EMAP II: a modulator of neovascularization in the developing lung

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Lung morphogenesis is a complex process that is instructed by master genes as well as by growth factor signals serving as induction and progression factors to modulate branching morphogenesis, cytodifferentiation, and neovascularization. Pulmonary vascular formation is an ongoing process that is highly regulated, involving not only proliferation and differentiation of vascular structures but also regression and stasis of these structures (20). Two distinct and separate processes, vasculogenesis and angiogenesis, are considered responsible for lung vascularization during fetal growth and development (2, 17). First, vasculogenesis is the transdifferentiation and organization of endothelial cells (ECs) into vascular structures. This occurs from randomly distributed mesodermal cells that transdifferentiate into ECs, proliferate, and organize into multicellular structures singularly arranged around a central lumen. Second, angiogenesis is the extension of previously formed vessels into undervascularized regions where differentiated ECs proliferate, sprout from previously formed vessels, and form new vascular structures. Within the mouse embryo (term 18.5 days), formation of the pulmonary circulation occurs predominantly during the canalicular stage (known as the vascular stage) from embryonic days (ED) 16.5 through 17.4 (1, 10, 14, 25, 26). During this period, there is an increase in the number of cells that express von Willebrand factor (vWF). These cells coalesce with other vWF-positive cells to form vascular structures by about ED16 (24). Furthermore, there is an increase in the EC-specific antigen flik-1. This protein is a receptor for vascular endothelial growth factor (VEGF) and is observed in mesenchymal cells surrounding the developing airways at about the same time (15). A recent morphological study by deMello et al. (4) demonstrated that peripheral vasculogenesis and central angiogenesis occur concurrently to form the lung vasculature. Communication between the two networks is rare early in lung development (ED13–14) but increases gradually until a full vascular circuit is established by ED17. Growth factors such as basic fibroblast growth factor (22) and VEGF (11, 16) are known stimulants of migration, proliferation, and transdifferentiation of ECs, leading to the appearance of vascular structures. However, nothing is known of the factors mediating the regression and stasis of the developing pulmonary vasculature.

Endothelial monocyte-activating polypeptide (EMAP) II is an ~34-kDa cytokine-like molecule first identified from murine methylcholanthrene A-induced fibrosarcomas (8). Although initial data suggested that EMAP II was likely to be a proinflammatory cytokine, in vivo experiments (9) elucidated only a transient and mild inflammatory response. Recently, in vivo studies (unpublished data) have shown EMAP II to have striking antiangiogenic properties defined by inhibition of vessel ingrowth in a Matrigel model and a corneal eye model. Delivery of EMAP II intraperitoneally to mice resulted in significant suppression of murine and human tumor growth. Suppression of tumor growth is likely due to perivascular apoptotic tissue injury caused by the direct targeting of EMAP II to the proliferating EC population (21). In addition, we found that EMAP II induced apoptosis only in ECs in vitro (unpublished data). Therefore, we hypothesized that EMAP II is an important regulator of lung vascularization.
In this study, we report an inverse correlation between EMAP II expression and vascularization within the developing lung. There is a significant decrease in EMAP II mRNA and protein expression in the developing lungs as it transitions from a poorly vascularized structure (ED14) to full fetal lung vascularization at the end of gestation (ED18.5) and through postnatal life into adulthood. This coincides with the neovascularization process. Furthermore, EMAP II is widely distributed, with significant expression in the myoepithelium when the lung is poorly vascularized, suggesting a broad inhibition of vascular development. Localized targeting of EMAP II to perivascular regions of the large arteries occurs later during the vascular stage, suggesting that EMAP II may play a role in maintaining stasis of larger angiogenic vessels while allowing further proliferation and differentiation of the peripheral vasculature. These data suggest a role for EMAP II as a director of neovascularization in the developing lung.

METHODS

RNA isolation, RT, and competitive PCR quantification of mRNA. To assess mRNA levels of EMAP II, we obtained fetal lungs from timed-gestation Swiss-Webster mice (Simonsen, Morgan Hill, CA) at ED14–18 of age (term 18.5 days). Specimens were obtained from at least three different litters, and multiple lungs from the same litter were pooled for each specimen. At least three independent RT products were thus evaluated for each data point. Medialistional structures were removed by microdissection, and total RNA from embryonic lungs was extracted by guanidium thiocyanate after homogenization utilizing a total RNA kit (Qiagen, Santa Clarita, CA). After spectrophotometric quantitation, total RNA was reverse transcribed with oligo(dT) primers as described previously by Zhao and Nishimoto (29). A competitive PCR assay was designed to quantitate subtle developmental differences in gene marker expression. Primers for murine EMAP II were primer I (upstream) 5'-GCATCGCGTCTGATGTTCTTGAAGCT-3' and primer II (downstream) 5'-GTATGTGGCACAACCTCATT-3' (GenBank accession no. U10118). Competitor primers were constructed based on the initial structure of the above designed EMAP II primers. Each composite primer had the target EMAP II gene primer sequence attached to a short stretch of sequence designed to hybridize to the opposite strand of a heterogeneous DNA fragment, thus incorporating itself into the heterogeneous fragment during the PCR amplification and ensuring that the competitor molecules had the same gene-specific primer sequences as the EMAP II cDNA. The heterogeneous DNA was derived from a piece of v-erbB DNA. The competitor PCR product was subsequently 484 bp in size compared with the EMAP II cDNA size of 411 bp, and identities of both EMAP II cDNA and its competitor were confirmed by DNA sequencing.

PCR amplification was carried out in a DNA Robocycler (Stratagene, La Jolla, CA) with a modification of a previously described assay for matrix Gla protein (30) (each time point was repeated 3–5 times). Each sample underwent 35 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 2 min, and extension at 72°C for 2 min after an initial 3-min denaturation at 94°C. The final cycle included a 5-min extension step. Subsequent electrophoresis was performed to separate the target and competitor PCR products that were 411 and 484 bp in size, respectively. The intensity of expression was determined by densitometric analysis of photo-graphed gels (Polaroid 667) with ImageQuant band-analyzing software (Molecular Dynamics, Sunnyvale, CA).

Protein isolation and Western blotting. Fetuses from timed-gestation Swiss-Webster mice at ED14–18.5 (term 18.5 days) or postnatal mice through adulthood were dissected. Afterward, the lungs were isolated and underwent protein analysis. Briefly, specimens from three different litters were obtained at each time point, and multiple lungs from the same litter were pooled for each specimen (for specimens in the fetal stage). In neonatal mice through adulthood, lungs were isolated from mice in different litters on three different occasions and subjected to protein analysis. After the mediastinal structures were removed by microdissection, the lungs were rinsed in 50 mM Tris, pH 7.4, 0.9 N NaCl, and 0.01% NaN₃. The lungs were then homogenized by sonification with a Branson sonifier and stored at −70°C. For Western analysis, homogenates were cleared by centrifugation at 14,000 g for 20 min, the protein concentration was determined by the bicinchoninic acid method, and the samples were normalized by protein content. Equal amounts of protein were then subjected to electrophoresis on a 12% SDS-PAGE gel, transferred to Immobilon-P, blocked overnight in a casein-based blocking solution (Boehringer Mannheim, Indianapolis, IN), and probed with a rabbit anti-EMAP II antibody (polyclonal monoclonal antibody based on immunoblotting of plasma and cell extracts; anti-EMAP II IgG blocked the activity of recombinant EMAP II (rEMAP II) in cell culture assays). Specific binding was detected with a chemiluminescence substrate (Pierce, Rockford, IL) and XAR-5 film (Eastman Kodak, Rochester, NY). Quantitative analysis was accomplished with a Molecular Dynamics personal densitometer.

Immunohistochemistry and histology. Developing lungs on ED14 and ED18 and postnatally through adulthood were fixed with 4% paraformaldehyde (PFA) and then progressively dehydrated before being embedded in paraffin and sectioned. For immunolocalization of EMAP II (polyclonal monospecific antibody based on immunoblotting of plasma and cell extracts; anti-EMAP II IgG blocked the activity of rEMAP II in cell culture assays) and platelet endothelial cell adhesion molecule (PECAM)-1 antigens (28), we employed rabbit anti-rEMAP II IgG (5 μg/ml) and rat anti-PECAM-1 antibody (4 μg/ml). Tissue sections were dehydrated and underwent peroxide quenching. After the sections were blocked, they were exposed to anti-EMAP II IgG or rat anti-mouse PECAM-1 overnight at 4°C with a histostain kit from Zymed (San Francisco, CA). Sections were then incubated with secondary biotinylated antibody according to the manufacturer’s protocol. A brief incubation with the streptavidin-horseradish peroxidase conjugate system (Zymed) was followed by development with the chromogen substrate 3-amino-9-ethylcarbazole. The preabsorbed immune control was accomplished by incubating the primary antibody with a 50-fold amount of EMAP II protein. Immunolocalization was accomplished with a Histostain-DS Kit from Zymed. Briefly, sections were prepared as described above, and then they were blocked and exposed first to anti-mouse PECAM-1 overnight at 4°C. After a brief incubation with a streptavidin–alkaline phosphatase, the second antibody was revealed with 5-bromo-1-chloro-3-indoyl phosphate/nitro blue tetrazolium. Sections underwent blocking and then exposure to the secondary primary antibody, anti-EMAP II, overnight at 4°C. After a brief incubation with the streptavidin-horseradish peroxidase conjugate system, the secondary primary antibody was developed with 3-aminon-9-ethylcarbazole.

In situ hybridization. Developing lungs (ED14 and 2 days postnatally) were obtained for use for in situ hybridization. The digoxigenin (Dig) RNA probe antisense and sense (con-
control) were made with the Dig RNA labeling kit (SP6/T7) from Boehringer Mannheim. Lungs were fixed in 4% PFA in diethyl pyrocarbonate (DEPC)-treated water, dehydrated with ethanol, and then embedded in paraffin. Using DEPC-treated equipment and solutions, we sectioned the paraffin-embedded specimens, and they were rehydrated and incubated in a prewarmed 5 µg/ml proteinase K solution. Slides were then reimmersed in 4% PFA, treated with 0.25% acetic anhydride, and dehydrated. Sections were exposed to a hybridization solution containing 50% formamide, 10% dextran sulfate, 1 mg/ml of tRNA, 1× Denhardt’s solution, 4× saline-sodium citrate (SSC), 50 mM Tris, and 5 mM EDTA that contained 150–300 ng/ml of Dig-labeled RNA probe at 50°C overnight. The RNA probe for EMAP II was 456 bp in size and obtained from a region that has minimal homology with other known proteins. The slides were washed at 55°C in 2× SSC-50% formamide, 1× SSC, and 0.1× SSC for 30 min before being incubated with RNase A (20 µg/ml) for 30 min at 37°C. Slides were then rinsed with 2× SSC, and Dig nucleic acid detection was accomplished with the Genius 3 kit from Boehringer Mannheim. Briefly, slides were incubated in 0.1 M maleic acid-0.15 M NaCl (pH 7.5) for 5 min, and then they underwent blocking in a 1% block reagent. After the blocking, the slides were incubated with anti-Dig-alkaline phosphatase conjugate at 4°C overnight. Slides were then rinsed and incubated with a dilute 5-bromo-1-chloro-3-indoyl phosphate/nitro blue tetrazolium solution for 3 h at room temperature. Afterward, slides were counterstained with a 0.02% fast green solution for 2 min, rinsed in water, air-dried, and mounted.

Statistical analysis. Statistical analysis was performed with Student’s t-test with the program Statview.

RESULTS

Temporal expression of EMAP II mRNA. To determine the level of expression of EMAP II mRNA in the developing lung, levels of EMAP II mRNA were quantitated by competitive RT-PCR. The EMAP II competitive PCR results are illustrated in Fig. 1. The amount of EMAP II was analyzed by comparison to the known 10-fg competitor. We found an inverse correlation between EMAP II expression and

Fig. 1. Competitive RT-PCR for endothelial monocyte-activating polypeptide (EMAP) II mRNA measurement showed that EMAP II mRNA levels decrease in developing murine lung with increasing gestational age. Competitive primer was constructed based on the base of EMAP II primer attached to a short stretch of sequence that hybridizes to opposite strand of a heterogeneous DNA fragment. Length of PCR product of EMAP II and EMAP II competitor are 411 and 484 bp, respectively. A: subsequent electrophoretic pattern for EMAP II competitive PCR, where 10 fg of EMAP II competitor were used to coamplify with a dilutional series of EMAP II cDNA (from lanes 1 to 10: 0, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, and 160 fg, respectively). Inverse relationship between intensities of EMAP II cDNA and its competitor PCR products was obtained. Ethidium bromide-stained gel was densitometrically scanned for band intensities and plotted against initial amount of EMAP II. A linear relationship with a high coefficient of correlation (R^2 = 0.99) was obtained (B). Next, 0, 5, 10, 20, 40, 80, and 160 ng of total RNA extracted from cultured embryonic lungs were reverse transcribed. Each reverse-transcribed mixture was also coamplified with 10 fg of EMAP II competitor template. Resultant PCR products were electrophoresed, stained, and densitometrically analyzed. Log(cDNA/competitor) was plotted against initial amount of total RNA being reverse transcribed (B). Slope of highly linear regression line (R^2 = 0.99) from reverse-transcribed RNA was almost identical to line derived from EMAP II cDNA. Embryonic mouse lungs were harvested for total RNA. Total RNA was reverse transcribed, and reverse-transcribed products were used in EMAP II competitive PCR. Amount of EMAP II mRNA in each assayed sample was subsequently normalized to its β-actin amount as an internal control (C). ED, embryonic day. EMAP II mRNA in ED14 lungs was arbitrarily set at 100%. There was a significant reduction in EMAP II mRNA from ED14 to ED19 (*P < 0.01) that was inversely proportional to increase in vascular formation within developing lung (D). Data are means ± SE of at least 3–6 different lungs for each assayed condition.
vascularization within the developing lung. As fetal lungs entered the vascular stage (ED16.5), there was a significant reduction in EMAP II mRNA. Furthermore, EMAP II mRNA was further reduced up to 83% (P < 0.01), comparing ED14 (a poorly vascularized stage) with ED18 (when there is full fetal vascular development; term 18.5 days; Fig. 1, C and D). This significant reduction in EMAP II mRNA was inversely proportional to the increase in vascular formation within the developing lung.

Temporal expression of EMAP II protein. To assess protein levels of EMAP II in the developing lung, we quantitated the levels of EMAP II by Western blot analysis. Equal amounts of lung protein extracts underwent Western blot analysis (performed in triplicate from three different litters or animals; Fig. 2) and showed a significant (fivefold) decrease in EMAP II protein from ED14 to ED18 (P < 0.001) that was maintained into adulthood (Fig. 2C). During the minimal vascular state, ED14–15, EMAP II was highly expressed. In contrast, there was a marked significant decrease by ED17, well into the vascular stage. This low level of expression, with one exception, was maintained throughout life. Interestingly, we noted that on postnatal days 8–16, there was a significant surge in the expression of EMAP II within the lungs, consistent with the period of time that murine lungs undergo microvascular maturation (Fig 2C). Thus EMAP II protein expression parallels the decrease in EMAP II mRNA.

Spatial expression of EMAP II mRNA and protein. In situ hybridization revealed that at ED14 in fetal lungs (Fig. 3, A and B), EMAP II was produced throughout the lung, with a predominance in the myoepithelium that lines the bronchioles. The location of EMAP II mRNA expression correlates with the same area of expression as other regulators of vessel formation, flt-1 and flk-1 (VEGF receptors localized by in situ hybridization (5)), that show increased expression over the same time period in which EMAP II production is significantly declining. Furthermore, the myoepithelial region is the area where the foregut splanchnopleuric mesoderm is located, from which the vascular supply is derived. In contrast, after full fetal vascular development is reached (postnatal day 2; Fig. 3, C and D), EMAP II is localized predominantly to the perivascular region, consistent with those results seen with immunohistochemistry. This result is consistent with that seen throughout the lung from ED18 into adulthood.

Immunolocalization of EMAP II in the lungs of a 14-day embryo indicates that EMAP II (Fig. 4, A and B) is widely expressed throughout the tissue (Fig. 4B, open arrows) and bronchioles, with marked amounts being produced in the myoepithelial region (Fig. 4B, open arrows).
Fig. 4. EMAP II is distributed widely throughout lung during early poorly vascularized stage of lung development. Immunolocalization of EMAP II in 14-day gestational murine lungs reveals EMAP II to be widely expressed throughout tissue and bronchioles (A; arrow, location of high-power view in B). Of note, there is marked increase in EMAP II located in myoepithelial region (B, closed arrows) consistent with location of EMAP II production as seen with use of in situ hybridization, with scattered diffuse expression noted throughout (B, open arrows). Sections obtained from same lung were stained with platelet endothelial cell adhesion molecule (PECAM)-1 to note vessel location (C; arrow, location of high-power view in D). D, arrows: areas of vessel formation. Coimmunolocalization (E) confirms location of EMAP II [3-amino-9-ethylcarbazole (red)] in relation to vessels. With EMAP II located in myoepithelial (E, open arrow) as well as perivascular region, noted by dual staining (E, closed arrows) with PECAM-1 [alkaline phosphatase (purple)], specificity of EMAP II was confirmed by preabsorbed antibody with excess protein where there was no signal for EMAP II (data not shown). Bar, 500 µm in A and C; 250 µm in B, D, and E.
formed vessels) organize the appearance of vascular proliferation and sprouting of vessels from previously vasculogenesis (the transdifferentiation and organiza-
devlopment of the fetal lung. Two distinct processes, (confirmed by comimunolocalization with the use of PECA-
mandalkine phosphatase; Fig. 4E, closed arrows) and diffusely throughout the lung. In contrast, by ED18, a time when EMAP II production is markedly reduced, EMAP II immunolocalizes predominantly to the large pulmonary vessels (Fig. 5, A and B), with minimal bronchial expression as defined by sequential sections with PECA-1 antibody for vessel identification (Fig. 5, C and D). Commmunolocalization of lungs from a mouse on postnatal day 2 (Fig. 5E) confirms the perivascular location of EMAP II (3-amin6-ethylcarbazole-labeled EMAP II (Fig. 5E, closed arrows) and PECA-1-alkaline phosphatase (Fig. 5E, open arrow)). Interestingly, it appears that EMAP II is localizing to the smooth muscle cell (Fig. 5E, closed arrows), consistent with other modulators of vessel growth such as FGF. With one exception (postnatal days 8–16), subsequent sections obtained from postnatal day 1 through adulthood are consistent with those seen on ED18. During days 8–16 postnatal murine lung (Fig. 6, A and B), coinciding with a surge of EMAP II protein (Fig. 2C), EMAP II distribution is widespread in both large and capillary vessels. Of note, EMAP II is distributed in a perivascular, subendothelial pattern in larger vessels (6B, arrows). This is confirmed through the use of PECA-1 immunochemical staining on sequential sections (Fig. 6, C and D), where PECA-1 is localized to endothelial cell junctions (Fig. 6D, arrows). This period of time is consistent with microvascular maturation of the lung within the mouse. EMAP II immunolocaliza-
tion specificity was confirmed through the use of a preabsorbed control (data not shown). Thus, when EMAP II is highly expressed in the early stages of lung development before significant vascularization of the lung, its distribution is throughout the lung. This is reflected in the spatial expression of EMAP II mRNA and protein, with its localization in the myoepithelial region (considered to be the forerm splanchnopleuric mesoderm responsible for vascular formation), suggesting that it may be functioning as a regulator of vascular development. However, during the later stages when there is rapid neovascularization of the lungs, EMAP II expression is limited to the large vessels. This suggests that the primary function of EMAP II later in gestation is to stabilize or cause stasis of vessel angiogenesis, whereas its decreased expression in the rest of the lung may facilitate the neovascularization process.

**DISCUSSION**

Neovascularization is critical to the growth and development of the fetal lung. Two distinct processes, vasculogenesis (the transdifferentiation and organization of cells into vasculature) and angiogenesis (the proliferation and sprouting of vessels from previously formed vessels) organize the appearance of vascular structures within the mesenchyme of embryonic lungs (2, 17). The development of a mature pulmonary circulation is dependent on the angiogenic ingrowth of central pulmonary vessels from the heart and linkage of these vessels to the distal capillary networks formed by vasculogenesis (3). These two separate embryonic pulmonary vascular systems connect before birth, forming a vascular circuit composed of the central pulmonary artery, branch arteries, capillaries, and veins (19). These two systems arise concurrently and gradually form a uniform vascular system by ED17 (4).

Regulation of new blood vessel growth is controlled by vasoactive agents, growth factors (13, 16, 22, 23), matrix proteins, and tissue interactions (7, 12, 18, 27). Effectors of blood vessel growth display a marked heterogeneity in the developmental responses of the large and small vessels. The initial characterization of neovascularization within the lung was limited by vessel markers that identified vessel development (vascular formation noted on ED16) such as vWF (24). However, Vecchiet al. (28) noted that PECAM-1 antibody was a reliable marker for the identification of murine embryonal ECs; it is expressed within the murine lung vasculature as early as the pseudoglandular stage. The presence of an active developing lung vasculature within the pseudoglandular stage was confirmed by the morphological study of deMello et al. (4). Furthermore, positive modulators of neovascularization such as VEGF have been noted to have an impact on the behavior of primordial ECs engaged in vasculogenesis and are important in the vascular patterning and regulation of vessel size (5).

Lung development, including neovascularization, is classically divided into five chronological stages in the mouse and is based on the growth and differentiation of specific pulmonary epithelial structures: 1) the embryonic stage is from ED9.5 to ED11.5 in the mouse, 2) airway construction occurs in the pseudoglandular stage from ED11.5 to ED16.5, 3) vessels develop in the canalicular stage from ED16.5 to ED17.4 (also known as the vascular stage), 4) the number of terminal sacs and vascularization increase and type I and II epithelial cells differentiate from ED17.4 to postnatal day 5 in the terminal sac stage, and 5) during the alveolar stage, from postnatal days 5–14, there is development and maturation of alveolar ducts and alveoli (1, 10, 14, 25, 26). Hence factors that enhance lung neovascular formation in the developing lung would be expected to have their greatest influence during the canalicular and terminal stages, whereas negative modulators of neovascularization would be expected to exert the greatest influence up to the canalicular stage.

EMAP II, originally identified as an antiangiogenic protein, has been shown to be a potent inhibitor of vessel growth and differentiation in vivo and in vitro (21; unpublished data). Thus EMAP II may play a role in lung vascularization. Consistent with the ability of EMAP II to modulate vessel growth and coinciding with the pseudoglandular stage, levels of EMAP II
mRNA (Fig. 1) and protein (Fig. 2) are significantly elevated. Furthermore, the wide distribution of EMAP II throughout the lung in the pseudoglandular stage (Fig. 4, A and B), within the mesenchymal cell population, bronchioles, and myoepithelial region, suggests that EMAP II exerts an antiangiogenic as well as an antivasculogenic effect during early lung development.

Fig. 5. Perivascular immunolocalization of EMAP II suggests that endothelium is a target of EMAP II. EMAP II immunolocalization in 18-day gestational murine lungs reveals EMAP II expression to be limited to pulmonary vessels (A; arrow, high-power view in B) as defined by sequential sections immunolocalized for PECAM-1 (C; arrow, high-power view in D). B and D, arrows: blood vessels. Immunolocalization reveals similar results in a 2-day postnatal mouse, with EMAP II [3-amino-9-ethylcarbazole (red)] being localized in a perivascular pattern (E, closed arrows). E, open arrows, PECAM-1 and alkaline phosphatase (purple). Interestingly, EMAP II appears to be distributed in a subendothelial cell fashion (E, closed arrows), indicating that it might be located within vascular smooth muscle surrounding vessels (E). Locations of EMAP II at these time points (ED18 and postnatal day 2) are consistent with those seen in mouse lung throughout adulthood. Bar, 500 µm in A and C; 250 µm in B, D, and E.
It has been shown that lung formation is derived from a composite of endodermal and mesodermal tissues. The vasculature of the lung is derived from the foregut splanchnopleuric mesoderm, which surrounds the epithelium as it grows out from the mediastinum into the pleural space, whereas the endoderm of the lung bud gives rise to the mucosal lining of the bronchi and epithelial cells of the alveoli. During the early stages of lung development, expression of EMAP II is increased within the mesenchyme surrounding the bronchioles, an area consistent with the mesoderm. Therefore, the spatial-temporal expression of EMAP II is consistent with its potential role as a negative modulator of vessel growth because vessels arise in the foregut splanchnopleuric mesoderm at later stages of lung development. Furthermore, EMAP II expression within the bronchioles may function as a traffic director for primordial ECs by preventing the development of capillaries within the bronchioles. We have shown that EMAP II has a direct suppressive effect on the dividing and growing ECs (21) and speculate that this suppressive effector is especially strong during fetal growth and development. As a negative modulator of vascular growth, EMAP II appears to be important early in lung development when cell proliferation, neovascularization, and cell dedifferentiation are the main emphasis. Consistent with this observation, Gebb and Shannon (6) recently showed that this same region, the area of the foregut splanchnopleuric mesoderm (myoepithelial layer), contains the VEGF receptors that increase significantly during the vascular stage within the rat, consistent with the time when EMAP II expression is undergoing marked reduction.

In contrast to high expression of EMAP II in the early stages of lung development, during the later vascular stage, there is a significant decline in EMAP II mRNA (Fig. 1) and protein (Fig. 2). This suggests that during rapid neovascularization, EMAP II is no longer present to exert a general negative regulatory effect on vascular formation. The level of EMAP II remains low until postnatal day 8 when there is a marked surge in its expression. This coincides with microvascular maturation within the murine lung. At this stage, EMAP II is...
found in capillaries within the air spaces and in the musculature surrounding large vessels, indicating that EMAP II is holding further vessel development in check. Furthermore, it is intriguing that by ED18 and later, EMAP II appears to be produced within the basement membrane of the blood vessel (Figs. 5 and 6). This is interesting because it is the same location where other positive regulators of vessel growth, such as FGF and VEGF, are made. Therefore, this further supports the modulatory role of EMAP II in vessel growth and stability.

Hence we have shown that during early embryological development when there is minimal vascular development, EMAP II is highly expressed in the developing lung. It is distributed throughout the mesenchyme and strongly concentrated within the myoepithelial layer. EMAP II localization is coincident with the region of the foregut splanchnopleuric mesoderm where the pulmonary vasculature develops. Within this region, we see an initial marked expression of EMAP II that decreases in correlation with the rise in vasculature. At the point of microvascular maturation, EMAP II expression increases and is associated with capillaries and large vessels. The localization of EMAP II to the large vessels during the vascular stage and through adulthood suggests that EMAP II may further influence vascular growth by stabilizing existing vascular structures; however, further investigation is needed.

Therefore, the striking elevation of EMAP II during the early minimal vascular stage of lung development and its rapid decline during the vascularization stage invite the speculation that EMAP II may be a director of endothelial dedifferentiation (vasclogenesis) and the angiogenic process of embryonic endothelial lung development.

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