in utero (d). The negative control (c) was treated with goat-anti-rabbit secondary antisera alone. These are serial sections to those used in Fig. 2 (scale bar=50 μm). Nuclei were counterstained with Hoechst stain (blue) to show histology. Data for these experiments were collected from both independently derived clones (clone 7 and clone 8).
developing bronchiole tree, restored epithelial cell proliferation to that seen in the WT lung and rescued the branching defect within null lungs.

**Discussion**

Lung morphogenesis is a dynamic process requiring cross-talk between the epithelium, the basement membrane and the surrounding mesenchyme, transmitted predominantly through growth factors and their receptors (Cardoso, 2000). Further interactions include the temporally and spatially regulated deposition of specific ECM proteins (Mollard and Dziadek, 1998; Roman, 1997; Schuger, 1997), accompanied by remodeling of the ECM by MMPs (Ganser et al., 1991; Gill et al., 2003; Kheradmand et al., 2002). Although the role of ECM in lung development has been well characterized, little is known about the specific mechanism through which it fulfills this function. Here, we provide evidence that FAK, and more specifically FN signaling through FAK, is required for bronchiole branching morphogenesis and that this signaling can be modulated by the enzymatic activity of MMPs.

We previously reported that loss of TIMP3 in the developing lung led to impaired bronchiole branching, and that this was accompanied by increased MMP activity and a reduction in FN protein abundance (Gill et al., 2003). Here we demonstrate that the reduction in FN abundance in TIMP3 null lungs is due to enhanced degradation of the FN protein by a TIMP3 sensitive metalloproteinase. As a consequence, we conclude that TIMP3 indirectly, through inhibition of metalloproteinases, regulates FN protein abundance in the developing lung. Furthermore, we show that treatment of TIMP3 null lungs in utero with a broad spectrum metalloproteinase inhibitor, GM6001, restored FN protein abundance and localization, enhanced FAK signaling, increased epithelial cell proliferation and rescued bronchiole branching to levels comparable to the developing WT lung. Therefore, we propose that TIMP3 normally functions to restrict metalloproteinase activity, leading to proper ECM-cell signaling through FN and FAK, ultimately resulting in proliferation of the epithelial cells in developing bronchioles and proper bronchiole branching morphogenesis.

In addition to measuring the extent of bronchiole branching, we also calculated the area of the left lobe in control or GM6001 treated WT and TIMP3 null left lung lobes. Control null lungs were found to be significantly smaller than control WT lungs. These data support our hypothesis that, in the null lungs, enhanced MMP activity leads to reduced epithelial proliferation, reduced bronchiole branching and therefore reduced total area of the lung. Treatment of WT lungs with GM6001 tended to reduce the size of the lung suggesting that excessive inhibition of MMPs is detrimental to proper lung growth. Alternatively, treatment of null lungs with the synthetic MMP inhibitor tended to increase the size of null lungs such that they were not significantly different from WT. Moderation of excessive MMP activity in the null lungs by GM6001 enhances epithelial proliferation and therefore restores both branching and size of the lung.

Our results with in utero GM6001 treatment differ from those of Kheradmand et al. (2002), who found that addition of GM6001...
blocked embryonic lung branching when given to pregnant dams on Ed10.5 through Ed12.5. This apparent discrepancy can be explained by the fact that we used five-fold less inhibitor in our studies. That is, at the dose used in our studies (20 mg/kg), GM6001 enhanced branching regardless of genotype, while at high dose (100 mg/kg; Kheradmand et al., 2002), the synthetic MMP inhibitor essentially blocked embryonic lung development. This concept is further supported by our previous studies where a low concentration of GM6001 added to cultured lung rudiments augmented bronchiole branching, while at high concentration GM6001 diminished branching (Gill et al., 2003). A potential mechanism detailing the role of TIMP3 and MMPs in lung branching morphogenesis is presented in Fig. 10.

To date, little work has been done to explore a specific function for FAK during lung morphogenesis; however, circumstantial evidence indirectly supports a role for FAK in regulating bronchiole branching. First, FAK is known to mediate ECM-cell signaling. Following ECM-integrin interaction, FAK is localized to focal adhesions within the cell and becomes autophosphorylated at tyrosine 397, leading to an increase in catalytic activity (Parsons, 2003; Schaller and Parsons, 1994). Second, integrin and ECM proteins that are capable of activating the FAK signaling pathway are required for bronchiole branching morphogenesis. Specifically, loss of α3β1 integrin, FN or laminin leads to impaired branching (Kreidberg et al., 1996; Roman et al., 1991; Sakai et al., 2003; Schuger et al., 1990, 1991). In our study, direct evidence supporting a function for FAK in murine lung morphogenesis was provided by treatment of developing WT lungs in organ culture with Fak siRNA. The impaired bronchiole branching observed as a consequence of reduced FAK signaling phenocopies the TIMP3 null defect previously observed in culture (Gill et al., 2003), and establishes that FAK is required for murine bronchiole branching morphogenesis.

Evidence for the regulation of lung epithelial cell proliferation by differential FAK activation is provided by our monolayer

![Figure 7](image_url) Fig. 7. FAK signaling is required for bronchiole branching in whole cultured lungs. (A) Photomicrograph of WT lungs after 72 h cultured in the presence of either scrambled siRNA (a; n=7) or Fak siRNA (b; n=5; scale bar=100 μm) demonstrating reduced branching in lungs exposed to Fak siRNA. (B) Quantification of the number of terminal bronchiole branches expressed as the percentage of the scrambled siRNA treated lung (set to 100%; *=significance; two-tailed, independent Student’s t test; P<0.04; error bars represent±SEM). (C) Down-regulation of FAK signaling after siRNA treatment of lungs in organ culture was confirmed via whole-mount immunofluorescent staining and confocal microscopy for pFAK. Lungs cultured in the presence of scrambled siRNA were immuno-positive for pFAK (a), while lungs cultured with Fak siRNA showed much less staining (c). Arrows indicate epithelial tubes of developing bronchioles. Down-regulation of FAK signaling by siRNA treatment did not reduce the abundance of the E-cadherin protein in organ culture lungs (b and d).
cell culture studies. We demonstrated that, in Ed13.5 primary cell co-culture, a reduction in FAK abundance by siRNA treatment was accompanied by a significant reduction in PCNA-positive cells and in epithelial cell number. Alternatively, cells cultured on FN had significantly increased FAK activation accompanied by a significant increase in PCNA abundance. The significant increase in epithelial cell number after FAK over-expression confirms a role for FAK in the control of epithelial cell proliferation. Finally, although we did not test this directly, siRNA-mediated down-regulation of FAK in lung organ culture appears to reduce epithelial cell number coincident with the reduced branching when compared to control lungs.
In the developing lung, regulation of proliferation has been demonstrated through two independent pathways, the MAPK and PI3K-Akt pathways (Kling et al., 2002; Wang et al., 2005b), both of which can be regulated through growth factor signaling (Ammit and Panettieri, 2001). FAK is capable of signaling through either of these pathways and thereby capable of participating in the regulation of proliferation during lung morphogenesis (Slack-Davis et al., 2003; Yamamoto et al., 2003).

Preliminary experiments designed to investigate Erk activation in WT and TIMP3 null lungs did not demonstrate differential Erk activation in response to differential FAK signaling (SEG and KJL unpublished data). Thus, the function of FAK in the regulation of proliferation is not via the Erk pathway in our system; however, FAK may regulate proliferation through interaction with the PI3K-Akt pathway.

We had originally postulated that activated FAK might be directly regulating the expression of E-cadherin in the lung. In this scenario, less activated FAK in the TIMP3 null lung could...

![Fig. 9. GM6001 treatment in utero rescues epithelial cell proliferation in TIMP3 null lungs. (A) Immunofluorescent analysis for PCNA (green) of WT (a) and TIMP3 null (b and d) lungs after treatment with either CMC (a and b) or GM6001 (d). Arrows indicate examples of proliferating epithelial cells. The negative control (c) was treated with goat-anti-rabbit secondary antisera alone. All sections were counterstained with propidium iodide (red; n=4 for both genotypes and treatments; scale bar=50 μm). (B) Graphical representation of the number of WT (set to 100%) or TIMP3 null epithelial cells expressing PCNA after treatment with CMC or GM6001 (n=4 separate lungs analyzed for both genotypes and treatments; two-way ANOVA; P<0.02; *=significance; error bars represent±SEM). Data for these experiments were collected from both independently derived clones (clone 7 and clone 8).

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![Fig. 8. FAK controls proliferation of lung epithelial cells in WT primary co-cultures. (A) Immunohistochemical analysis of primary lung cells for cytokeratin (a and c) or PCNA (b and d) after treatment with either scrambled siRNA (a and b) or Fak siRNA (c and d; n=3 experiments from different litters; scale bars=250 μm). Fewer cytokeratin positive cells (red stain) are evident in the Fak siRNA treated cultures. Most cells stain positive for PCNA in cultures treated with scrambled siRNA, while only one cell in the Fak siRNA treated field was positive for PCNA (arrow, d). (B) Western blot analysis of primary lung epithelial and mesenchymal cells cultured for 24 h in the presence of scrambled siRNA or Fak siRNA for FAK and E-cadherin. Numbers below lanes represent the densitometric ratio of protein abundance normalized for GAPDH signal demonstrating a significant reduction in abundance of both proteins (*=significance; two-tailed, independent Student’s t test; P<0.03; n=4). (C) Graphical representation of the number of cells positive for cytokeratin or PCNA after treatment with scrambled siRNA (set to 100%) or Fak siRNA (*=significance; two-tailed, independent Student’s t test; P<0.02; error bars represent±SEM, n=4). (D) Western blot analysis of cells cultured for 24 h on either BSA or FN, or in the presence or absence of a Fak expression vector. Numbers below lanes represent the densitometric ratio of protein abundance normalized for GAPDH signal where controls are set to one (=significance; two-tailed, independent Student’s t test; P<0.04; n=3). (E) Graphical representation of the number of cells expressing cytokeratin after treatment with an empty vector (set to 100%) or a Fak-containing vector (=significance; two-tailed, independent Student’s t test; P<0.001; error bars represent±SEM; n=3).
result in reduced expression of E-cadherin in potential cleft forming regions, resulting in fewer branch points and a subsequent reduction in bronchiole branch numbers observed. However, down-regulation of FAK signaling by siRNA in whole lung organ culture, while decreasing bronchiole branching, did not reduce E-cadherin abundance measured by immunofluorescence. Thus, the most plausible explanation for reduced E-cadherin abundance in the TIMP3 null lung is as a direct result of impaired proliferation observed in the bronchiole epithelium. Less epithelial cells in smaller and fewer bronchi within the developing TIMP3 null lungs (vs. WT lungs) would logically lead to the decrease in E-cadherin abundance detected by Western blot analysis. In support of this idea, primary lung cell cultures treated with Fak siRNA demonstrated a reduction in PCNA positive cells, a reduction in the total number of epithelial cells in the cultures and a reduction in E-cadherin abundance. Conversely, over-expressing FAK demonstrate increased proliferation and hence, increased epithelial cell number and increased E-cadherin abundance.

It is also known that FAK is involved in the regulation of cell migration, but this function is dependent on cell type or on the type of migration being examined (migration towards a chemo-attractant vs. random migration). Genetic studies have demonstrated reduced migration in FAK-deficient cells suggesting that FAK is required for cell migration (Ilic et al., 1995). Conversely, reduction of FAK through siRNA leads to a reduction in N-cadherin and an increase in random HeLa cell migration (Schaller, 2004; Yano et al., 2004). In the MCF-7 breast cancer cell line, inhibition of SHP-2 (a src homology two-containing phosphotyrosine phosphatase that inactivates FAK) leads to an increase in FAK activation and E-cadherin expression accompanied by a decrease in migration (Wang et al., 2005a). Regardless of these seemingly contradictory results, all support a role for FAK signaling in the regulation of cellular migration, and aberrant cell migration in the developing lung has been shown to disrupt branching (Shannon et al., 2003). Thus, it is tempting to speculate that in the TIMP3 null lungs, down-regulation of FAK signaling may lead to a disruption of either epithelial cell invasion or collective migration of the epithelial sheet during bronchiole branching morphogenesis.

Recent evidence has broadened the function of metalloproteinases beyond simple digestion of ECM molecules to include activation or degradation of bioactive molecules such as growth factors and their receptors (Mott and Werb, 2004; Parks et al., 2004). We have not ruled out the possibility of aberrant growth factor processing or signaling contributing to the anomalous bronchiole branching in the TIMP3 null lung. However, our examination of defective lung development in the TIMP3 null embryo has identified ECM-cell signaling through FAK as a vital aspect of branching morphogenesis, and maintenance of the correct balance between active MMPs and TIMPs is a key regulator of this signaling. We demonstrate here, by a
combination of in vivo and biochemical techniques, that MMPs can degrade FN in the developing lung. While FN degradation has been described in the TIMP3 null involving mammary gland, the net result in the mammary gland was an increase in epithelial apoptosis (Fata et al., 2001). We tested for, but could not detect, enhanced epithelial apoptosis in the null lung. Conversely, we did detect a FN/pFAK-dependent reduction in proliferation in vivo. Thus, our current model is that the absence of TIMP3 in the developing lung leads to enhanced degradation of FN, resulting in reduced ECM-cell signaling through FAK. The reduction in FAK signaling decreases epithelial cell proliferation, culminating in the impaired bronchiole branching observed in TIMP3 null lungs (Fig. 10). Future experiments will allow us to identify the downstream mediators of FAK signaling in lung morphogenesis.

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References


