RESEARCH ARTICLE

Thyroid follicle development requires Smad1/5- and endothelial cell-dependent basement membrane assembly

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ABSTRACT

Thyroid follicles, the functional units of the thyroid gland, are delineated by a monolayer of thyrocytes resting on a continuous basement membrane. The developmental mechanisms of folliculogenesis, whereby follicles are formed by the reorganization of a non-structured mass of non-polarized epithelial cells, are largely unknown. Here we show that assembly of the epithelial basement membrane is crucial for folliculogenesis and is controlled by endothelial cell invasion and by BMP-Smad signaling in thyrocytes. Thyroid-specific Smad1 and Smad5 double-knockout (Smad1/5KO) mice displayed growth retardation, hypothyroidism and defective follicular architecture. In Smad1/5KO embryonic thyroids, epithelial cells remained associated in large clusters and formed small follicles. Although similar follicular defects are found in Vegfa knockout (VegfaKO) thyroids, Smad1/5KO thyroids had normal endothelial cell density yet impaired endothelial differentiation. Interestingly, both VegfaKO and Smad1/5KO thyroids displayed impaired basement membrane assembly. Furthermore, conditioned medium (CM) from embryonic endothelial progenitor cells (eEPCs) rescued the folliculogenesis defects of both Smad1/5KO and VegfaKO thyroids. Laminin α1, β1 and γ1, abundantly released by eEPCs into CM, were crucial for folliculogenesis. Thus, epithelial Smad signaling and endothelial cell invasion promote folliculogenesis via assembly of the basement membrane.

KEY WORDS: Thyroid, Smad1, Smad5, Follicle, Epithelium, Extracellular matrix, Mouse

INTRODUCTION

Thyroid follicles are the functional units of the thyroid gland. Each follicle is composed of a polarized monolayer of epithelial cells delineating a lumen where thyroglobulin (Tg) is stored as colloid. Thyrocytes differentiate from thyroid progenitors that bud from the foregut endoderm as a mass of non-polarized epithelial cells, which reorganize to form prefolicular structures by acquisition of apicobasal polarity (Fagman et al., 2006; Hick et al., 2013). Differentiated thyrocytes face the lumen at the apical pole, and are attached to the basement membrane at their basal pole. In the mature thyroid, a dense network of blood vessels surrounds each follicle. Together, they form angiofollicular units responsible for T3 and T4 thyroid hormone synthesis and storage within luminal Tg, and then hormone secretion into the bloodstream (Colin et al., 2013; Nilsson and Fagman, 2013). A limited number of transcription factors (Nkx2.1, Pax8, Foxe1 and Hhex) and signaling molecules are known to control thyroid development (Fagman and Nilsson, 2010), but the molecular and subcellular morphogenetic machineries regulating follicle formation remain essentially unknown.

Several studies have demonstrated the importance of epithelial-endothelial interactions in organs developing from the endoderm (Hick et al., 2013; Lammert et al., 2001; Lazarus et al., 2011; Pierreux et al., 2010). In the thyroid, we found that endothelial cells are recruited in response to epithelial-derived Vegfa and are essential for the organization of the thyroid epithelial cell mass into follicles (Hick et al., 2013). However, the identity and mode of action of folliculogenic factor(s) are still unknown.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor beta (TGFβ) family that control many developmental processes such as epithelial differentiation and tissue morphogenesis (Macias et al., 2015). Upon BMP ligand binding to its receptor, the intracellular signal transducers Smad5/5/8 become activated by C-terminal phosphorylation. Phosphorylated Smad1/5/8 bind Smad4 and translocate to the nucleus where they regulate the expression of target genes, such as the Id genes. BMPs also interact with members of the apicobasal polarity complex, as exemplified by early events of neural tube closure (Eom et al., 2011). Loss of the twisted gastrulation (Twsg1) gene, which encodes a BMP modulator, causes craniofacial defects, affects foregut endoderm and reduces the expression of Hhex, a transcription factor required for early thyroid development (Petryk et al., 2004). Interestingly, reports on embryonic stem cell (ESC) differentiation have shown that modulation of BMP signaling is crucial for efficient lineage specification of Nkx2.1+ endodermal progenitors (Green et al., 2011; Longmire et al., 2012; Kurmann et al., 2015).

Since Smads often function redundantly, we studied the role of BMP signaling in the thyroid gland by combined genetic inactivation of Smad1 and Smad5. We found that the Smad1 and Smad5 double-knockout (Smad1/5KO) mice have retarded growth, suffer from hypothyroidism and display a major perturbation in follicle development. Defective folliculogenesis, highly reminiscent of that observed in Vegfa knockout (VegfaKO) mice, was associated with impaired assembly of basement membrane proteins and could be rescued by laminin-rich conditioned medium. Our results indicate that epithelial BMP-Smad1/5 signaling and endothelial...
cells of the thyroid promote folliculogenesis via assembly of the basement membrane.

RESULTS

Active BMP signaling in thyrocyte progenitors during thyroid development

We first analyzed BMP signaling during normal thyroid development at the time of follicle formation (from E14.5 to E18.5). Most BMP ligands were expressed in the developing thyroid and, among these, Bmp2, Bmp4, Bmp5 and Bmp7 were the most abundant (Fig. 1A). Genes encoding BMP type I and type II receptors, intracellular Smad1, Smad5 and Smad8, as well as the common mediator Smad4, were also well expressed (Fig. 1A). Measurement of the absolute copy number of Smad1/5/8 in E14.5 thyroid glands (Fig. 1B) showed that Smad8 mRNA is only ∼25% as abundant as Smad1 and Smad5.

We next assessed whether BMP signaling is active during thyroid development. We first recorded in vivo BMP signaling activity in the thyroid region using a transgenic line expressing green fluorescent protein (GFP) under the control of BMP-responsive elements (BREs) (Monteiro et al., 2008). At E14.5, GFP expression was not detected in E-cadherin+ thyroid epithelial cells, but was found in the parathyroid and in PECAM+ endothelial cells that form blood vessels running along and in between the thyroid epithelial cell masses (Fig. 1C). One day later, GFP was detected in some thyroid epithelial cells, and by E16.5 in almost all of the E-cadherin+/Tg+ cells (Fig. 1C). Conversely, GFP colocalization with PECAM was weak at that stage.

We further monitored phosphorylation of Smad1/5 (pSmad1/5) by whole-mount analysis of microdissected thyroid lobes (Fig. 1D). In untreated thyroid lobes, we could only detect pSmad1/5 at E15.5 in some E-cadherin+ and E-cadherin− cells. However, incubation of E14.5 and E15.5 thyroid lobes in Bmp4 induced Smad1/5 phosphorylation and nuclear translocation in epithelial cells (Fig. 1D). This indicated that thyroid epithelial cells are responsive to BMP signaling as early as E14.5 and that signaling is turned on in the developing thyroid between E14.5 and E15.5, i.e. at the start of, and during, follicle development.

Smad1/5−/− mice display growth retardation, abnormal follicles and hypothyroidism

To determine the role of BMP signaling in the thyroid, we deleted the most abundant Smads, namely Smad1 and Smad5, in the thyroid by crossing mice bearing conditional alleles of Smad1 (Smad1fl/fl)
and Smad5 (Smad5^fl/fl) with Pax8-Cre mice (Bouchard et al., 2004).
Single-knockout (Smad1^KO and Smad5^KO) as well as double-
knockout (Smad1/5^dKO) mice were viable and born at normal
Mendelian ratios. Only the Smad1/5^dKO displayed a smaller body
size and significantly reduced body weight at 10 weeks (Fig. 2A).

Histological analysis of control thyroids at 10 weeks showed a
collection of large follicles composed of a monolayer of epithelial
cells delineating a round lumen (Fig. 2Ba). Smad1^KO and Smad5^KO
thyroid appeared comparable to that of the control, although the
follicles in Smad5^KO were somewhat smaller (Fig. 2Bb,c). In
marked contrast, the thyroid structure of Smad1/5^dKO was
completely disorganized, with substantially fewer, irregular
follicles delineated by taller thyrocytes (Fig. 2Bd). This was
accompanied by adipogenesis in Smad1/5^dKO, which also occurred
to a lesser extent in Smad1^KO (data not shown). To evaluate thyroid
function, we immunostained sections for iodinated Tg. This led to
homogeneous staining filling the colloid of all follicles of control
and single-knockout thyroids (Fig. 2Be-g). In Smad1/5^dKO,
iodinated Tg was limited to a peripheral rim, suggesting colloid
depletion due to high turnover rate (Fig. 2Bh). These data suggested
that, although some thyroid differentiation can occur in Smad1/
5^dKO, functionality was dramatically affected.

Because Smad1/5^dKO displayed growth retardation and
histological alterations of the thyroid glands, we next analyzed
the thyroid hormonal status at 10 weeks. Plasma T3 and
T4 concentrations were decreased by 80% in Smad1/5^dKO
(Fig. 2C) and, accordingly, thyroid-stimulating hormone (TSH)
concentrations were dramatically increased up to 20,000-fold
(Fig. 2C). Thus, this major feedback response was insufficient to
maintain plasma T3 and T4 concentrations (Fig. 2C), demonstrating
thyroid unresponsiveness. Taken together, these data indicated that
Smad1 and Smad5 are redundantly required for thyroid follicle
formation and function.

Smad1/5 are efficiently inactivated in thyrocyte progenitors

The severe follicular alterations and major hypothyroidism of
adult Smad1/5^dKO mice suggested that follicle formation and/or
differentiation might be affected in embryonic thyroid. We first
verified Cre-mediated Smad1 and Smad5 recombination by
measuring the abundance of Smad1/5/8 mRNA during early
stages of embryonic thyroid development (Fig. 3A). Compared
with controls, Smad1/5^dKO thyroid showed a 50% reduction in the
abundance of Smad1 and Smad5 mRNA as early as E14.5, and
this reduction persisted until the end of gestation (Fig. 3A). Smad8
expression, used as a control Smad, was not affected at
E14.5 or E16.5. Surprisingly, its level was significantly decreased
at E18.5, suggesting positive regulation by BMP-Smad1/5
signaling during thyroid development. Based on epithelial-
specific Pax8-Cre expression, we hypothesized that the 50%
global reduction in Smad1/5 reflected loss of expression in the
developing thyrocytes but persistent expression in other cell
types. Further, treating cultured thyroid lobes with Bmp4 revealed
phosphorylation of Smad1 and Smad5 in control but not in the
vast majority of Smad1/5^dKO epithelial cells (Fig. 3B). These data
indicated that Smad1/5 were successfully inactivated in thyroid
epithelial cells and suggest that the remaining 50% expression in

![Fig. 2. Smad1/5^dKO mice display growth retardation, abnormal follicles and hypothyroidism.](image-url)
Ten-week-old single- and double-knockout mice were
compared with age-matched controls. (A) Smad1/5^dKO mice are of smaller body size and lower body weight compared with control. (B) H&E staining of control (a), Smad1^KO (b) and Smad5^KO (c) thyroids reveals abundant mature follicles lined with flat thyrocytes, whereas Smad1/5^dKO (d) exhibits severe disorganization of the thyroid parenchyma, with fewer and abnormal follicular structures lined with thicker thyrocytes, suggesting an abnormally high TSH level. Control (e), Smad1^KO (f) and Smad5^KO (g) thyroids show homogeneous and dense signal for iodinated Tg in the colloid, whereas in the Smad1/5^dKO (h), iodinated Tg is limited to a peripheral rim. (C) TSH, T3 and T4 concentrations were measured in plasma of control and Smad1/5^dKO mice (n≥15). Most Smad1/5^dKO mice exhibit very high plasma or serum TSH and low T3 and T4. **P<0.001, ***P<0.0001, Mann–Whitney U-test. Data are presented as mean±s.e.m. Scale bar: 100 µm.
the thyroid can be attributed to other cell types where Cre is not induced.

**Smad1/5 inactivation impairs thyroid folliculogenesis**

Follicle formation from an original mass of non-polarized epithelial cells involves a complex morphogenetic process starting at ~E14.5. Careful analysis of thyroid gland development reveals progressive fragmentation of the epithelial mass, organization of the epithelial cells in independent spherical structures or rosettes, acquisition of apicobasal polarity and finally lumen expansion (Fagman et al., 2006; Hick et al., 2013). To determine when BMP signaling impacts on follicle formation, we...
analyzed thyroid development in control and Smad1/5\(^{dKO}\) mouse embryos from E14.5 to E18.5 (Fig. 3C,E).

In controls, punctate immunofluorescent labeling for the apical marker ezrin first became apparent in the thyroid mass at E14.5, just underneath or fused with the membrane of epithelial progenitors (Fig. 3Ca). We interpret these puncta as ezrin-coated vesicles en route to predefined plasma membrane domains in order to initiate apical pole formation. In sections we frequently observed two such vesicles closely apposed, face to face, next to the plasma membrane of adjacent cells (arrows in Fig. 3C,E). At this stage, the basal marker laminin was only found between thyroid peripheral cells and the stroma (Fig. 3Ea). By E16.5, coordinated fusion of vesicles from several epithelial cells initiated the creation of small lumina. Thryocyte progenitors organized in microfollicles progressively assembled a laminin-rich basement membrane, appearing as a dense meshwork that fragmented the mass (Fig. 3Cb,Ed). By the end of gestation, most follicular structures were individualized, i.e. each completely surrounded by laminin (basal pole) and enclosing an expanded lumen circumscribed by ezrin\(^{-}\) membrane (apical pole) (Fig. 3CcEc).

In the absence of Smad1 and Smad5, the thyroid mass at E14.5 was delineated by a single laminin shell and thyroid epithelial cells contained small puncta of ezrin, as in wild-type controls (Fig. 3Cd,Ed). By E16.5, fusion of ezrin\(^{-}\) vesicles with the membrane also occurred, but the lumina of microfollicles were much smaller than in wild type (Fig. 3Ce,Ed). In addition, there was a 50% reduction in epithelial cells showing ezrin\(^{-}\) membrane (83.9±13.8% in wild type versus 35.5±11.7% in Smad1/5\(^{dKO}\), Fig. 3F). Fragmentation of the thyroid mass was also impaired: a large fraction of epithelial cells remained associated in large clusters so that their contact with the basement membrane (pan-laminin) was reduced by about half (92.2±7.7% in wild type versus 56.7±9.5% in Smad1/5\(^{dKO}\); Fig. 3F). By E18.5, the difference between control and Smad1/5\(^{dKO}\) was even more evident. Most epithelial cells remained associated, microfollicles were composed of a reduced number of cells, were only partially delineated by laminin and their lumen did not appreciably expand (Fig. 3Cc,Ed). Quantification of sectioned lumen size revealed a 4-fold difference in Smad1/5\(^{dKO}\) compared with control (33.9±2.9 \(\mu m^2\) in Smad1/5\(^{dKO}\) versus 137.1±11.2 \(\mu m^2\) in wild type), thus an 8-fold difference in volume. However, the global thyroid size, the thyrocyte proliferation rate and the number of open ezrin\(^{-}\) structures were not affected by Smad1/5 inactivation (data not shown). Altogether, these results indicated that BMP signaling is essential for epithelial reorganization of the thyroid progenitor mass into follicles with large lumina.

Despite the reduced number of ezrin\(^{-}\) epithelial cells and the smaller lumina, we noticed that, when present, ezrin was correctly localized to the apical pole in Smad1/5\(^{dKO}\). This suggested that apical polarity was preserved in these cells. To further test this interpretation, we analyzed control and Smad1/5\(^{dKO}\) thyroid for Par3, a key component of the apical polarization machinery, and F-actin for cytoskeleton organization, and found no obvious difference between controls and Smad1/5\(^{dKO}\) (Fig. S1A). Furthermore, transmission electron microscopy revealed that epithelial cells involved in immature microfollicles in Smad1/5\(^{dKO}\) still developed tight junctions and microvilli projecting into the lumen space, as in control follicles (Fig. S1B). However, intracellular microvilli inclusion bodies were observed in Smad1/5\(^{dKO}\), suggesting defective apical delivery of intracellular vesicles (Fig. S1B, inset).

Altogether, these data indicated that BMP-Smad signaling controls folliculogenesis downstream of the initial apical specification but upstream of basement membrane assembly, epithelial reorganization and lumen expansion.

**Smad1/5 signaling in thyrocytes stimulates Vegfa expression and affects gene expression in neighboring endothelial cells**

Previous work from our group has shown that endothelial cell recruitment into the developing thyroid is required for follicle formation. The folliculogenic effect of endothelial cells was contact independent and could be mimicked with medium conditioned by endothelial cells (Hick et al., 2013). We thus tested whether the defective folliculogenesis in Smad1/5\(^{dKO}\) could be due to a lack of either endothelial cells or a signal derived therefrom. We identified endothelial cells by immunofluorescence for PECAM (Pecam1), a cell adhesion molecule specifically expressed by endothelial cells, in control and Smad1/5\(^{dKO}\) thyroids from E14.5 to E18.5 (Fig. 4A). In control and Smad1/5\(^{dKO}\) thyroids, the abundance and localization of blood vessels were unaffected at the three stages analyzed (Fig. 4A). Quantification of endothelial sectioned surface and the epithelial-to-endothelial sectioned surface ratio did not reveal any significant differences between Smad1/5\(^{dKO}\) and the control.

To evaluate endothelial cell differentiation in Smad1/5\(^{dKO}\), we measured the expression of selected endothelial-specific genes. Expression of vascular endothelial cadherin [cadherin 5 (Cdh5)] and vascular endothelial cadherin 2 [protocadherin 12 (Pcdh12)] as well as of Tie1 was significantly reduced at E16.5 (Fig. 4B), but expression of Vegfr2, Pecam1 and endothelial cell-specific molecule 1 (Esm1) was normal. Vegfa controls endothelial cell development and BMP-Smad1/5 signaling is known to control Vegfa expression in several cell types (Bai et al., 2013; Deckers et al., 2002; Shao et al., 2009; Shimizu et al., 2012). We measured Vegfa in developing Smad1/5\(^{dKO}\) thyroids and found that it was reduced starting at E14.5; this reduction reached statistical significance starting at E16.5 (Fig. 4C). We thus directly analyzed the effect of BMP addition on Vegfa expression in microdissected thyroid lobes at E14.5 and in the thyroid follicular cell line FRTL-5. Treatment of thyroid lobes with the specific BMP inhibitor D MH1 (Hao et al., 2010) led to a reduction in expression of Vegfa and of the BMP-Smad1/5 signaling target gene Id2 (Fig. 4D). Incubation of wild-type thyroid lobes with BMP ligands induced Id2, but did not modify the already high Vegfa expression (Hick et al., 2013). To circumvent any BMP accessibility issues, we incubated the thyroid cell line FRTL-5 with a combination of the most abundant BMP ligands in the thyroid (Bmp2, 4, 5 and 7) and noticed a dose-dependent stimulation of the expression of Vegfa and Id2 (Fig. 4E). These results indicated that epithelial Smad1/5 signaling regulates Vegfa and endothelial gene expression.

**Production of the basement membrane proteins laminin and type IV collagen is decreased in Smad1/5\(^{dKO}\) and Vegfa\(^{KO}\) thyroid glands**

We next turned to the identification of the follicle-promoting factor(s). Knowing that thyrocytes and endothelial cells form their own continuous basement membrane, that extracellular matrix components promote epithelial cell organization, and that contact with laminin was partly impaired in Smad1/5\(^{dKO}\), we postulated that the basement membrane could stimulate folliculogenesis. To test this hypothesis, we first looked for a possible defect in laminin and type IV collagen expression in Smad1/5\(^{dKO}\) and Vegfa\(^{KO}\) (Fig. S2). Laminins are a family of five \(\alpha\), three \(\beta\) and three \(\gamma\) chains forming \(\alpha\beta\gamma\) heterotrimers that span the basement membrane. The collagen...
type IV family has six α chains forming trimers interconnected with the laminin network.

In Smad1/5ko, expression of laminin α2, α3, β1 and β3 chains was significantly, albeit transiently, reduced (Fig. S2A,B). Expression of laminin α3 and α4 was significantly reduced in VegfaKO (Fig. S2C). Of note, laminin α4 displays an endothelial-specific pattern in E16.5 embryos (The Matrixome Project, Osaka University, Japan). A similar analysis revealed a reduction of most type IV collagen genes in Smad1/5ko (Fig. S2D). There was a substantial and sustained reduction in collagen α3-6(IV) chains. Collagen α1(IV) and α2(IV) were modestly reduced or delayed. In VegfaKO, expression of collagen α1(IV) and α2(IV) was significantly reduced (Fig. S2E); both genes were also reported as endothelial specific (The Matrixome Project). In conclusion, gene expression analysis revealed decreased expression of several basement membrane proteins in Smad1/5ko and VegfaKO.

We next examined by immunofluorescence whether decreased gene expression resulted in impaired basement membrane assembly. In line with the gene expression analysis, collagen type IV signals were severely affected in both Smad1/5ko and VegfaKO (Fig. 5Ab,d). Pan-laminin signals were also reduced in Smad1/5ko and only modestly in VegfaKO (Fig. 5Af,h). Taken together, these data indicated that expression and/or deposition of basement membrane proteins depends on endothelial cells and Smad1/5 signaling in thyrocytes, and suggested an essential role for basement membrane assembly in folliculogenesis.
Epithelial laminin production is affected in Smad1/5\textsuperscript{dKO}

Mature angiofollicular units are characterized by close proximity between endothelial and epithelial cells. To determine the respective contribution of epithelial and endothelial cells to basement membrane protein synthesis, we analyzed the expression of laminin and collagen type IV genes in FACS-sorted epithelial populations (Fig. S3). Epithelial thyrocyte progenitors were selected based on YFP expression after Pax8-Cre-mediated recombination of the ROSA-STOP-YFP locus. This population was compared with YFP\textsuperscript{−} cells, which thus included C-cells, endothelial and mesenchymal cells but also non-recombined thyrocyte progenitors. Yfp mRNA was only found in the YFP\textsuperscript{+} cells. Pax8 and the epithelial marker E-cadherin showed a 2-fold enrichment in this population, while Vegfr2 was enriched 4-fold in the YFP\textsuperscript{−} population, as expected (Fig. S3A). mRNAs for all collagen type IV genes as well as laminins α3, β1-3 and γ1,2 were equally distributed in YFP\textsuperscript{+} and YFP\textsuperscript{−} thyroid cells. By contrast, laminin α1 and α5 chains were enriched in the YFP\textsuperscript{+} epithelial population, whereas laminin α2 and α4 chains showed the strongest expression in the YFP\textsuperscript{+} population (Fig. S3B).

Since laminins α1 and α5 were enriched in the epithelial population, we studied their expression by immunofluorescence using isoform-specific antibodies. In control thyroid glands, epithelial follicular structures were surrounded by almost continuous laminin α1 and α5 signal. In Smad1/5\textsuperscript{dKO} thyroids, laminin α1 signal was severely reduced (Fig. 5B), supporting the RT-qPCR analysis (Fig. S2A). Laminin α5 signal was comparable to that of controls, although the pattern was affected owing to the epithelial organization defect (Fig. 5C). These data confirmed that the key basement membrane proteins, laminin and collagen type IV,
are produced by both epithelial and endothelial components of the angiofollicular units, and indicated that Smad1/5dKO epithelial cells have an impaired ability to produce and assemble laminin α1-containing heterotrimers.

**Medium conditioned by endothelial cells contains folliculogenic factor(s) and rescues the follicle formation defects of Smad1/5dKO and VegfaKO**

To investigate the mechanism of follicle formation, we resorted to an ex vivo culture system of thyroid lobes. We had previously reported that pharmacological ablation of endothelial cells in E12.5 thyroid explants prevented folliculogenesis; conversely, addition of medium conditioned by embryonic endothelial progenitor cells (eEPCs), herein referred to as eEPC-CM, was able to rescue, and even overstimulate, follicle formation (Hick et al., 2013). Using an improved thyroid lobe culture system (Delmarcelle et al., 2014), we found that addition of eEPC-CM to non-ablated wild-type thyroid lobes promotes follicle development by up to 6-fold after 3 days of culture (Fig. 6A,C). This medium thus contains folliculogenic factor(s).

We then addressed whether eEPC-CM could rescue the defective folliculogenesis of Smad1/5dKO and VegfaKO. We first verified that E14.5 Smad1/5dKO thyroid lobes reproduce impaired follicle formation upon ex vivo culture: only very small ezrin+ structures could be observed after 3 days (Fig. 6B). Importantly, eEPC-CM significantly induced follicle formation in Smad1/5dKO thyroid lobes (Fig. 6B,C), and the same was found for VegfaKO (Fig. S4). The similar rescue of immature microfollicle development of Smad1/5dKO and VegfaKO thyroid glands by folliculogenic factor(s) in eEPC-CM suggested that these factor(s) act downstream of epithelial Smad1/5 signaling and of endothelial cell invasion.

**Laminins deposited and assembled around thyroid epithelial cells promote folliculogenesis**

Among potential folliculogenic factors, we examined whether eEPCs expressed and released basement membrane proteins into their CM. We first determined the expression profile of laminin subunit and collagen type IV genes in eEPCs. We found that eEPCs express very high levels of laminin α1, β1 and γ1 mRNAs, which almost reached the level of the β-actin (Actb) housekeeping gene (Fig. 7A). Among collagen type IV genes, α6 showed the highest level of expression, albeit ~100× lower than laminin β1.

We next verified that eEPCs can secrete laminins into the CM, in particular laminin-111 (heterotrimer of α1, β1 and γ1), by silver staining, western blotting and mass spectrometry. Silver staining after SDS-PAGE of CM revealed a well-defined band migrating at a similar Mr to purified laminin-111 (Fig. 7B). Western blotting with laminin α1-specific antibody identified this subunit (Fig. 7B). Mass spectrometry further easily detected laminin α1, β1 and γ1 peptides in the CM (Table S1). Finally, we asked whether laminin-111 from eEPC-CM was deposited on cultured thyroid explants and form small follicles. Addition of eEPC-CM to the culture stimulates polarization and follicle formation. (B) Compared with control, ezrin+ structures are smaller in untreated Smad1/5dKO thyroid lobes, but are enlarged when cultured with eEPC-CM. (C) The number of large ezrin+ luminal follicles per thyroid explant section was calculated. ***P<0.0001, one-way ANOVA followed by Bonferroni’s multiple comparison test (n≥23). Data are presented as mean±s.e.m. Scale bars: 20 µm in A; 50 µm in B. See also Fig. S4.
results were obtained upon laminin β1 and γ1 silencing (data not shown). These data indicated that laminin-111 in eEPC-CM is a key actor of thyroid folliculogenesis.

DISCUSSION

Here, we examined if and how BMP-Smad signaling is required for thyroid follicle formation. We found that BMP signaling was activated in thyroid epithelial cells during folliculogenesis. Inactivation of Smad1/5 in thyroid epithelium caused major alterations to follicular structures, hypothyroidism and growth retardation but no loss of viability. Defective follicular development was associated with impaired basement membrane assembly, highly reminiscent of the folliculogenesis defect of VegfaKO embryos in which endothelial cells are, however, absent. Follicle formation defects in Smad1/5KO and VegfaKO could be rescued with laminin-rich CM. Taken together, our findings support a model in which thyroid follicle formation depends on basement membrane assembly, a process controlled by epithelial Smad1/5 signaling and endothelial cells.

Ten-week-old Smad1/5KO mice were smaller and of reduced body weight compared with control littermates. Failure to grow is readily explained by the very low levels of thyroid hormones T3 and T4 and the extremely high TSH values, which demonstrated non-compensated hypothyroidism. Thyroid unresponsiveness despite huge TSH values is well known in mouse knockouts for the TSH receptor (Tshr), Nkx2.1, Foxe1 and Pax8 (De Felice and Di Lauro, 2004). Smad1/5KO thyroid lobes were normal in size at birth, and the functional impairment of adult thyroid probably results from the embryonic folliculogenesis defects. However, we cannot exclude a role for BMP signaling in postnatal thyroid homeostasis.

Our laboratory has provided evidence that follicle formation occurs by reorganization of the thyroid primordium epithelial cell mass into polarized monolayers surrounding a central lumen, which is comparable to the cord hollowing model of de novo lumen formation (Andrew and Ewald, 2010) and the process of apicobasal polarization with lumen formation, as observed in MDCK cells when cultured in a 3D Matrigel. In this 3D culture system, fusion of vesicles to the apical membrane initiation site (AMIS) allows apical

![Fig. 7. Laminins deposited and assembled around thyroid epithelial cells promote folliculogenesis.](image)

(A) eEPCs express high level of selected laminin genes and much lower levels of collagen IV genes. All mRNAs were normalized to Actb. Data are presented as means±s.e.m. (n≥3). NA, non-amplified. (B) Laminin α1 (400 kDa) is secreted by eEPCs and accumulates in eEPC-CM. WB, western blot. (C) E14.5 thyroid lobes cultured with eEPC-CM show intense signal for laminin α1 around thyroid epithelial cells (E-cadherin) and show larger follicles (ezrin) as compared with untreated lobes. (D) shRNA-mediated laminin α1 silencing in eEPCs decreases laminin α1 in eEPC extract and in eEPC-CM, as compared with control shRNA. Ezrin is used as a loading control for eEPC extracts. (E) Compared with thyroid lobes cultured with CM from control shRNA-treated eEPCs, lobes cultured in the presence of CM from laminin α1 shRNA-treated eEPCs do not show laminin α1 deposition and have small follicles, similar to those of untreated lobes. Scale bars: 20 µm.

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lumen opening and cyst formation (Bryant et al., 2010). In the thyroid, we also observed pre-apical intracellular vesicles (ezrin) and their coordinate exocytosis to generate apical lumen. Immature microfollicles can then expand or enlarge, while segregating from adjacent microfollicles and becoming vascularized by invading endothelial cells. In Smad1/5KO, despite the formation of ezrin vesicles in some cells and endothelial invasion, the exocytosis of vesicles with membranes and lumen expansion were clearly impaired, as was fragmentation of the mass, leading to the individualization of microfollicles.

The occurrence of immature microfollicles in Smad1/5KO thyroid suggests that the basal machineries for apical polarization and for the creation of sealed lumina are preserved. However, this does not mean that these machineries are quantitatively fully operative. Indeed, we found that substantially fewer epithelial cells participate in microfollicle formation (Fig. 3D), develop an ezrin apical pole (Fig. 3F) and assemble a laminin+ basal pole (Fig. 3F). Altogether, these data indicate that, although some epithelial cells in Smad1/5KO thyroid engage in polarization and the formation of a sealed lumen, substantially fewer participate in this concerted process when BMP signaling is inactivated. These observations suggest either that BMP signaling is not required for apical polarization or that the genetic inactivation using Cre recombinase is not 100% effective in all Pax8+ cells at the same time and for all four Smad alleles.

Progression of immature microfollicles into larger structures, as clearly affected in Smad1/5KO, cannot be explained by impaired epithelial proliferation (normal rate in Smad1/5KO). Conceivably, impaired follicle enlargement could be related to defective differentiation and thus a lower accumulation of Tg that would otherwise force expansion of the colloid lumen. However, VegfaKO mice, which also display immature microfollicles, express Tg at an almost normal level (90% of wild type; Hick et al., 2013). This argues that Tg accumulation is not sufficient for lumen expansion. Comparison of Smad1/5KO and VegfaKO rather suggests that lumen enlargement depends on correct apicobasal polarization. Indeed, as compared with aged-matched control littermates, we found in both Smad1/5KO and VegfaKO reduced basement membrane deposition and the persistence of intracellular ezrin vesicles, suggesting a relationship between basement membrane-triggered polarization and impaired fusion with the apical membrane (Fig. S1B and Fig. 3C,E, arrows).

The impaired follicle formation observed in the absence of Smad1 and Smad5 is reminiscent of the defect observed in the absence of endothelial cells upon Vegfa inactivation in thyroid epithelium (Hick et al., 2013). Although endothelial cell density in epithelial-specific Smad1/5KO was comparable to that of control thyroid glands, expression of endothelial cell identity markers (Cd31, Pecdh12 and Tie1) and endothelial-enriched basement membrane proteins (type IV collagen α1, α2 and laminin α3) was impaired, consistent with ongoing reciprocal paracrine communications between epithelial and endothelial cells (Hick et al., 2013). In this report, we further found that BMP-Smad signaling in thyroid epithelial cells controls the expression of Vegfa, in agreement with reports in other cell types (Bai et al., 2013; Deckers et al., 2002; Shao et al., 2009; Shimizu et al., 2012). Of note, this regulation in zebrafish could be either positive (for Smad1) or negative (for Smad5) (He and Chen, 2005). Our findings in developing thyroid suggest instead that BMPs could control the endothelial gene expression program indirectly, i.e. by regulating Vegfa expression. Changes in gene expression in endothelial cells could in turn impact on follicle formation. Several studies have emphasized the crucial role played by endothelial cells via angiocrine factors in the regulation of organ morphogenesis (Ramasamy et al., 2015). Integrating available knowledge supports a reciprocal paracrine communication in which thyrocyte progenitors, under the influence of BMP, participate in the recruitment and maturation of thyroid endothelial cells, via Vegfa. In turn, endothelial cell invasion and maturation in the thyroid mass would create a microenvironment permissive for folliculogenesis.

The importance of the extracellular environment for cell polarization in tissue culture experiments has long been recognized (Wang et al., 1990a,b). Culture of MDCK epithelial cells in collagen-rich gel or Matrigel triggers the production of laminin and assembly of a basement membrane, which in turn promotes cystic structures (Bryant et al., 2010), a process that can be directly compared to the formation of thyroid follicles. In addition, ESCs, when forced to transiently co-express transcription factors Pax8 and Nkx2.1, differentiate into thyrocyte progenitors that can form functional follicles if further cultured in Matrigel (Antonica et al., 2012; Ma et al., 2015). These two convergent in vitro studies emphasize the essential role of basement membrane and extracellular matrix proteins for the development of a 3D, cyst-like or follicular structure.

In vivo, we observed reduced expression of laminin α1 and collagen type IV in Smad1/5KO. Few reports have suggested a direct role for BMP signaling in basement membrane synthesis and deposition. During ductus arteriosus closure, Bmp9 and Bmp10 are required to stimulate endothelial cell differentiation and extracellular matrix deposition (Levet et al., 2015). In kidney mesangial cells, Smad1 directly controls the expression of collagen type IV α1 and α2, which leads to glomerular basement membrane thickening (Abe et al., 2004; Matsubara et al., 2015). Thus, cumulative evidence indicates that BMP-Smad signaling can regulate tissue morphogenesis by controlling the expression and/or deposition of extracellular matrix proteins.

Analysis of basement membrane proteins in VegfaKO also revealed decreased type IV collagen signal (Fig. 5A), most probably owing to the absence of endothelial cells (Hick et al., 2013). In addition, we found impaired epithelial laminin α1 assembly in VegfaKO (data not shown). This suggests that endothelial cell invasion into the epithelial mass, by disrupting multidirectional epithelial cell-cell contacts and inducing interstitial matrix deposition that individualizes cell cords then microfollicles, would indirectly stimulate epithelial basement membrane assembly, as in MDCK cells grown in Matrigel.

Laminin deposition is the primary event of basement membrane formation, and induces the secondary assembly of the type IV collagen meshwork (Morrissey and Sherwood, 2015; Pöschl et al., 2004). In turn, the collagen IV meshwork is essential for integrity, stability and functionality of the basement membrane (Pöschl et al., 2004; Yurchenco, 2011). We thus propose that the addition of exogenous, eEPC-derived laminin-111 was sufficient to stimulate the organization of epithelial cells in follicles in control thyroid lobes, and to rescue follicle formation defects in Smad1/5KO and VegfaKO, by promoting the assembly of endogenous laminin and type IV collagen scaffolds. Rescue of folliculogenesis in Smad1/5KO and VegfaKO thus indicates that basement membrane assembly is a critical step in folliculogenesis, downstream of epithelial BMP signaling and of endothelial cell invasion.

In conclusion, we propose a model that places epithelial basement membrane assembly in the developing thyroid gland as a crucial signal to orient epithelial cell polarization and promote their organization into follicles. First, epithelial cells from the thyroid...
primordium produce Vegfa; at this step, epithelial BMP-Smad signaling participates in Vegfa gene expression. Second, upon Vegfa-dependent endothelial cell recruitment and invasion into the thyroid epithelial cell mass, multiregional epithelial-cell cell contacts are disrupted while lateral contacts are preserved. This triggers, together with continued BMP-Smad signaling, epithelial cell production and deposition of their own basement membrane proteins. Finally, epithelial basement membrane assembly orients the epithelial cells, promotes the apical delivery of ezrin+ vesicles and the individualization of follicles.

MATERIALS AND METHODS

Animals

Smad1/5 floxed, Vegfa floxed, Pax8-Cre and BRE-GFP mice were obtained from A. Zwijsen and E. J. Robertson (Arnold et al., 2006; Umans et al., 2003), N. Ferrara (Genentech) (Gerber et al., 1999), M. Busslinger (Bouchard et al., 2004) and S.C.d.S.S.L. (Monteiro et al., 2008), respectively. All other mice were of the CD1 strain. Mice were raised and treated according to the guidelines of laboratory animal care of the University Animal Welfare Committee, Université Catholique de Louvain.

Dissection and culture of thyroid explants

Thyroid lobes were microdissected from E14.5 mouse embryos and processed as described (Delmarcelle et al., 2014). DMH1 (Sigma-Aldrich) was dissolved in DMSO and used at 3 or 10 µM. Recombinant mouse Bmp4 (R&D Systems) was reconstituted at 100 µg/ml in 4 mM HCl containing 0.1% BSA and added at 20 ng/ml to the culture medium. Control explants were exposed to the same concentration of vehicle as the test samples. Thyroid lobes were maintained in culture for 3 or 4 days, and processed for RNA extraction and immunolabeling as described (Delmarcelle et al., 2014).

Histology, immunolabeling and morphometric analyses

Histology was performed on 6µm cryosections of thyroid lobes or FRTL-5 cells using Hematoxylin and Eosin (H&E). Immunofluorescence on thyroid gland sections from embryos or explants was performed as described previously (Pierreux et al., 2006). Antibodies and dilutions are shown in Table S2. Nuclei were counterstained with Hoechst (Sigma) in PBS during incubation with secondary antibody. For immunoperoxidase, the Envision system (Dako) was used. Whole histological sections were recorded with Mirax Scan (Zeiss). Fluorescence on sections was observed with a Zeiss Cell Axiocvert 200 inverted fluorescence microscope or with a Zeiss Cell Observer spinning disk (COSD) microscope. For quantification of follicles, at least 18 sections in four independent explants were counted. Endothelial and epithelial surface was quantified using Axiovision 4.8.2 software (Zeiss). Briefly, images were prepared as a binary mask, filled and the surface area was calculated on six images/section spanning 360 µm of three controls and three Smad1/5KO thyroids. The epithelial to endothelial surface ratio was then calculated.

T3, T4 and TSH level measurements

T3 and T4 and TSH concentrations were determined by radioimmunoassay (RIA) (Siemens Medical Solution Diagnostics). Plasma TSH concentrations were measured using a sensitive, heterologous, diselenium double-antibody precipitation RIA as described previously (Pohlzen et al., 1999). Hormone levels were measured in 16 female and 15 male mice.

RNA extraction and real-time RT-PCR

Total RNA was extracted from microdissected thyroid lobes, cultured explants or FRTL-5 cells using Trizol reagent (Thermo Scientific) and phenol/chloroform extraction (Delmarcelle et al., 2014). RNA (0.5-1 µg) was reverse transcribed with random hexamers using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed using the KAPA SYBR Fast qPCR Kit (Sopachem). Primer sequences are described in Table S3. Actb and Rpl27 were used as reference genes and relative changes in the target gene to reference gene mRNA ratio were determined by transformation of threshold cycles to absolute mRNA numbers (Dupasquier et al., 2014) or using the ΔΔCt method.

Embryonic endothelial progenitor cell-conditioned medium (eEPC-CM)

Previously established mouse eEPCs (Hatzopoulos et al., 1998; Kupatt et al., 2005) were used for no more than 6 months after being thawed, as described (Shab et al., 2006). The preparation of CM was adapted from Hick et al. (2013). Briefly, 90% confluent cells were incubated for 24 h in M199 medium (Thermo Scientific) without serum (6 ml for a 7.8 cm2 dish). Supernatant was first centrifuged at 1900 g for 5 min to remove floating cells or large debris, then at 17,000 g for 20 min and finally concentrated 10× by centrifugation at 1900 g for 5 min in Amicon Ultra 50 K units. The 10× concentrated medium was supplemented with 10% FCS before use. Mass spectrometry was performed as described (Cho et al., 2014).

Cell culture

Fisher rat thyroid follicular FRTL-5 cells were cultured in Coon’s modified F12 medium as previously described (Crapo et al., 2015) and were used within 6 months of purchase. Cells were grown in 12-well tissue culture dishes for 24 h before treatment with BMP ligands. Recombinant mouse Bmp2, 4, 5 and 7 (R&D Systems) were reconstituted in 4 mM HCl containing 0.1% BSA at 100-150 µg/ml and used as a mix of 25 ng/ml (Bmp2), 5 ng/ml (Bmp4), 150 ng/ml (Bmp5) and 75 ng/ml (Bmp7). After 4 h, cells were lysed in Trizol reagent and RNA was extracted.

Lentiviral infection

Lentiviral constructs driving expression of shRNA against laminin α1 were generated as described previously (Gerin et al., 2010). Briefly, the pGIPZ lentivector containing a shRNA against laminin α1, α5, β1 and γ1 or the pGIPZ empty vector (Open Biosystems) were transfected into HEK293T cells for lentiviral production. Packaging was performed using a second-generation plasmid system (psPAX2 and pMD2.G; Addgene plasmids 12,259 and 12,260, respectively) by transient transfection using the calcium phosphate co-precipitation method. We used 8.4 µg lentiviral vectors, 8.4 µg psPAX2 and 4.2 µg pMD2.G per 6-cm tissue culture dish. After 24 h of transfection, eEPCs were infected with filtrated lentiviral particles and 4 µg/ml Polybrene (Sigma). Infected cells were selected for 4 days with 2 µg/ml puromycin (Merck). CM was prepared as above.

Western blotting

Western blotting was performed as described (Cominelli et al., 2014). Briefly, cells were lysed in RIPA buffer and protein concentration was measured by bicinchoninic acid assay. Then, 30 µg cell protein extract or a corresponding volume of 10×CM was loaded with 2% SDS and 10 mM DTT sample buffer on 5% polyacrylamide gels (4% stacking gel), resolved by SDS-PAGE and transferred onto PVDF membrane (Polyscreen PVDF transfer membrane, PerkinElmer). Blots were blocked and incubated overnight at 4°C with primary antibody: rabbit anti-laminin α1 (1/1000; Sasaki et al., 2002); mouse anti-α1 (1/1000; Thermo Scientific; Table S2). After washes and incubation with the appropriate secondary antibody, immunoreactive bands were visualized by chemiluminescence (Supersignal West Femto maximum sensitivity substrate mixed with Supersignal West Pico chemiluminescent substrate, Thermo Scientific) and acquired using a 4000MM Image Station (Eastman Kodak). For silver staining, gels were stained using the Silverquest Staining Kit (Invitrogen).

Electron microscopy

Control and Smad1/5KO thyroid lobes were rinsed with 155 mM NaCl, fixed by 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 for 30 min at 4°C, and washed three times with 50 mM Tris-HCl pH 6.0 for 10 min. Cells were post-fixed with 1% (v/v) OsO4/2% KFe(CN)6 solution (reduced osmium) and stained en bloc with 1% uranyl acetate for 2 h and pelleted in 2% agar. Pellets were dehydrated in a graded ethanol series and embedded in Spurr resin. Ultrathin (70 nm nominal) sections were obtained.
with a Reichert ultramicrotome, collected on copper/rhodium 400 mesh Mantaform grids (EMS, UK) and contrasted with 3% uranyl acetate followed by lead citrate, each for 10 min. Grids were washed with water, dried, and analyzed in an FEI CM12 electron microscope operating at 80 kV.

Statistics
Data are presented as mean±s.e.m. Quantification was performed on multiple sections from at least three thyroid lobes for each genotype. Mann–Whitney U-test and one-way ANOVA followed by Bonferroni’s multiple comparison tests were performed using Prism software (GraphPad).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
M.V. and A.-S.D. designed and performed the experiments and wrote the manuscript. M.L., M.B., P.L., J.B., L.U., P.V.D.S. and S.R. performed experiments. S.c.d.S.L. provided the BRE mouse line. T.S., G.B., P.H., F.P.L., S.R., A.Z. and P.J.C. contributed intellectually and edited the manuscript. C.E.P. conceived the project, contributed experimental and intellectual advice and edited the manuscript.

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References


