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INTUSSUSCEPTIVE-LIKE ANGIOGENESIS IN HUMAN FETAL LUNG XENOGRAPTS: LINK WITH BRONCHOPULMONARY DYSPLASIA-ASSOCIATED MICROVASCULAR DYSANGIOGENESIS?

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Abstract

Background—Human fetal lung xenografts display an unusual pattern of non-sprouting, plexus-forming angiogenesis that is reminiscent of the dysmorphic angioarchitecture described in bronchopulmonary dysplasia (BPD). The aim of this study was to determine the clinicopathological correlates, growth characteristics and molecular regulation of this aberrant form of graft angiogenesis.

Methods—Fetal lung xenografts, derived from 12 previable fetuses (15 to 22 weeks' gestation) and engrafted in the renal subcapsular space of SCID-beige mice, were analyzed 4 weeks post-transplantation for morphology, vascularization, proliferative activity and gene expression.

Results—Focal plexus-forming angiogenesis (PFA) was observed in 60/230 (26%) of xenografts. PFA was characterized by a complex network of tortuous non-sprouting vascular structures with low endothelial proliferative activity, suggestive of intussusceptive-type angiogenesis. There was no correlation between the occurrence of PFA and gestational age or time interval between delivery and engraftment. PFA was preferentially localized in the relatively hypoxic central subcapsular area. Microarray analysis suggested altered expression of 15 genes in graft regions with PFA, of which 7 are known angiogenic/lymphangiogenic regulators and 5 are known hypoxia-inducible genes. qRT-PCR analysis confirmed significant upregulation of *SULF2*, *IGF2* and *HMOX1* in graft regions with PFA.

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DECLARATION OF INTERESTS.

The authors declare no conflict of interest.

Conclusion—These observations in human fetal lungs *ex vivo* suggest that postcanalicular lungs can switch from sprouting angiogenesis to an aberrant intussusceptive-type of angiogenesis that is highly reminiscent of BPD-associated dysangiogenesis. While circumstantial evidence suggests hypoxia may be implicated, the exact triggering mechanisms, molecular regulation and clinical implications of this angiogenic switch in preterm lungs *in vivo* remain to be determined.

Keywords

chronic lung disease of newborn; BPD; angiogenesis; insulin-like growth factor; sulfatase 2; heme oxygenase 1

INTRODUCTION

Premature infants with structurally immature lungs born between 23 and 28 weeks gestation are at risk for bronchopulmonary dysplasia (BPD), or chronic lung disease of the preterm newborn, a complex condition associated with high perinatal morbidity and mortality¹. In spite of increased use of exogenous surfactant and antenatal steroids, improved ventilatory strategies, and changes in neonatal intensive care, the proportion of surviving infants with BPD has remained unchanged between 1995 and 2006². An estimated 30% of very low birth weight infants (weighing less than 1,500 g) will develop BPD and are predisposed to its long term complications, including asthma, emphysema, and poor neurodevelopmental outcome.^{3, 4}

The main pathological hallmark of BPD at autopsy is an arrest of alveolar development, characterized by diminished alveolar septation and significantly reduced secondary crest formation, resulting in pseudo-emphysematous, large and simplified distal airspaces separated by septa showing various degrees of interstitial fibrosis^{5, 6}. In addition to these well described architectural characteristics of the distal lung parenchyma in BPD, recent attention has been given to the pulmonary microvascular alterations in infants with BPD^{6–10} or in BPD-like animal models such as chronically ventilated premature baboons¹¹. Lungs of premature infants with early or fully established BPD who succumbed with or due to their lung disease show tortuous, non-sprouting pulmonary microvasculature, described as “BPD-associated dysmorphic microvasculature” or “BPD-associated dysangiogenesis”^{6, 9, 10}. Disruption of normal microvascular development in premature lungs has been implicated in the arrested alveolar development that is typical of BPD¹², as proper formation of the pulmonary microvasculature is believed to be required for normal alveolar development¹³.

The mechanisms underlying this BPD-associated dysangiogenesis remain poorly understood, which is attributable, in large part, to the lack of a valid model system. Existing *in vivo* animal model systems of BPD include rodents, rabbits and baboons exposed to a wide range of injurious factors, such as hypoxia, hyperoxia, invasive mechanical ventilation, inflammation and genetic manipulation [summarized in^{14–17}]. The lungs of most of these animal models exhibit simplified, enlarged airspaces, diminished alveolar septation, and thin septa and thus provide a faithful replication of the pseudo-emphysematous alterations typical of BPD [^{14–17}]. The pulmonary microvasculature in these animal models has been relatively neglected or, when studied, has only been reported to be attenuated or diminished in size.

Similarly, in our previously described animal models of BPD, which were based on perinatal induction of respiratory epithelial apoptosis¹⁸ or neonatal hyperoxia exposure¹⁹, the vasculature was linear and non-sprouting within thin septa. To our knowledge, there is at present no valid animal model that replicates the complex and tortuous dysmorphic microvascular alterations observed in infants with severe BPD at postmortem examination⁶.

In the course of recent studies that employed a human-to-rodent fetal lung xenograft model^{20, 21}, we identified unusual patterns of microvascular growth in a subset of human fetal lung grafts. The microscopic appearance of these aberrant vascular patterns was strongly reminiscent of the microvascular dysmorphism previously described in postmortem studies of premature ventilated infants and infants with BPD⁶. In view of the exciting possibility that human fetal lung xenografts represent a much sought-after model of BPD-associated dysangiogenesis, the present study was undertaken to further characterize this intriguing phenomenon. The aim of this study was to determine the morphologic and growth characteristics, clinicopathologic correlates and molecular regulation of the dysmorphic graft microvasculature of the human fetal lung xenograft as a potentially unique model of BPD-associated dysangiogenesis.

MATERIALS AND METHODS

Patients

This study was centered on 12 xenograft experiments, which used lung tissues derived from 12 fetuses who underwent postmortem examination at Women and Infants Hospital (Providence, RI). Lung samples were obtained from previable (< 22 weeks' gestation) second trimester fetuses. The study protocol was approved by the institutional review board and full informed written consent was obtained in compliance with institutional guidelines. The study was limited to fetuses delivered following spontaneous (non-induced) pregnancy loss. Fetuses delivered by elective or medical abortions and fetuses with congenital, chromosomal, pulmonary, or cardiac anomalies were excluded. In addition, fetuses with evidence of any degree of maceration, reflecting a prolonged interval between fetal demise and delivery²², were excluded. Records were reviewed for postmenstrual age (PMA) at delivery and likely cause of death and/or preterm delivery. The post-delivery interval (i.e. time between delivery and grafting of the lung tissue) was recorded. Morphologic findings in xenografted lungs were compared with postmortem lungs of premature infants with neonatal lung disease, retrieved from the archives of the Department of Pathology at Women and Infants Hospital.

Harvesting, transplantation and processing of fetal lung

The fetal examinations were performed by the Perinatal Pathology staff at Women and Infants Hospital according to standard methods. The gestational age was obtained from the records and confirmed by fetal foot length measurements. Samples for transplantation (1–2 mm³ pieces) were taken from the peripheral parenchyma of the right lung. Transplantation was performed at the Xenotransplant Core Facility at Brown University (Providence, RI), as previously described^{20, 21}. Recipients of the renal subcapsular xenografts were 6 to 12-week-old male SCID-beige mice (Fox Chase SCID beige, Charles River, Wilmington, MA).

For each of the 12 experiments, 3 to 9 mice were transplanted with 3 to 5 lung tissue fragments per kidney each. The xenografts were analyzed 4 weeks after implantation. The animals were euthanized by an overdose of isoflurane. The kidneys were formalin-fixed and paraffin-embedded *in toto* for histologic and immunohistochemical studies. All animal research was conducted in accordance with Brown University institutional guidelines for the care and use of laboratory animals in compliance with National Institute of Health guidelines.

Analysis of graft morphology, vascularization and proliferation

Graft vascularization was studied by immunohistochemistry using an antibody against the endothelial marker, CD31 (PECAM-1). The antibody used in this study is specific for human CD31 antigen and does not cross-react with the mouse antigen (DakoCytomation, Glostrup, Denmark). Additional immunohistochemical analyses were performed using anti-carbonic anhydrase IX antibody (Abcam, Cambridge, MA) as marker of tissue hypoxia and anti-Ki-67 antibody (DakoCytomation) as proliferation marker. The CD31, CAIX and Ki-67 immunohistochemical studies were performed using an avidin-biotin-peroxidase system, as described before⁶. Endothelial cell proliferation was assessed by double immunofluorescence studies combining labeling for Ki-67 and CD31, as described before^{6, 20}. Controls for specificity consisted of omission of the primary antibody, which abolished all staining.

Laser-capture microdissection, RNA extraction, microarray and qRT-PCR analysis of gene expression in xenografts

For gene expression studies, graft areas with or without altered angiogenesis were selectively captured on LCM Macro CapSure caps (Applied Biosystems, Foster City, CA) using the Arcturus XT LCM instrument (Applied Biosystems). Graft regions without PFA, derived from the same graft, served as control tissue for graft regions with PFA. Total RNA was extracted from the captured areas using the RecoverAll Total Nucleic Acid Extraction kits for formalin-fixed, paraffin-embedded (FFPE) tissues (Ambion, Austin, TX). RNA was further purified and concentrated using the RNEasy Minelute Cleanup kit (Qiagen, Valencia, CA) and then evaluated by the Agilent Bioanalyzer using an RNA 6000 Nano or Pico LabChip (Agilent Technologies, Santa Clara, CA).

Total RNA (150 ng) extracted from FFPE blocks was used with the Affymetrix SensationPlus™FFPE Amplification and WT labeling Kit (cat # 902042) according to the manufacturer's instructions. The *in vitro* transcription step yielded between 62 and 112 µg of total RNA per sample. 25 µg of RNA was used as input in the 2nd cycle resulting in single stranded cDNA (sscDNA) amounts ranging between 4.1 and 6.4 µg. For each sample, 3.5 µg of sscDNA was hybridized overnight at 47°C and 60 rpm to Human Gene ST 1.0 arrays. Overnight hybridizations were followed by the standard Affymetrix wash and stain protocol, according to the manufacturer's instructions, and visualized using an Affymetrix 3000 7G scanner. Quality control and data analysis to detect differentially expressed genes were carried out using Partek Genomics Suite version 6.6 using the RMA (robust multiarray) and default settings.

50 ng total RNA was used for qRT-PCR analysis. Synthesis of cDNA was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Bedford, MA) according to the manufacturer's instructions. The TaqMan PreAmp Master Mix kit (Applied Biosystems) was used to effectively increase the sensitivity of the real-time PCR analysis. The preamplified cDNA was used to run real-time qRT-PCR using the TaqMan Gene Expression Master mix in the ABI 7500 Fast Real Time PCR system (Applied Biosystems). Primer and probe sets used were premade by and purchased from Applied Biosystems. The data were first normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and then analyzed using the $\Delta\Delta C_t$ method. The mRNA expression was reported as fold change over control (calibrator).

Data analysis

Values are expressed as mean $\pm$ standard deviation (SD) or standard error of mean (SEM). Statistical analyses were performed using standard one-way ANOVA with Dunnett's multiple comparison test (GraphPad Prism; GraphPad Software, Inc., San Diego, CA). The significance of difference between groups was determined by unpaired Student *t* test where applicable. The significance level was set at $P < 0.05$.

RESULTS

Clinical data

The clinical data of the 12 fetuses whose lung tissues were used in the xenograft experiments are summarized in Table 1. The mean gestational age was 18.8 ± 2.7 weeks (range: 15 to 22 weeks). The vast majority (10/12) were male. Seven of twelve fetuses were stillborn (all 20 weeks' gestation or less); the remaining 5 patients (all between 20 and 22 weeks' gestation) were liveborn and lived for less than one hour. In 6/12 cases, preterm delivery was attributable to an infectious etiology/acute chorioamnionitis. In 5 of these 6 cases the placenta showed histopathologic evidence of associated fetal inflammatory response (funisitis and/or chorionic vasculitis). In the remaining 6/12 cases, there was no histopathologic evidence of infection/inflammation upon examination of the placenta. The post-delivery interval ranged between 12 and 36 h (median: 20.5 h).

Analysis of graft morphology and vascularization

Between 6 and 43 (median: 14.5) renal subcapsular grafts were available for morphologic analysis for each of the 12 xenograft experiments. Most grafts, examined at post-transplantation week 4, showed relatively large, angulated airspaces lined by low cuboidal or flat epithelium (Fig. 1). In most grafts, the airspace septa were relatively thin throughout with inconspicuous microvasculature by hematoxylin-eosin staining (Fig. 1B,G bottom). The airspace lining focally had an irregular serrated 'pseudo-saccular' appearance caused by occasional epithelial projections from the wall. A subset of grafts showed regional widening and expansion of the septa associated with prominent, abundant and congested capillaries (Fig. 1B,G top). This distinct zonal hypervascularity, seen in 60/230 grafts (26%), was only seen in the central subcapsular region (Fig. 1B,C,G,H).

Delineation of the endothelial cells by anti-CD31 immunohistochemistry allowed more detailed examination of the pulmonary microvasculature in normal and hypervascular areas of the xenografts (Fig. 1D,E,I,J). Consistent with their ‘pseudo-saccular’ or late canalicular microscopic appearance, the capillaries in normal appearing xenograft areas generally displayed a linear double-track network with focal evidence of sprouting (Fig. 1D,I bottom). In contrast, the vessels in the subcapsular hypervascular areas formed more complex networks, composed of interdigitating tortuous and abundant capillaries that spanned the entire width of the distended septa. The capillaries in these networks were often engorged with red blood cells and displayed prominent bulbous or sinusoidal dilation without evidence of classic monopodial sprouting (Fig. 1D,E, I, J top). The transition between normal, age-appropriate double-track linear vasculature and aberrant plexus-forming vasculature was usually abrupt (Fig. 1I,J). From here on, these subcapsular hypervascular regions will be described as displaying ‘plexus-forming’ angiogenesis.

Vascularization of human preterm lungs

The vasculature of xenografts, both in normal areas and in areas displaying plexus-forming angiogenesis, was compared with that of archival postmortem lungs from age-matched preterm infants (Fig. 2). Lungs of infants between 22 and 26 weeks’ gestation who lived less than 24 hours were studied as non-ventilated controls. Consistent with the late canalicular/early saccular stage of development, the alveolar septa of control lungs were relatively thin and displayed occasional secondary crest formation (Fig. 2A–B). Anti-CD31 immunostaining demonstrated the typical linear arrangement of slender vessels in subepithelial location, forming a single- or double-track pattern within the septa (Fig. 2C–D).

Lungs of infants born between 23–24 weeks who had lived for 1–2 weeks were used to demonstrate the findings after short-term ventilation and exposure to other potentially lung-modifying events associated with preterm birth and subsequent neonatal care. As shown in Fig. 2E–H, the microvasculature of preterm short-term ventilated infants had several morphologic features in common with the observed plexus-forming angiogenesis in xenografts. As described before⁶, the lungs of short-term ventilated infants, at least of those who succumbed with or due to lung disease, demonstrated widening and conspicuous vascular congestion of the septa (Fig. 2E–F). Within these distended septa, the microvasculature was complex and tortuous, and demonstrated an admixture of aneurysmally dilated vessels alternating with smaller ones, as highlighted by anti-CD31 staining (Fig. 2G–H). The vessels were engorged with red blood cells and formed an irregular network that occupied the entire width of the distended septa, rather than being restricted to the subepithelial zone in a strict linear configuration.

Correlation between clinical data and frequency of plexus-forming angiogenesis in xenografts

Plexus-forming angiogenesis was observed in grafts derived from all 12 individual source lungs, although the proportion of grafts exhibiting plexus-forming angiogenesis was variable, ranging between 12% and 45% per experiment (median: 26.5%) (Table 1). There was no correlation between the frequency of PFA and any of the clinical variables

examined, including gestational age or post-delivery interval. Similarly, the fraction of PFA-displaying xenografts was equivalent between stillborns and liveborns (median: 26% (N = 7) versus 27% (N = 5), respectively) and between fetuses with or without acute chorioamnionitis (median: 27.5% (N = 6) versus 23% (N = 6), respectively). Finally, there appeared to be no correlation between frequency of PFA and gender, although only two xenografts were derived from female fetuses (median PFA frequency: 26.5 % in males (N = 10) versus 27.5% in females (N = 2)). These findings suggest that the propensity of xenografted fetal lung tissue to undergo plexus-forming angiogenesis was independent of these fetal or pregnancy-associated clinical characteristics.

Analysis of proliferative activity in fetal lung xenografts

The proliferative activity in graft regions with normal or plexus-forming angiogenesis was studied by anti-Ki-67 immunohistochemistry. As shown in Fig. 3, subcapsular foci with plexus-forming angiogenesis displayed brisk proliferative activity. The proliferative activity of endothelial cells was assessed by double immunofluorescence staining, combining anti-Ki-67 (proliferation marker) and anti-CD31 (endothelial cell marker) antibodies. Colocalization of nuclear Ki-67 and cytoplasmic/membranous CD31 signals, indicative of endothelial cell proliferation, was only rarely observed (Fig. 3A2, arrows). Instead, most Ki-67 immunoreactivity was localized to CD31-negative, non-endothelial cells (Fig. 3B2). Based on their location and curved nuclei, many of these proliferating cells are consistent with pericytes.

Analysis of relative tissue hypoxia by carbonic anhydrase IX (CAIX) immunohistochemical analysis

Plexus-forming angiogenesis in human fetal lung xenografts was localized to the central subcapsular zone. This zone is farthest away from the host renal cortex which, through vascular connections between graft and host organ, provides perfusion and oxygenation of the graft. Based on this preferential location, we speculated that the development of plexus-forming angiogenesis was induced or mediated, in part, by relative tissue hypoxia. To test this hypothesis, we studied the regional distribution of hypoxic regions in the human fetal lung xenografts by immunohistochemical analysis of expression of carbonic anhydrase (CA) IX as marker of tissue hypoxia²³. As shown in Fig. 3C, there was a distinct spatial gradient of CAIX immunoreactivity within xenografts, with most intense CAIX staining in the immediate subcapsular graft stroma and epithelium, suggestive of relative tissue hypoxia.

Analysis of gene expression in xenograft areas with or without plexus-forming angiogenesis

To understand the molecular regulation of altered, plexus-forming angiogenesis in human fetal lung xenografts, we performed global gene expression studies using the available formalin-fixed, paraffin-embedded material. For each xenograft, areas with or without plexus-forming angiogenesis were selectively captured by laser-capture microdissection (Fig. 4A–D). Tissues were pooled to yield 4 groups with or without PFA, each representing 3–4 different grafts from different experiments. Microarray analysis identified 15 genes suggestive of differential regulation ($P < 0.05$) (Table 2). Of these 15 genes, 14 were found

to be upregulated in areas with PFA, while a single gene, *HBE1*, was found to be down-regulated.

The gene expression differences between graft areas with or without plexus-forming angiogenesis detected by microarray analysis were tested further by qRT-PCR analysis of a larger number of individual grafts, derived from at least 6 lung samples from different experiments. Genes significantly upregulated in areas with plexus-forming angiogenesis compared with graft areas without plexus-forming angiogenesis included *IGF2*, *HMOX1* and *SULF2* (Fig. 4E). Expression of the following genes was determined to be equivalent between graft areas with or without plexus-forming angiogenesis: *PGF*, *ENO1*, *SIPPI1*, *LYVE1*, and *BNIP3*. Other genes identified as potentially differentially regulated by microarray analysis could not be evaluated by qRT-PCR due to the limited RNA amounts available.

DISCUSSION

In this study, we have described an unusual microvascular growth pattern observed in human fetal lung-to-rodent xenograft experiments. This aberrant form of angiogenesis, characterized by complex branching and interdigitating networks of tortuous, bulbously dilated and congested capillaries, was easily distinguished from the more usual sprouting type of angiogenesis that is age-appropriate for second-trimester human fetal lungs²⁴. The abnormal vascular growth pattern in human fetal lung xenografts, for which we elected the descriptive term “plexus-forming angiogenesis”, bears a striking resemblance to the dysmorphic microvasculature previously described in postmortem lung samples of premature infants^{6, 9}. This suggests a deeper understanding of the mechanisms underlying the observed dysangiogenesis in fetal lung xenografts can provide insights into the apparently analogous phenomenon described in preterm human lungs *in situ*.

The morphologic features of the plexus-forming angiogenesis seen in human fetal lung xenografts, including the complexity of the vascular networks, the tortuosity and sinusoidal or bulbous dilation of the vascular structures, and the low proliferative activity of the endothelial cells, suggested a variant form of intussusceptive angiogenesis. Intussusceptive angiogenesis was formally described for the first time in the developing rat lung²⁵. The basic event in intussusceptive angiogenesis (alternatively described as “longitudinal splitting” or “luminal division”) is the formation of transvascular (intraluminal) tissue pillars, formed by invagination of the capillary walls into the vascular lumen^{25, 26}. Pillar formation in capillaries results in rapid expansion of the capillary bed in size and complexity (intussusceptive microvascular growth) and also represents a mechanism for pruning redundant vessels and for remodeling the existing microvasculature^{26, 27}. While the light microscopic features of plexus-forming angiogenesis in xenografts are strongly suggestive of intussusceptive angiogenesis, it needs to be emphasized that the categorization of this type of angiogenesis depends on visualization of transluminal pillars by high-resolution three-dimensional imaging techniques, which were not available for the current studies²⁶. For now, therefore, the aberrant plexus-forming angiogenesis seen in human fetal lung xenografts is best described as “intussusceptive-like”.

It is reasonable to postulate that, under appropriate circumstances, human preterm lungs have the capacity to undergo a switch from sprouting to intussusceptive angiogenesis. Intussusceptive angiogenesis is recognized as a common mechanism of blood vessel growth during development, and a switch from initial sprouting to subsequent intussusceptive angiogenesis has been described in developing chick chorioallantoic membrane, lungs, bone, retina, muscle, kidney, ovary and mammary gland [reviewed in²⁸]. In addition, intussusceptive angiogenesis has been described in physiological adaptations, such as post-pneumectomy regenerative lung growth²⁹ and exercised muscles³⁰, and in pathological conditions, such as tumorigenesis^{26, 31}. Intussusceptive angiogenesis has further been observed in different murine disease models, including models of liver cirrhosis, models of inflammation, and the hypoxic mouse retina, and is suspected of participation in the excessive angiogenesis that characterizes psoriasis, rheumatic disease, and retinopathy³².

In this study, grafts derived from all 12 individuals demonstrated plexus-forming angiogenesis, irrespective of their clinical background. Specifically, we found no correlation between PFA frequency and clinical variables such as gestational age, time interval between delivery and graft implantation, presence or absence of chorioamnionitis, and live birth or stillbirth. For each individual experiment (i.e. source lung), however, the fraction of grafts demonstrating PFA was highly variable, ranging between 12% and 45%. It is unclear why only a subset of grafts, derived from the same fetal lung, displayed this angiogenic pattern. It is possible that the topographic derivation of the grafts (such as subpleural versus central location) may have contributed to the observed angiogenic diversity. Indeed, as demonstrated in a murine model of post-pneumectomy lung growth, the patterns of compensatory alveolar angiogenesis show regional differences in the lung, with subpleural regions, in particular, displaying the potential to undergo intussusceptive angiogenesis²⁹.

The mechanisms underlying the switch from sprouting to intussusceptive-like angiogenesis observed in a subset of human fetal lung xenografts remain unclear. The foci of plexus-forming angiogenesis were localized to the central subcapsular region of the grafts. This preferential location, at a maximal distance from the graft-kidney interface, indicates that deficient perfusion/relative tissue hypoxia could be the angiogenic switch. In agreement with this hypothesis, the subcapsular region was found to be relatively hypoxic in comparison to the remainder of the graft, as indicated by intense immunoreactivity with the tissue hypoxia marker carbonic anhydrase IX²³. By analogy, switching from sprouting to intussusceptive angiogenesis has been recognized as an adaptation to hypoxia in the adult retina³³, in tumors³⁴ and in a murine model of ovarian pedicle repair following ovariectomy³⁵. While it is plausible that hypoxia played a major role in the angiogenic switch in xenografts, other factors, such as mechanical factors specific to the subcapsular region, could be contributory. By analogy, flow alterations affecting shear stress (in endothelial cells) and wall stress (in smooth muscle cells) have been implicated in intussusceptive angiogenesis in various clinical and experimental conditions [reviewed in²⁷].

The molecular regulation of intussusceptive angiogenesis remains poorly understood, although several growth factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor-B (PDGF-B), angiopoietins and their Tie receptors, and others, have been implicated [reviewed in²⁷]. To begin to understand the molecular

regulation of plexus-forming angiogenesis in human lung xenografts, we compared the global gene expression patterns of graft areas with plexus-forming angiogenesis with those of graft areas displaying normal, age-appropriate sprouting angiogenesis. Of the fifteen genes determined to be differentially regulated, at least 7 have been shown to have angiogenic or lymphangiogenic regulatory functions; in particular: *SULF2*³⁶, *ARHGDIB* (RhoGDI2)³⁷, *HMOX1*³⁸, *PGF*³⁹, *ENO1*⁴⁰, *IGF2*⁴¹ and *LYVE1*⁴². In addition, at least 5 of the 15 genes determined to be upregulated in areas of plexus-forming angiogenesis are known to be hypoxia-inducible, at least under some conditions; namely: *BNIP-3*, *HMOX1*, *PGF*, *ENO1* and *IGF2*. Three genes identified as differentially regulated by microarray analysis were confirmed to be significantly upregulated in areas of plexus-forming angiogenesis by confirmatory qRT-PCR analysis, namely: *IGF2*, *HMOX-1*, and *SULF-2*. Interestingly, *IGF2* may be involved in the pathological neovascularization that characterizes proliferative diabetic retinopathy and retinopathy of prematurity⁴³, complex vascular dysangiogenetic processes that exhibit similarities to the plexus-forming angiogenesis observed in fetal lung xenografts.

This study had several limitations. First, microarray analysis was based on RNA extracted from small amounts of formalin-fixed, paraffin-embedded tissue, and was therefore limited by both RNA quantity and quality. Second, as stated before, we were not able to formally categorize the pattern of aberrant angiogenesis in the lung xenografts. Whereas various morphologic features, including the complex networks of tortuous, non-sprouting vessels and the low endothelial cell proliferation, are highly suggestive of intussusception, unequivocal classification as such will require higher resolution three-dimensional imaging techniques such as scanning electron microscopy of vascular corrosion casts or serial sectioning for light or transmission electron microscopy followed by three-dimensional reconstruction, confocal laser scanning microscopy or high-resolution micro computerized tomography for visualization of the complex spatial structure of transluminal pillars²⁶.

In conclusion, we have described an aberrant non-sprouting, “intussusceptive-like” type of angiogenesis in human fetal lung xenografts that strongly resembles BPD-associated dysangiogenesis⁶. The occurrence of this phenomenon in human fetal lung xenografts underscores the developmental plasticity of postcanalicular lungs and supports the notion that, under certain conditions, human preterm lungs have the capacity to switch from sprouting microvascular angiogenesis to a plexus-forming, intussusceptive-type angiogenesis. Circumstantial evidence suggests hypoxia and dysregulated IGF signaling play a role in this angiogenic switch in human fetal lung grafts *ex vivo*. The clinical correlates and detailed molecular mechanisms of the presumed analogous angiogenic switch in human preterm lungs *in vivo* remain to be determined.

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Figure 1. Vascularization in xenografts

A–E. Representative preimplantation lung (A) and corresponding renal xenograft (B–E) (graft obtained at 19 weeks' gestation, studied at post-transplant week 4). The preimplantation lung shows elongated, branching tubular structures placed in abundant, immature appearing mesenchymal stroma, consistent with early canalicular stage of development (A). In the xenograft derived from this lung, examined 4 weeks post-engraftment, a distinct hypervascular region is present in the central subcapsular zone (B–C). Higher magnification shows two different patterns of angiogenesis: the top of the figure displays complex networks of engorged capillaries that span the entire width of the distended airspace septa (“plexus-forming angiogenesis”); the bottom demonstrates the linear, mainly subepithelial capillary structures that are appropriate for postcanalicular lung development (C–E).

F–J. Representative preimplantation lung (F) and corresponding renal xenograft (graft obtained at 22 weeks' gestation, studied at post-transplant week 4). The preimplantation lung shows more prominent and irregular airspaces, separated by thinning septa, consistent with late canalicular stage of development (F). As in figure 1A–E, the xenograft shows regional hypervascularity, limited to the central subcapsular zone. In this case, there is an abrupt transition from plexus-forming angiogenesis (H–J, top) to normal angiogenesis (H–J, bottom).

D, E, I, J: CD-31 (PECAM-1) immunohistochemical analysis (DAB-peroxidase staining with hematoxylin counterstain); all other panels: hematoxylin-eosin staining.

B, G: original magnification: $\times 40$; A, F: $\times 100$; C, D, H, I: $\times 200$; E, J: $\times 400$.

Figure 2. Vascularization in control human lungs

A–D. Representative control lung (infant born at 24 weeks' gestation, lived for 2h). The lungs display the relatively narrow septa, irregular airspaces, and predominantly subepithelial capillary network organized in a double tram-track pattern characteristic of post-canalicular, early saccular lung development.

E–H. Representative lung of short-term ventilated infant (infant born at 23 weeks' gestation, lived for 8d, ventilated). Compared to age-matched control lungs, these lungs demonstrated widened airspace septa and larger, simpler airspaces. Abundant bulbously or sinusoidally dilated capillaries are randomly scattered throughout the entire width of the septa.

C, D, G, H: CD-31 (PECAM-1) immunohistochemical analysis (DAB-peroxidase staining with hematoxylin counterstain); other panels: hematoxylin-eosin staining.

A, E: original magnification; $\times 100$; B, C, F, G: $\times 200$; D, H: $\times 400$.

Figure 3. Analysis of proliferation and tissue hypoxia in xenografts

A1. Renal xenograft (obtained at 22 weeks' gestation, studied at post-transplant week 4). Numerous Ki-67-positive nuclei are seen in this subcapsular zone of plexus-forming angiogenesis, consistent with brisk proliferative activity.

A2. In addition to proliferating endothelial cells demonstrating double immunoreactivity for Ki-67 (green, nuclear) and CD31 (red, cytoplasmic) (arrows), numerous non-endothelial proliferating cells (CD31-negative, Ki-67-positive) are present immediately adjacent to the capillary structures.

B1–B2. Renal xenograft (obtained at 20 weeks' gestation, studied at post-transplant week 4). Similar to the graft shown in A1–2, the region of plexus-forming angiogenesis in this slightly younger lung shows prominent proliferative activity, mainly localized to non-endothelial cells.

C. Renal xenograft (obtained at 19 weeks' gestation, studied at post-transplant week 4). Immunostaining for carbonic anhydrase (CA) IX, a tissue hypoxia marker, demonstrates more intense CAIX immunoreactivity in the immediate subcapsular region compared with the graft-kidney interface, suggestive of a hypoxic gradient within the graft.

A1–B1: hematoxylin-eosin staining (left panel); CD31 immunostaining (middle panel) and Ki-67 immunostaining (right panel); A2–B2: Ki-67 (Alexafluor-green) and CD31 (Cy3-red) double immunofluorescence; C: carbonic anhydrase (CA) IX immunostaining.

A1 and B1: original magnification $\times 400$; A2: $\times 400$ (left panel) and $\times 800$ (right panel); C: $\times 100$ (left panel) and $\times 200$ (right panel).

Figure 4. Laser capture microdissection (LCM) and qRT-PCR analysis of gene expression

A–B. Renal xenograft (obtained at 20 weeks' gestation, studied at post-transplant week 4) before (A) and after (B) laser capture microdissection of the region of plexus-forming angiogenesis. The remaining graft tissue is sampled in subsequent steps, and collected separately. The overlying renal (murine) capsule is not sampled.

C–D. Higher magnification of fig. 4A illustrating the complex capillary network of plexus-forming angiogenesis with, in this case, apparent extension into the overlying renal capsule.

A: Original magnification $\times 100$; B: $\times 60$, C–D: $\times 200$.

A, C: hematoxylin-eosin staining; D: CD-31 (PECAM-1) immunohistochemical analysis (DAB-peroxidase staining with hematoxylin counterstain).

E. Results of qRT-PCR analysis of mRNA expression of *IGF2*, *SULF2* and *HMOX1* in graft regions without (“control”) or with (“PFA”) plexus-forming angiogenesis.

Data represent mean $\pm$ SD.

*: $P < 0.05$; **: $P < 0.01$ versus control.

Table 1

Clinical data.

Case #	Age (wks)	Sex	SB/LB	ACA	PDI (hrs)	Fraction (%) of grafts with PFA
1	15	M	SB	Y	14	2/8 (25)
2	15	M	SB	Y	12	4/14 (29)
3	16	F	SB	Y	30	5/13 (38)
4	16	M	SB	Y	33	5/43 (12)
5	17	M	SB	N	36	3/9 (33)
6	20	M	SB	Y	12	6/23 (26)
7	20	M	SB	N	17	7/37 (19)
8	20	M	LB	N	12	15/33 (45)
9	21	M	LB	Y	36	6/17 (35)
10	21	F	LB	N	20	2/12 (17)
11	22	M	LB	N	21	1/6 (17)
12	22	M	LB	N	21	4/15 (27)

M: male; F: female; SB: stillborn; LB: live-born; ACA: acute chorioamnionitis; PDI: post-delivery interval; PFA: plexus-forming angiogenesis

Table 2

Differentially expressed genes in areas with plexus-forming angiogenesis ($P < 0.05$).

Gene symbol	Gene ID	Fold change	P value	Description
Genes upregulated in areas with plexus-forming angiogenesis				
<i>Fam185A</i>	NM_001145268	1.59	5.62E-05	Family with sequence similarity 185, member A
<i>SULF2</i>	NM_018837	1.62	29.96 E-05	Sulfatase 2
<i>BNIP3</i>	NM_004052	1.52	0.00127	Bcl2/adenovirus E1B 19 kDa interacting protein 3
<i>ARHGD1B</i>	NM_001175	1.56	0.00643	Rho GDP dissociation inhibitor (GDI) beta
<i>ABCC9</i>	NM_005691	1.80	0.01045	ATP-binding cassette, sub-family C (CFTR/MRP), member 9
<i>LDHA</i>	NM_005566	1.91	0.01103	Lactate dehydrogenase A
<i>HMOX1</i>	NM_002133	1.51	0.01549	Heme oxygenase (decycling) 1
<i>PGF</i>	NM_002632	1.53	0.02391	Placental growth factor
<i>ENO1</i>	NM_001428	1.56	0.01451	Enolase 1, (alpha)
<i>IGF2</i>	NM_000612	2.51	0.02531	Insulin-like growth factor 2 (somatomedin A)
<i>LYVE1</i>	NM_006691	1.66	0.03086	Lymphatic vessel endothelial hyaluronan receptor 1
<i>SNORD45B</i>	NR_002748	1.78	0.03156	Small nucleolar RNA, C/D box 45B
<i>VTRNA1-2</i>	NR_026704	1.59	0.04112	Vault RNA 1–2
<i>SPP1</i>	NM_001040058	1.87	0.04748	Secreted phosphoprotein 1
Genes downregulated in areas with plexus-forming angiogenesis				
<i>HBE1</i>	NM_005330	1.62	0.01366	Hemoglobin, epsilon 1