Epithelial cell integrin β1 is required for developmental angiogenesis in the pituitary gland

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As a key component of the vertebrate neuroendocrine system, the pituitary gland relies on the progressive and coordinated development of distinct hormone-producing cell types and an invading vascular network. The molecular mechanisms that drive formation of the pituitary vasculature, which is necessary for regulated synthesis and secretion of hormones that maintain homeostasis, metabolism, and endocrine function, remain poorly understood. Here, we report that expression of integrin β1 in embryonic pituitary epithelial cells is required for angiogenesis in the developing mouse pituitary gland. Deletion of pituitary epithelial integrin β1 before the onset of angiogenesis resulted in failure of invading endothelial cells to recruit pericytes efficiently, whereas deletion later in embryogenesis led to decreased vascular density and lumen formation. In both cases, lack of epithelial integrin β1 was associated with a complete absence of vasculature in the pituitary gland at birth. Within pituitary epithelial cells, integrin β1 directs a large transcriptional program that includes components of the extracellular matrix and associated signaling factors that are linked to the observed non–cell-autonomous effects on angiogenesis. We conclude that epithelial integrin β1 functions as a critical and canonical regulator of developmental angiogenesis in the pituitary gland, thus providing insight into the long-standing systems biology conundrum of how vascular invasion is coordinated with tissue development.

angiogenesis | integrin β1 | pituitary gland | mice

The mammalian pituitary consists of glandular anterior and intermediate lobes that arise from invaginated oral ectoderm termed Rathke's pouch and a neural posterior lobe that develops in tandem as an outgrowth of the ventral diencephalon (1). A dense vascular network delivers hypothalamic regulatory factors and target organ feedback signals that control pituitary endocrine function (2). Angiogenesis gives rise to this network beginning on mouse embryonic day 13.5 (e13.5) as epithelial cells in the expanding anterior wall of Rathke's pouch delaminate and intercalate with invading endothelial and mesenchymal cells (3). As embryonic blood vessels branch and spread laterally throughout the expanding anterior lobe, they form fenestrated capillaries that surround developing endocrine cells (4). Before birth, portal vessels from the hypothalamus begin to deliver regulatory hormones, target organ feedback, and afferent blood flow to this capillary network (5). Anterior pituitary hormones secreted in response to these signals are then delivered into the general circulation via venous drainage (6).

Vascular development depends on integrins, a large family of glycoprotein type I transmembrane receptors that function as αβ heterodimers to mediate cell adhesion to the extracellular matrix (ECM) (7). In turn, integrin binding to ECM affects deposition, organization, and remodeling of individual ECM components into functional supramolecular structures (8). Integrin β1 pairs with twelve separate α-chains to form the largest integrin subfamily, and its role in vascular development has been the subject of significant investigation. Although integrin β1-null embryos died before assembly of vessel primordia (vasculogenesis), integrin

β1-null teratomas and embryoid bodies suggested an endothelial cell-autonomous requirement for integrin β1 in angiogenesis [formation of new vessels from preexisting vessels (7)].

Targeted deletions proved directly that endothelial cell integrin β1 was dispensable for vasculogenesis but required for subsequent angiogenic sprouting, branching, adhesion, migration, and cell survival (9). Later in embryogenesis, integrin β1 was necessary in arterioles for Par3-dependent endothelial cell polarity and lumen formation (10). In postnatal retinal vasculature, integrin β1-mediated endothelial cell–ECM interactions were required for production and assembly of the ECM, as well as formation of stable, non-leaky blood vessels (11). Mural cells (pericytes and vascular smooth muscle cells) that encase blood vessels also must express integrin β1 for adhesion and spreading, as well as assembly of ECM proteins that stabilize blood vessel walls (12, 13).

Here, we report that expression of integrin β1 in embryonic pituitary gland epithelial cells is absolutely required for the actions of the stromal cells that constitute the developing vasculature. Based on the phenotypes of mice harboring two temporally distinct, tissue-specific disruptions of *Itgb1* in embryonic pituitary gland epithelial cells, we conclude that the non–cell-autonomous function of integrin β1 is critical for pericyte recruitment, lumen formation, and persistence of the vasculature. Analysis of the epithelial cell transcriptome revealed an integrin β1-dependent gene expression program encoding ECM and associated signaling molecules that exert pleiotropic effects on the coordinated development of the pituitary gland and its invading vascular system during organogenesis.

Significance

During embryogenesis, a dense vascular network develops in the pituitary gland through the process of angiogenesis. In tandem, pituitary gland precursor cells differentiate into hormone-producing cells that will rely on the vasculature to carry out regulated endocrine function. Our data show that expression of the cell surface adhesion molecule, integrin β1, in the epithelial-derived precursor cells is required for development of the vasculature and coordinated terminal differentiation of endocrine cells.

Results

Integrin β1 Is Expressed in the Pituitary Gland Throughout Embryonic Development. Integrin β1 protein was detected in all three lobes of the pituitary from e10.5 through birth, in oral ectoderm-derived epithelial cells that comprise the parenchyma of the developing gland, and in endothelial and supporting mesenchymal cells that form the vasculature (Fig. S1 A and B). Laminin, an ECM glycoprotein that is bound by a subset of integrin β1 heterodimers, was abundant in the basement membrane that separates the epithelial tissue of the early embryonic pituitary gland from adjacent mesenchyme (3, 14). As angiogenesis proceeds, this demarcation persists in the form of a vascular basement membrane that surrounds nascent vessels in the anterior lobe (3) (Fig. S1B). Quantitative PCR analyses of integrin β1 mRNA in the pituitary gland from e12.5 to adulthood detected continuous low levels, with a spike in expression at postnatal day 0 (p0). Validity of these measurements was provided by quantitation of previously characterized *Prop1* and *prolactin* (Prl) mRNAs (15) (Fig. S1C).

Targeted Deletions of Integrin β1 in Embryonic Pituitary Gland Epithelial Cells at e10.5 and e14.5. To examine integrin β1 function in pituitary epithelial cells before cell-type differentiation or angiogenesis, we crossed *Pitx1-cre* transgenic mice to *Itgb1^flox* mice, resulting in complete loss of integrin β1 protein throughout Rathke's pouch by e10.5 (16, 17) (Fig. 1A). To study the role of integrin β1 after the initiation of cell-type differentiation and angiogenesis, we crossed *Prop1-cre* transgenic mice to *Itgb1^flox* mice, causing progressive loss of integrin β1 protein in the parenchyma of the developing anterior and intermediate lobes that began on e13.5 and was complete by e14.5 (18) (Fig. 1B and Fig. S1D). Importantly, coimmunostaining of integrin β1 and CD31 in *Prop1-cre* embryos demonstrated that expression of integrin β1 in invading endothelial cells was unaffected (Fig. S1E).

Itgb1^f/f; Pitx1-cre Pups Die at Birth but Itgb1^f/f; Prop1-cre Mice Are Viable. *Itgb1^f/f; Pitx1-cre* mice were born in Mendelian ratios, but all mutant pups died at birth. At e15.5, hematoxylin and eosin (H&E)-stained midline sagittal sections revealed a smaller gland with a
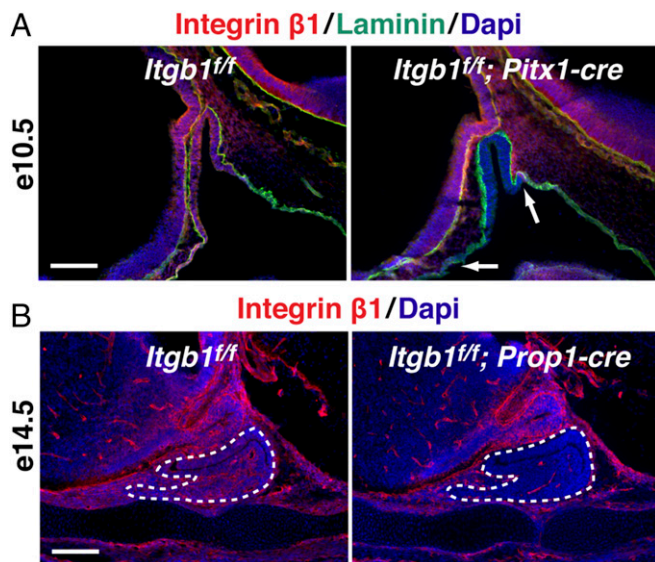
Fig. 1. Targeted deletions of *Itgb1* in the pituitary gland. (A) *Pitx1-cre* eliminates integrin β1 at e10.5 in Rathke's pouch epithelium (*Right*, between arrows). Laminin marks basement membrane. (Scale bar: 130 μm.) Image on the left is shown again as the first panel in an ontogenic series in Fig. S1B. (B) *Prop1-cre* eliminates integrin β1 at e14.5 in pituitary gland epithelium (enclosed by dotted lines). (Scale bar: 130 μm.) Midsagittal sections are shown in A and B.

shortened pituitary cleft. By p0, the anterior and intermediate lobes were significantly smaller and displayed altered morphology, the posterior lobe was displaced in the rostral direction, there was poor anatomical definition of the intermediate lobe because of progressive shortening of the cleft, red blood cells (RBCs) were absent from the anterior lobe, and the secondary palate had failed to fuse along the midline (Fig. S2 A–D).

Itgb1^f/f; Prop1-cre animals were born in Mendelian ratios and survived normally into adulthood. H&E staining at p2 showed signs of hemorrhage or hematoma in the lateral wings of the anterior lobes that was confirmed by microCT scans (Fig. S2 E and F). Although H&E staining showed normal placement of the three pituitary lobes, *Itgb1^f/f; Prop1-cre* pituitaries dissected at p2 revealed a decreased size of the anterior lobe that became dramatic by p25 (Fig. S2G). Given that normal postnatal growth relies on secretion of growth hormone (GH) from anterior lobe somatotropes, we compared the weights of *Itgb1^f/f; Prop1-cre* mice with the weights of *Itgb1^f/f* littermates in the 10-d period following weaning at p21. In four separate litters, all *Itgb1^f/f; Prop1-cre* animals weighed less at each time point, and by p31, their weights were, on average, 66% of controls (Fig. S2H). These results suggested diminished somatotrope function due to decreased expression, secretion, and/or delivery of GH.

Endocrine Cell-Type Differentiation. To assess the role of integrin β1 in endocrine cell-type differentiation, we examined expression of pituitary hormone genes. Pit-1 is a key transcription factor that is required for specification of the somatotrope, lactotrope, and thyrotrope cells that, upon terminal differentiation, express growth hormone (GH), Prl, and thyroid-stimulating hormone-β (TSH-β), respectively. These three hormone-encoding genes are also direct transcriptional targets of Pit-1, which functions in combination with hypothalamic regulatory signals and target organ feedback to achieve regulated physiological expression (15). Although normal temporal expression of pro-opiomelanocortin (POMC) at e13.5, TSH-β at e15.5, and POMC and GH at p0 was observed, differences in spatial expression patterns were noted at each stage in *Itgb1^f/f; Pitx1-cre* embryos (Fig. S3 A–C). At p0, expression of *Pit-1* mRNA was normal but *GH* and *Prl* were significantly down-regulated, whereas *TSH-β* was up-regulated (Fig. S3D). At p18, *Itgb1^f/f; Prop1-cre* pituitary glands were significantly smaller, expressed less GH and Prl protein, and contained mislocalized dorsal thyrotropes (Fig. S3E). At p17, *Pit-1* mRNA expression was normal but levels of *GH* and *Prl* were diminished (Fig. S3F). In both *Itgb1^f/f; Pitx1-cre* and *Itgb1^f/f; Prop1-cre* pituitaries, normal expression of Pit-1 was combined with significantly altered expression of its target genes, suggesting that failure to receive additional critical hypothalamic regulatory signals and target organ feedback via the circulatory system might be the explanation.

Blood Vessels Are Absent in Both Itgb1^f/f; Pitx1-cre and Itgb1^f/f; Prop1-cre Mice at Birth. Development of the vascular network that delivers hypothalamic regulatory signals and target organ feedback to pituitary endocrine cell types was examined at p0. In control pituitaries, CD31 immunostained abundant blood vessels in the anterior lobe (the intermediate lobe is notably avascular). In contrast, both *Itgb1^f/f; Pitx1-cre* and *Itgb1^f/f; Prop1-cre* pituitaries displayed a remarkable absence of CD31 immunostaining, suggesting that the entire dense network of blood vessels had failed to form and/or stabilize in the absence of epithelial integrin β1 (Fig. 2A). This result was verified with a second, independent endothelial cell marker, PLVAP (plasmalemmal vesicle-associated protein) (19) (Fig. 2B). Coimmunostaining of CD31 and laminin showed laminin-rich vascular basement membranes in control animals but failed to detect laminin-rich "empty basement membrane sleeves" in either *Itgb1^f/f; Pitx1-cre* or *Itgb1^f/f; Prop1-cre* pituitary glands, suggesting that even if vessels had formed, they had been absent for several days (20) (Fig. S4A). Coimmunostaining of integrin β1 and laminin

in control animals demonstrated that the highest level of integrin β1 expression occurred in cells within the laminin-rich vascular basement membrane (Fig. S4B).

Angiogenesis Begins at e13.5 but Invading Endothelial Cells Fail to Recruit Pericytes in *Itgb1^f/f; Pitx1-cre* Pituitaries. To assess how the initial steps in angiogenesis proceeded in the absence of epithelial integrin β1, we examined *Itgb1^f/f; Pitx1-cre* pituitaries at e13.5, the time point at which we observed angiogenesis beginning in the anterior lobe. CD31 and integrin β1(+) endothelial cells were present in control and *Itgb1^f/f; Pitx1-cre* pituitaries (Fig. S5A). Co-immunostaining with CD31 and laminin revealed that they deposited laminin-rich basement membranes at e13.5. By e14.5, however, neither endothelial cells nor their basement membranes were detected in *Itgb1^f/f; Pitx1-cre* pituitaries (Fig. S5B).

To determine whether lumen formation was related to the disappearance of endothelial cells, we looked for evidence of RBCs using H&E staining. In both control and *Itgb1^f/f; Pitx1-cre* embryos at e13.5, RBCs were present in vessels surrounding the pituitary but were not detected within the gland, suggesting that lumen
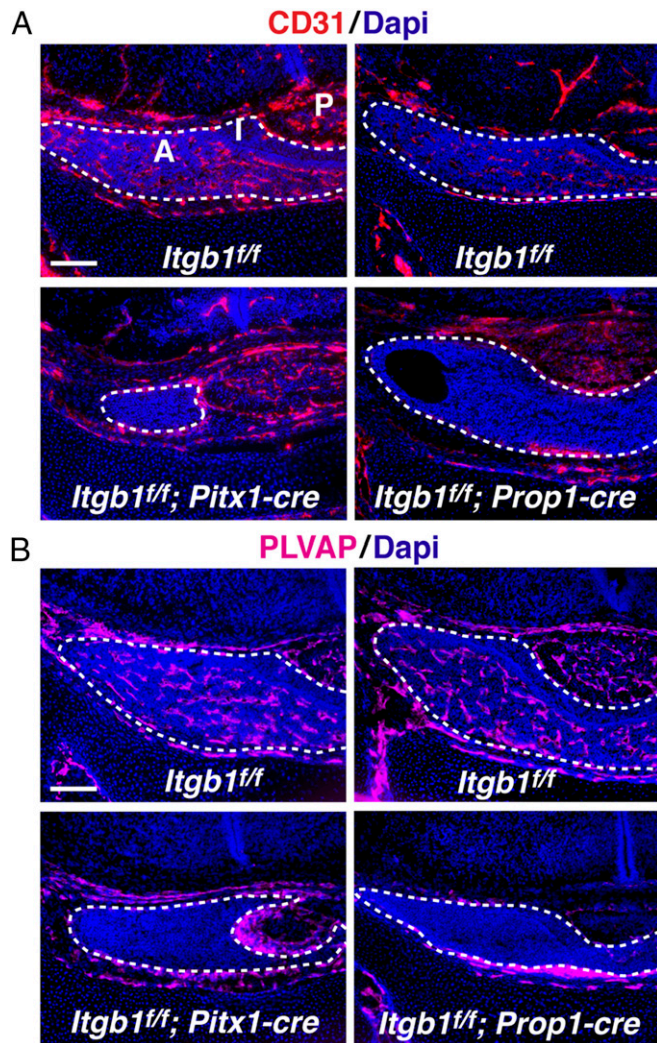

Fig. 2. Absence of vasculature at p0 in both *Itgb1^f/f; Pitx1-cre* and *Itgb1^f/f; Prop1-cre* pituitary glands. (A and B) Immunostaining of coronal sections with independent markers, CD31 and PLVAP, failed to detect endothelial cells in *Itgb1^f/f; Pitx1-cre* and *Itgb1^f/f; Prop1-cre* pituitary glands at birth. A, anterior lobe; I, intermediate lobe; P, posterior lobe; PLVAP, plasmalemmal vesicle-associated protein. (Scale bars: 130 μm.)

formation begins after e13.5 (Fig. S5C). Therefore, failure of vascular development in the *Itgb1^f/f; Pitx1-cre* pituitaries likely preceded lumen formation.

Next, we considered recruitment of pericytes that normally envelop nascent microvessels, span endothelial cell junctions, and embed within the vascular basement membrane to provide structural support and signals that control endothelial cell growth versus quiescence (21). In the pituitary, rostral neural crest-derived mesenchyme gives rise to pericytes that surround anterior lobe capillaries (22). At e13.5, we coimmunostained with the pericyte marker PDGF receptor β (PDGFRβ) and vascular endothelial growth factor receptor 2 (VEGFR2) which labels endothelial cells (21). We observed densely packed PDGFRβ(+) cells surrounding the developing glands, and in controls, pericytes were associated with invading endothelial cells. In *Itgb1^f/f; Pitx1-cre* pituitaries, however, endothelial cells lacked associated pericytes at e13.5, and by e14.5, endothelial cells were also absent (Fig. 3 and Fig. S5 D and E).

A well-characterized mechanism of pericyte recruitment involves secretion of PDGF-B by endothelial tip cells in angiogenic sprouts. Subsequent tethering of secreted PDGF-B to heparan sulfate and heparan sulfate proteoglycans (HSPGs) in the ECM retains it at relatively high local concentrations for binding to PDGFRβ on pericytes. In the absence of PDGF-B signaling, lack of pericyte recruitment has been correlated with increased vessel diameter, hemorrhage, and edema in many tissues (21). In the pituitary, genetic ablation of neural crest-derived pericytes resulted in vessels that survived and formed lumens but were dilated and leaky (22, 23). Based on these findings, we examined vascular development in the pituitary glands of PDGFRβ^−/− mice (24). At e14.5, immunostaining with VEGFR2 revealed that blood vessels were present but highly dilated, suggesting that PDGF-B signaling plays a role in pericyte recruitment in the embryonic pituitary (Fig. S5F). By extension, defective PDGF-B signaling may explain failure of pericyte recruitment in e13.5 *Itgb1^f/f; Pitx1-cre* pituitaries, but it does not fully explain the absence of endothelial cells observed at e14.5.
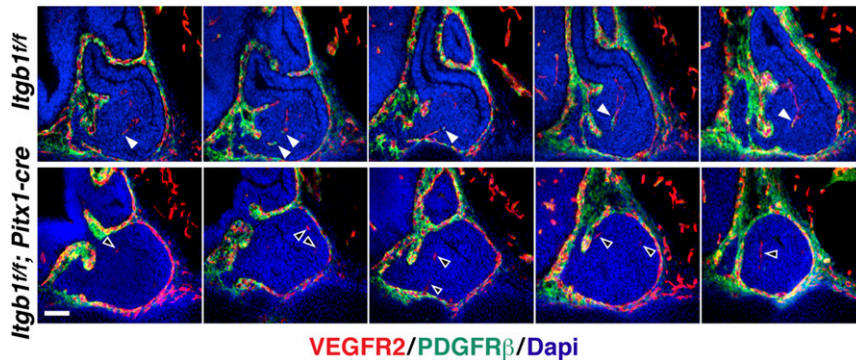
Reduced Vessel Density and Lumen Formation in *Itgb1^f/f; Prop1-cre* Pituitaries at e14.5. The role of epithelial integrin β1 in later steps in angiogenesis was examined in *Itgb1^f/f; Prop1-cre* embryos. Coimmunostaining of CD31 and laminin at e14.5 and e15.5 demonstrated that endothelial cells invaded the anterior lobe and deposited laminin-rich basement membranes equivalently in control and *Itgb1^f/f; Prop1-cre* pituitaries (Fig. S6A). Immunostaining of CD31 and NG2 (Fig. S6B), or PDGFRβ (Fig. S6C), demonstrated that pericytes were associated with endothelial cells in *Itgb1^f/f; Prop1-cre* pituitary glands at e15.5. We next examined lumen formation. H&E staining at e14.5 detected RBCs in both control and *Itgb1^f/f; Prop1-cre* pituitaries in the rostral anterior lobes where endothelial cells first invade. RBCs were also present within the central and caudal anterior lobe in control pituitaries, thus identifying e14.5 as the time at which lumen formation normally begins. In *Itgb1^f/f; Prop1-cre* pituitaries, however, RBCs were rarely observed in the central and caudal anterior lobe, suggesting compromised lumen formation and/or reduced vessel number (Fig. S6D).

To investigate vessel number and lumen formation further, e14.5 coronal sections were immunostained with integrin β1 and CD31. At this stage, cords of invading endothelial cells appeared as tight clusters with or without visible lumens (Fig. 4A and Fig. S6E). In *Itgb1^f/f; Prop1-cre* pituitaries, endothelial cell cluster number was reduced by >50%, suggesting a defect in vessel branching (Fig. 4 B and D), and the number with a detectable lumen was ~25% of normal (Fig. 4 C and D). Within clusters that formed lumens at e14.5, median lumen diameter in controls was 6.0 μm, with a range from 2.0 μm to 14.9 μm, but it was only 3.5 μm, with a range from 1.1 μm to 10.1 μm in *Itgb1^f/f; Prop1-cre* pituitary glands (Fig. 4E and Fig. S6 E and F). Hence, loss of epithelial integrin β1 by e14.5 resulted in a decreased number of nascent blood vessels with compromised lumen formation.

Fig. 3. Endothelial cells invade *Itgb1^f/f^; Pitx1-cre* pituitary glands at e13.5 but fail to recruit pericytes. Serial sagittal sections beginning at the midline and progressing through the lateral anterior lobes immunostained with VEGFR2 (endothelial cells) and PDGFRβ (pericytes) are shown. Pericytes recruited to endothelial cells in *Itgb1^f/f^* control pituitary glands (filled arrowheads) are shown. Endothelial cells that failed to recruit pericytes in *Itgb1^f/f^; Pitx1-cre* pituitary glands (empty arrowheads) are shown. Magnified images of the same panels are provided in Fig. S5D. (Scale bar: 62.5 μm.)

RNA-Sequencing Identified an Integrin β1-Dependent Transcriptional Program Encoding ECM and Associated Molecules. In *Itgb1^f/f^; Pitx1-cre* mice, e10.5 inactivation of integrin β1 provided a homogeneous population of epithelial cells before initiation of angiogenesis at e13.5. We hypothesized that profiling gene expression at e12.5 in cells that uniformly lacked integrin β1 might provide insight into why angiogenesis failed (Fig. 5A). Massively parallel RNA-sequencing (RNA-seq) was used to compare the transcriptomes of pituitaries dissected at e12.5 from *Itgb1^f/f^* and *Itgb1^f/f^; Pitx1-cre* littermate embryos. Deletion of exon 3 in the *Itgb1^f/f^; Pitx1-cre* RNA samples was validated by viewing sequence tags mapped to the *Itgb1* locus (Fig. S7A).

In *Itgb1^f/f^; Pitx1-cre* pituitaries, RNA-seq data revealed significantly altered expression (>1.5-fold change) of 1,138 genes ($P \leq 0.01$). Expression of 496 genes decreased, and expression of 642 genes increased. Gene ontology analysis revealed changes in both cell-intrinsic and cell-extrinsic gene programs (Fig. 5B). Heat maps representing selected genes showed explicit membership in four categories of extrinsic factors that may be involved in the non–cell-autonomous effect of epithelial cells on angiogenesis. Genes in these categories included components of the ECM, ECM-associated intercellular signaling molecules, several integrin subunits that heterodimerize with integrin β1 (α3, α7, and α9), and a number of disintegrins/metalloproteinases (Fig. 5C). Curiously, integrins α3β1 and α7β1 bind specifically to laminin, a major component of vascular basement membranes in the embryonic pituitary (3, 14).

In the ECM category, expression of the core matrisome components fibronectin (FN), six of 28 collagens, and laminin C1 were significantly altered (8) (Fig. 5C). FN, which is important for embryonic blood vessel morphogenesis, was the most down-regulated, as seen in mice null for integrin α5 (25, 26). In addition to stimulating expression of FN, integrin α5β1 is required to bind to FN to initiate assembly of the fibrillar matrix, which functions as a reservoir for locally secreted growth factors such as VEGF-A (8). Immunohistochemical staining of FN in e12.5 *Itgb1^f/f^; Pitx1-cre* pituitaries revealed diminished expression along the rostral aspect of the anterior lobe where endothelial cells first invade and a punctate, rather than fibrillar, pattern, suggesting diminished integrin β1-induced conversion of compact FN into extended fibrils (Fig. S7B).

In the retina, FN deposited by astrocytes localized VEGF-A ahead of migrating tip cells in angiogenic sprouts (27). Interestingly, VEGF-A is highly expressed in e14.5 embryonic pituitaries (GenePaint.org) and has been implicated in vascular development in the mouse pituitary gland (28). Based on these findings, we asked if deletion of VEGF-A might phenocopy the absence of endothelial cells observed in e14.5 *Itgb1^f/f^; Pitx1-cre* pituitaries. *VEGF-A^f/f^; Pitx1-cre* mice were generated, and pituitaries were immunostained with VEGFR2 and PDGFRβ, but angiogenesis appeared normal at e14.5 (29) (Fig. S7C). One explanation is that VEGF-A may have escaped deletion in a small percentage of cells such as folliculostellate cells (30). However, given that *Pitx1-cre* deleted *Itgb1^flox^* alleles with complete penetrance in the mouse pituitary
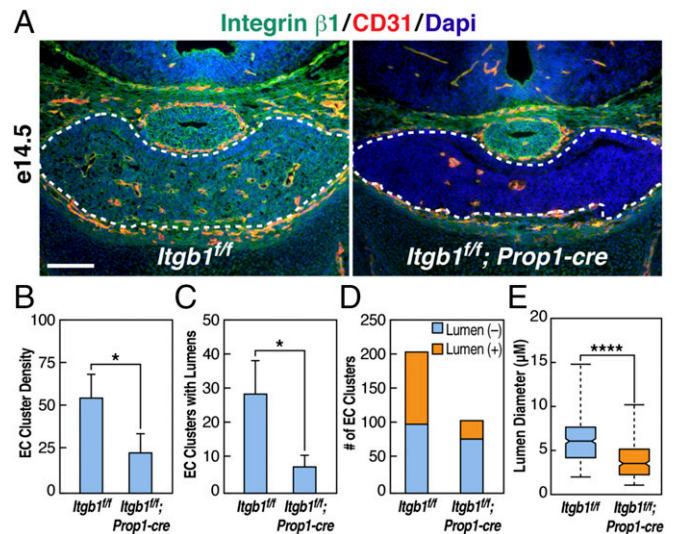
Fig. 4. Endothelial cell (EC) cluster number and lumen formation are reduced in *Itgb1^f/f^; Prop1-cre* pituitary glands at e14.5. (A) Coronal sections immunostained with integrin β1 and CD31 reveal that EC cluster number is reduced in *Itgb1^f/f^; Prop1-cre* pituitary glands at e14.5. (Scale bar: 130 μm.) Higher magnification images of the panel on the left and of a section adjacent to the panel on the right are shown in Fig. S6E. (B) Average number of EC clusters in coronal sections of e14.5 pituitary glands. Three sections of equivalent size and anatomical position from each of four *Itgb1^f/f^* and *Itgb1^f/f^; Prop1-cre* embryos were analyzed. The sum of EC clusters counted in the three sections for each animal is shown. Error bars represent SEM. *$P = 0.0408$ determined by t test. (C) Average number of EC clusters with lumens in the coronal sections analyzed in B. Error bars represent SEM. *$P = 0.0266$ determined by t test. (D) Sum of all EC clusters counted without lumens (blue) and with lumens (orange) as shown in B and C. (E) Mean diameter of blood vessel lumens determined using the straight-line tool in ImageJ (NIH) to measure the shortest distance across lumens in four *Itgb1^f/f^* and four *Itgb1^f/f^; Prop1-cre* pituitary glands at e14.5. Measurements were made in three sections from each pituitary for a total of 104 lumens in *Itgb1^f/f^* and 27 in *Itgb1^f/f^; Prop1-cre*. Whisker ends represent minimum and maximum data points. ****$P < 0.0001$ determined by Mann–Whitney test. Magnified images are provided in Fig. S5 E and F.
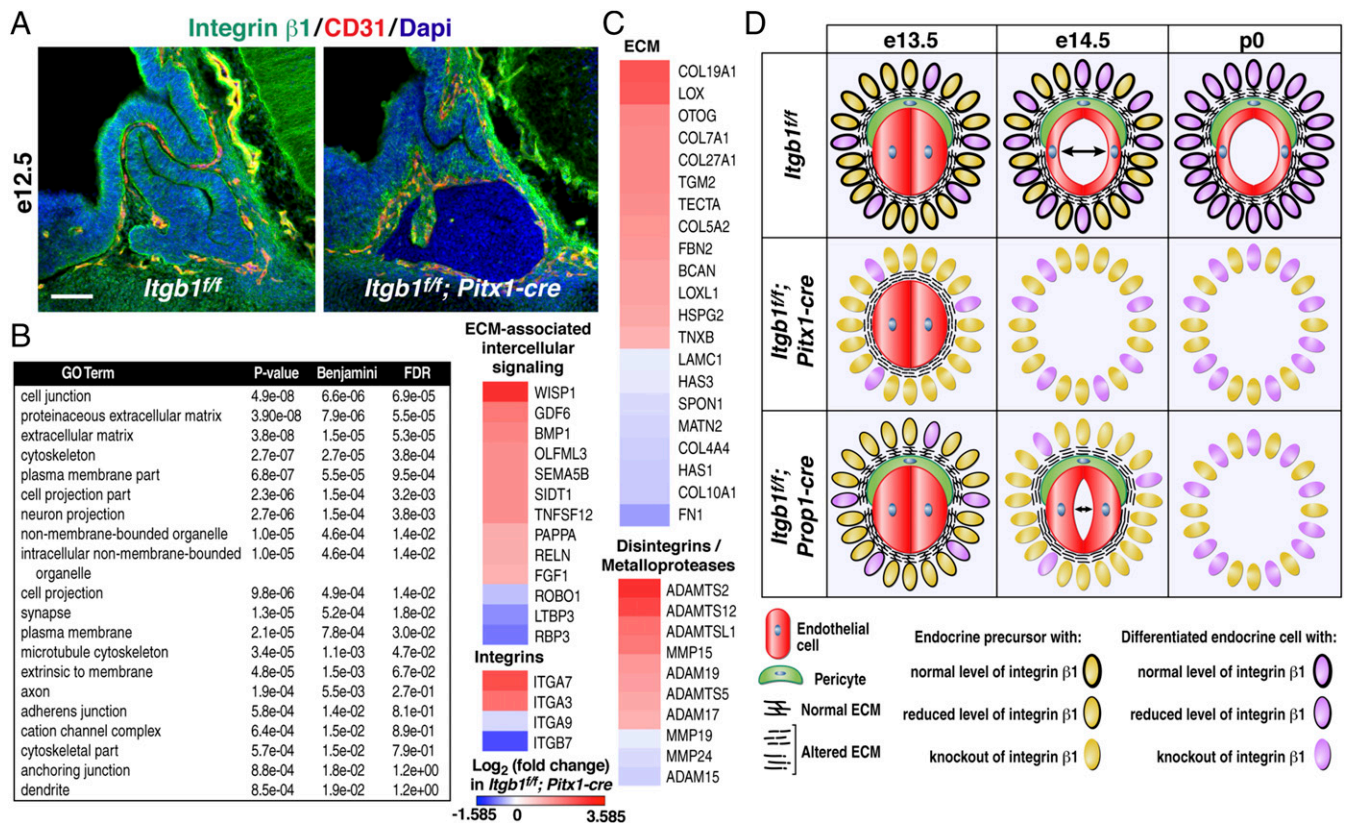
Fig. 5. Before initiation of angiogenesis in the pituitary gland, integrin β1-dependent transcriptome encodes ECM and associated factors critical for angiogenesis. (*A*) Midsagittal sections of e12.5 *Itgb1^f/f* and *Itgb1^f/f*; *Pitx1-cre* pituitaries immunostained with integrin β1 and CD31 1 d before initiation of angiogenesis. (Scale bar: 62.5 μm.) (*B*) RNA-seq analysis of pituitaries dissected from *Itgb1^f/f* and *Itgb1^f/f*; *Pitx1-cre* embryos at e12.5 identified 496 down-regulated and 642 up-regulated genes. Gene ontology analysis revealed significant changes in categories of genes encoding intrinsic and extrinsic factors. (*C*) Heat maps with identity of genes that encode ECM and related proteins. (*D*) Model for function of pituitary gland epithelial cell integrin β1 in developmental angiogenesis.

gland, it seems unlikely. A more probable explanation is functional redundancy with VEGF-C, which is also highly expressed in the e14.5 pituitary gland (GenePaint.org) and can bind to VEGFR2 (Fig. S7*D*).

Discussion

Our findings provide unambiguous evidence of critical non–cell-autonomous roles for integrin β1 in vascular development that contrast with numerous reports of other tissue-specific integrin β1 knockouts (31–34), and complement established vascular cell-autonomous functions of integrin β1 (9–13). Our data support the idea that there are distinct tissue and temporal context-dependent requirements for specific integrin heterodimers in vascular development (11). Indeed, the only other well-defined example of a non–cell-autonomous role played by integrins in vascular development involves integrin αvβ8 in the CNS and retina, where failure to activate latent ECM-bound TGF-β was an underlying cause of the observed vascular defects (35, 36). Interestingly, the palate defect in the *Pitx1-cre; Itgb1^f/f* embryos phenocopies double-knockouts of integrin β6/β8 and single-knockouts of TGF-β3, suggesting integrin β1-dependent TGF-β signaling in the palate (37, 38).

We observed that multiple steps in developmental angiogenesis required expression of epithelial integrin β1 in the embryonic pituitary gland. In its absence, expression of genes encoding core components of the ECM, ECM-associated signaling factors, and remodeling molecules are changed, suggesting that alterations in ECM function might underlie the observed phenotypes. In addition, integrin β1 initiates self-assembly of secreted FN dimers into a multimeric fibrillar matrix that subsequently affects formation of collagen fibrils (8). Ultimately, changes in the deposition and

assembly of the ECM in the absence of epithelial integrin β1 may have altered its ability to provide structural support, and to function as a scaffold that binds and presents secreted growth factors to vascular cells (8).

PDGF-B, VEGF, and TGF-β are secreted growth factors that regulate blood vessel development through binding to the ECM in a manner that controls their local concentration and bioavailability (8). Examination of PDGFRβ-null mice indicated that PDGF-B signaling plays a role in pericyte recruitment in the embryonic pituitary gland. Failure to recruit pericytes, however, led to dilated and leaky vessels but not to the loss of endothelial cells observed in the e14.5 *Pitx1-cre; Itgb1^f/f* pituitaries (21). Therefore, additional endothelial cell migration, retention, and/or survival signals must also be compromised. Candidate molecules with such function include VEGF-A and VEGF-C, both of which are highly expressed in the embryonic pituitary gland at e14.5. Deletion of VEGF-A alone in *VEGF-A^f/f*; *Pitx1-cre* pituitaries surprisingly showed no effect on endothelial cell invasion or pericyte recruitment, and so raised the possibility of compensation by VEGF-C (39). Interestingly, vascularization defects in the embryonic pituitaries of Prop^df/df^ mice (Ames dwarf) that lack the transcription factor, Prop1, have been attributed to altered expression of VEGF-A protein (28). Although this finding appeared to contrast with the apparent lack of vascular phenotype in our deletion of VEGF-A, it was separately noted that VEGF-C mRNA was also decreased more than threefold in pituitaries from e12.5 Prop1 knockout mice (Fig. S8). Together, these data suggest that simultaneous down-regulation of both VEGF-A and VEGF-C may cause the vascular defects seen in the embryonic pituitary glands of mice

lacking Prop1. In the case of *Pitx1-cre; Itgb1^{f/f}* mice, and perhaps *Prop1-cre; Itgb1^{f/f}* mice, interaction of both VEGF-A and VEGF-C with the ECM would likely be compromised. This outcome suggests that the absence of vasculature observed with inactivation of pituitary epithelial integrin β1 might result from the failure of a series of signals, including PDGF-B, VEGF-A, and VEGF-C, that, together, orchestrate vascular development.

In total, our data support the conclusion that integrin β1 in the epithelial cells of the embryonic pituitary gland is required for normal expression of numerous ECM and associated molecules that function in critical non–cell-autonomous roles during vascular development. In addition, the cell-autonomous function of integrin β1 in epithelial cells is required for normal expression of endocrine cell terminal differentiation markers such as GH and Prl. Together, these effects reveal that epithelial integrin β1 coordinates development of the embryonic pituitary gland and its invading vascular system. Whether reciprocal heterotypic interactions between the parenchyma and nascent vasculature extend beyond development of a circulatory system that delivers regulatory signals to hormone-producing cells remains an open question.

Materials and Methods

Conditional Deletions of the *Itgb1* and *VEGF-A* Genes. The mouse strain B6;129-*Itgb1^{tm1Efu/J}* (*Itgb1^{flox}*) was obtained from The Jackson Laboratory (17). VEGF *loxP* mice (*VEGF-A^{flox}*) were provided by Genentech (29). *Itgb1^{f/f}* and *VEGF-A^{flox}* mice were bred to *Pitx1-cre* (16) or *Prop1-cre* transgenic mice (18). *PDGFRβ^{−/−}* embryos were generously provided by Lorin Olson, Oklahoma Medical Research Foundation, Oklahoma City (24). All procedures were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

Accession Number. For RNA-seq data deposited in the National Center for Biotechnology Information's Gene Expression Omnibus databank, the accession number is GSE89171 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89171).

Additional information is provided in *SI Materials and Methods*.

1. Kerr T (1946) The development of the pituitary of the laboratory mouse. *Q J Microsc Sci* 87:3–29.
2. Szabó K, Csányi K (1982) The vascular architecture of the developing pituitary-median eminence complex in the rat. *Cell Tissue Res* 224(3):563–577.
3. Hashimoto H, Ishikawa H, Kusakabe M (1998) Three-dimensional analysis of the developing pituitary gland in the mouse. *Dev Dyn* 212(1):157–166.
4. Farquhar MG (1961) Fine structure and function in capillaries of the anterior pituitary gland. *Angiology* 12:270–292.
5. Dearden NM, Holmes RL (1976) Cyto-differentiation and portal vascular development in the mouse adenohypophysis. *J Anat* 121(Pt 3):551–569.
6. Schaeffer M, et al. (2011) Influence of estrogens on GH-cell network dynamics in females: A live in situ imaging approach. *Endocrinology* 152(12):4789–4799.
7. Plow EF, Meller J, Byzova TV (2014) Integrin function in vascular biology: A view from 2013. *Curr Opin Hematol* 21(3):241–247.
8. Hynes RO, Naba A (2012) Overview of the matrisome–an inventory of extracellular matrix constituents and functions. *Cold Spring Harb Perspect Biol* 4(1):a004903.
9. Malan D, et al. (2010) Endothelial beta1 integrins regulate sprouting and network formation during vascular development. *Development* 137(6):993–1002.
10. Zovein AC, et al. (2010) Beta1 integrin establishes endothelial cell polarity and arteriolar lumen formation via a Par3-dependent mechanism. *Dev Cell* 18(1):39–51.
11. Yamamoto H, et al. (2015) Integrin β1 controls VE-cadherin localization and blood vessel stability. *Nat Commun* 6:6429.
12. Turlo KA, et al. (2012) An essential requirement for β1 integrin in the assembly of extracellular matrix proteins within the vascular wall. *Dev Biol* 365(1):23–35.
13. Abraham S, Kogata N, Fässler R, Adams RH (2008) Integrin beta1 subunit controls mural cell adhesion, spreading, and blood vessel wall stability. *Circ Res* 102(5):562–570.
14. Hynes RO (2002) Integrins: Bidirectional, allosteric signaling machines. *Cell* 110(6):673–687.
15. Zhu X, Gleiberman AS, Rosenfeld MG (2007) Molecular physiology of pituitary development: Signaling and transcriptional networks. *Physiol Rev* 87(3):933–963.
16. Treier M, et al. (2001) Hedgehog signaling is required for pituitary gland development. *Development* 128(3):377–386.
17. Raghavan S, Bauer C, Mundschau G, Li Q, Fuchs E (2000) Conditional ablation of beta1 integrin in skin. Severe defects in epidermal proliferation, basement membrane formation, and hair follicle invagination. *J Cell Biol* 150(5):1149–1160.
18. Zhu X, Tollkuhn J, Taylor H, Rosenfeld MG (2015) Notch-dependent pituitary SOX2(+) stem cells exhibit a timed functional extinction in regulation of the postnatal gland. *Stem Cell Rep* 5(6):1196–1209.
19. Stan RV, et al. (2012) The diaphragms of fenestrated endothelia: Gatekeepers of vascular permeability and blood composition. *Dev Cell* 23(6):1203–1218.
20. Baluk P, Morikawa S, Haskell A, Mancuso M, McDonald DM (2003) Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. *Am J Pathol* 163(5):1801–1815.
21. Armulik A, Genové G, Betsholtz C (2011) Pericytes: Developmental, physiological, and pathological perspectives, problems, and promises. *Dev Cell* 21(2):193–215.
22. Etchevers HC, Vincent C, Le Douarin NM, Couly GF (2001) The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. *Development* 128(7):1059–1068.
23. Davis SW, et al. (2016) β-catenin is required in the neural crest and mesencephalon for pituitary gland organogenesis. *BMC Dev Biol* 16(1):16.
24. Soriano P (1994) Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes Dev* 8(16):1888–1896.
25. Astrof S, Hynes RO (2009) Fibronectins in vascular morphogenesis. *Angiogenesis* 12(2):165–175.
26. Francis SE, et al. (2002) Central roles of alpha5beta1 integrin and fibronectin in vascular development in mouse embryos and embryoid bodies. *Arterioscler Thromb Vasc Biol* 22(6):927–933.
27. Stenzel D, et al. (2011) Integrin-dependent and -independent functions of astrocytic fibronectin in retinal angiogenesis. *Development* 138(20):4451–4463.
28. Ward RD, Stone BM, Raetzman LT, Camper SA (2006) Cell proliferation and vascularization in mouse models of pituitary hormone deficiency. *Mol Endocrinol* 20(6):1378–1390.
29. Gerber HP, et al. (1999) VEGF is required for growth and survival in neonatal mice. *Development* 126(6):1149–1159.
30. Ferrara N, Henzel WJ (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 161(2):851–858.
31. Diaferia GR, et al. (2013) β1 integrin is a crucial regulator of pancreatic β-cell expansion. *Development* 140(16):3360–3372.
32. Taddei I, et al. (2008) Beta1 integrin deletion from the basal compartment of the mammary epithelium affects stem cells. *Nat Cell Biol* 10(6):716–722.
33. Plosa EJ, et al. (2014) Epithelial β1 integrin is required for lung branching morphogenesis and alveolarization. *Development* 141(24):4751–4762.
34. Graus-Porta D, et al. (2001) Beta1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex. *Neuron* 31(3):367–379.
35. Hirota S, et al. (2011) The astrocyte-expressed integrin αvβ8 governs blood vessel sprouting in the developing retina. *Development* 138(23):5157–5166.
36. Arnold TD, et al. (2014) Excessive vascular sprouting underlies cerebral hemorrhage in mice lacking αVβ8-TGFβ signaling in the brain. *Development* 141(23):4489–4499.
37. Tudela C, et al. (2002) TGF-beta3 is required for the adhesion and intercalation of medial edge epithelial cells during palate fusion. *Int J Dev Biol* 46(3):333–336.
38. Aluwihare P, et al. (2009) Mice that lack activity of alphavbeta6- and alphavbeta8-integrins reproduce the abnormalities of Tgfb1- and Tgfb3-null mice. *J Cell Sci* 122(Pt 2):227–232.
39. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L (2006) VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol* 7(5):359–371.
40. Committee on Care and Use of Laboratory Animals (1996) *Guide for the Care and Use of Laboratory Animals* (Natl Inst Health, Bethesda), DHHS Publ No (NIH) 85-23.
41. Simmons DM, et al. (1990) Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. *Genes Dev* 4:695–711.
42. Cole SW, Galic Z, Zack JA (2003) Controlling false-negative errors in microarray differential expression analysis: A PRIM approach. *Bioinformatics* 19(14):1808–1816.
43. Olson LE, et al. (2006) Homeodomain-mediated beta-catenin-dependent switching events dictate cell-lineage determination. *Cell* 125(3):593–605.

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