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Smooth muscle: a stiff sculptor of epithelial shapes

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Smooth muscle is increasingly recognized as a key mechanical sculptor of epithelia during embryonic development. Smooth muscle is a mesenchymal tissue that surrounds the epithelia of organs including the gut, blood vessels, lungs, bladder, ureter, uterus, oviduct and epididymis. Smooth muscle is stiffer than its adjacent epithelium and often serves its morphogenetic function by physically constraining the growth of a proliferating epithelial layer. This constraint leads to mechanical instabilities and epithelial morphogenesis through buckling. Smooth muscle stiffness alone, without smooth muscle cell shortening, seems to be sufficient to drive epithelial morphogenesis. Fully understanding the development of organs that use smooth muscle stiffness as a driver of morphogenesis requires investigating how smooth muscle develops, a key aspect of which is distinguishing smooth muscle-like tissues from one another *in vivo* and in culture. This necessitates a comprehensive appreciation of the genetic, anatomical and functional markers that are used to distinguish the different subtypes of smooth muscle (for example, vascular versus visceral) from similar cell types (including myofibroblasts and myoepithelial cells). Here, we review how smooth muscle acts as a mechanical driver of morphogenesis and discuss ways of identifying smooth muscle, which is critical for understanding these morphogenetic events.

This article is part of the Theo Murphy meeting issue 'Mechanics of Development'.

1. Introduction

Smooth muscle is now recognized as a key contributor to the morphogenesis of branched and folded organs. As a mesenchymal tissue, smooth muscle can participate in reciprocal signalling with the epithelium to generate complex patterns of epithelial folds [1]. Smooth muscle can also apply mechanical forces to physically sculpt epithelia. By directly inducing epithelial folding through mechanical instabilities, physical mechanisms using smooth muscle can effect changes over longer length scales than morphogen diffusion and may require simpler regulatory schemes than biochemical mechanisms that rely on complex morphogen gradients. For example, starting from a uniform, unbranched tube, a single buckling event can create branches throughout an epithelium simply by homogeneously tuning the proliferation rate of the entire epithelium relative to that of the adjacent smooth muscle [2]. Creating this architecture through diffusion, however, would require multiple short-range foci of high morphogen concentration to specify the location of each branch through local changes in proliferation or cell shape [3].

The physical contributions of smooth muscle to tissue morphogenesis have been investigated in the small intestine [4], oesophagus [5], lung [6], oviduct [7] and epididymis [8] of developing organisms, through both experiments and modelling. When smooth muscle is knocked out in the developing ureter [9] or blood vessels [10], their epithelial or endothelial tubes become dilated or

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develop aneurysms, suggesting that smooth muscle also supports the physical structure of these organs as they develop. Although smooth muscle is primarily known for its contractile properties, smooth muscle cell shortening does not seem to be critical for its morphogenetic effects. Instead, computational models have suggested that epithelial folding can be driven by a stiff, static smooth muscle layer surrounding the growing epithelium [4,5,7,11]. Therefore, the feature of smooth muscle most critical for directing epithelial morphogenesis appears to be its high mechanical stiffness relative to the neighbouring epithelium.

In smooth muscle-induced physical mechanisms of morphogenesis, there is a complex interplay of physical and biochemical mechanisms, and understanding this interplay is key to building a complete picture of the morphogenetic events. Further investigating these phenomena will require a combination of *in vivo* and cell and tissue culture assays to understand how the differentiation of smooth muscle is controlled. Since there are several smooth muscle-like tissues [12,13], interpretation of these assays depends heavily on understanding how to distinguish among these similar tissues.

Here, we review the definition of smooth muscle and its key properties for physically directing epithelial morphogenesis. We then discuss organs in which smooth muscle is a critical mechanical sculptor of tissue architecture. Finally, because understanding the control of smooth muscle differentiation in each of these tissues is the next step for developing a complete model of how they are constructed, we review markers and phenotypes used to distinguish amongst various smooth muscle-like cells.

2. Definition and properties of smooth muscle

Smooth muscle is an involuntary contractile tissue found in almost every part of the body, from the intestines to blood vessels and hair follicles. Although it has been known that the intestines can move independently since at least the time of Galen in the second century [14], it was not until the sixteenth century that Fallopius provided one of the earliest descriptions of the muscle fibres responsible for this motion by grossly dissecting them from the stomach [15]. Similar tissues were still being discovered by gross dissection in the early nineteenth century when Reisseisen found muscle fibres in the lung [16,17]. There remained considerable debate, however, about whether arteries contained a muscle sheath [18] until the work of Henle and von Kölliker [19–21]. Henle, a histologist, recognized that the arteries were surrounded by a layer of muscle tissue that lacked the striations found in voluntary muscles [20]. Von Kölliker isolated the spindle-shaped muscle cells within the vasculature and those that formed the involuntary muscles of other organs [19], thus demonstrating that these tissues comprise smooth muscle.

Today, smooth muscle is identified as tissue containing α-smooth muscle actin (α-SMA)-expressing cells [22]. More specific markers are used to distinguish between different types of smooth muscle (vascular versus visceral) or similar cell types (such as myofibroblasts and myoepithelial cells). These markers include specific proteins, contractile function [23] and histological features such as appearance and anatomical location. The primary physiological function of smooth muscle cells is involuntary contraction. Smooth muscle tissues typically contain several cells, contraction of which induces movements ranging from peristalsis of digested food along the gut to piloerection of body hair. Although all cell types are capable of some form of actomyosin-based contractility, smooth muscle cells have a specialized contractile apparatus (consisting of smooth muscle-specific actin and myosin isoforms) that is usually calcium-dependent but that is activated by a mechanism different from that for skeletal muscle: calcium influx leads to a signalling cascade that results in the phosphorylation of myosin light chain, which activates cross-bridge cycling of myosin heads on actin [24].

When smooth muscle cells contract, they can both shorten and increase their stiffnesses [25,26] through actomyosin contractility and through rearrangements of the cytoskeleton, for example, by repolymerizing actin [27]. The stiffness of a smooth muscle cell arises from its contractile network of actin fibres and myosin, along with its intermediate filament cytoskeleton of vimentin and desmin [28–31] (figure 1). Atomic force microscopy (AFM) studies on relaxed, dissected embryonic chicken gut sections have found that the muscular layer has a lower stiffness than the epithelium [32,33], but after contractile stimulation, individual smooth muscle cells can become at least twice as stiff as epithelial cells, even when muscle cells are prevented from physically shortening [34,35]. These measurements likely underestimate the tissue-scale stiffness of smooth muscle because individual cells, tissue sections, and short length-scale AFM measurements cannot account for the cellular and matrix organization of native smooth muscle tissue. Unlike striated muscle, stretching smooth muscle tissues do not decrease their ability to generate force or enhance their stiffness. Because the contractile apparatus is made up of a fibre meshwork, myosin heads can interact with nearby actin filaments no matter how stretched the cell is; this structure contrasts with that of skeletal muscle where the highly regular configurations of actin and myosin cannot interact if the cell is stretched or compressed too far [24]. The highly developed cytoskeletal network and their ability to maintain stiffness over a variety of cell lengths make smooth muscle cells especially well poised for directing epithelial morphogenesis.

Another potentially useful property of smooth muscle for driving morphogenesis is its geometric anisotropy: muscle fibres tend to orient in one direction within a tissue. Smooth muscle tissues shorten primarily in the direction of their fibres, but whether they are, in fact, stiffer in that direction remains unclear. Studies of the stiffness of individual smooth muscle cells and whole smooth muscle tissues in different organisms have yielded conflicting results, with some reporting that tissues are stiffer along the long axis of the cells [36] and others reporting higher stiffness along the short axis [37]; significant changes in stiffness anisotropy occur after contraction [38,39]. This is even less well studied in embryonic smooth muscle. Many smooth muscle-containing organs have multiple smooth muscle layers, often with one circumferentially and one longitudinally oriented layer, which may be particularly important in tissues where these different layers differentiate at different times.

As more research has focused on the physical mechanisms of morphogenesis, it has become clear that these properties make smooth muscle an effective tool for changing the shapes of embryonic epithelial tissues. This is most evident in the gastrointestinal tract, the lung, and both the male and female reproductive systems.

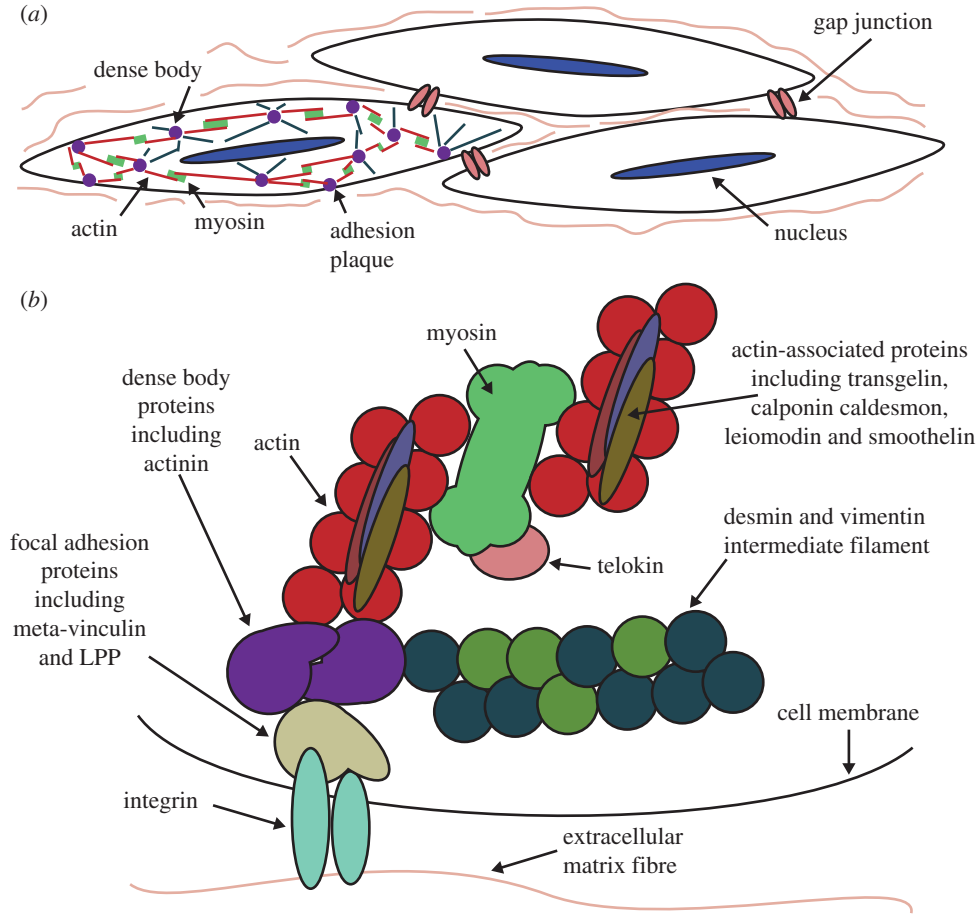

Figure 1. (*a*) The structure of a smooth muscle cell. The contractile apparatus of smooth muscle consists of a meshwork of actin and myosin fibres that undergo cross-bridge cycling upon activation of the cell. This meshwork is interconnected with the cytoskeletal network including intermediate filaments, cell–matrix and cell–cell adhesions. (*b*) Detailed view of these interconnections, focusing on structural proteins that are frequently used as markers of smooth muscle.

3. Gastrointestinal system

(a) Intestine

In the adult chicken gut, the mucosa and its epithelium are folded into thousands of projections known as villi that increase the intestinal absorptive surface area [4]. The mucosa is, in turn, surrounded by two major layers of smooth muscle that contribute to digestive mobility: an inner circular layer and an outer longitudinal layer [40–42]. There is also a thin layer of smooth muscle known as the muscularis mucosa, which sits at the boundary of the mucosa and the submucosa and has less clear functional importance in the adult (figure 2*a*,*b*) [22]. This anatomy is similar to that of the mammalian gut, but some observers classify the chicken circular smooth muscle into distinct inner and outer circular layers. Additionally, the chicken submucosal connective tissue is much thinner than the mammalian submucosa (and is absent in some parts of the intestine), and the chicken small intestine does not have the submucosal folds (plicae circulares) present in the human gut [22,40,41].

In the white leghorn chicken embryo, the three muscle layers form during a few days of development beginning with the circular layer at embryonic day 8 (E8), followed by the longitudinal layer at E13, and finally the muscularis mucosa at E16. The appearance of each of these layers is accompanied by changes in the morphology of the epithelium and, after the final muscle layer appears, the formation of villi (figure 2*c*) [4] (other timeframes for these events have been reported for different chicken breeds [43]). After each differentiation event, inhibiting the differentiation of smooth muscle abolishes the change in epithelial morphology. It is possible that this is a purely mechanical interaction because dissecting the smooth muscle layer off the gut epithelium removes the folds, and encasing the epithelium in a silk tube can rescue the folded epithelial phenotype. Consistent with this conclusion, finite element-based computational modelling has shown that differences in stiffness between the muscle layers and proliferating epithelium are sufficient to drive epithelial folding into the structures observed following smooth muscle differentiation [4,44,45]. This process appears to depend mostly on the static stiffness of the smooth muscle rather than its shortening because reducing gut motility with sodium nitroprusside [46] does not abrogate epithelial folding. Consistently, the computational model generates epithelial folds when the smooth muscle is treated as a stiff, static tissue. However, although the mechanical model eventually recapitulates the observed geometry, it falls into several local energy minima on its path to the final configuration. *In vivo*, the system may need to be perturbed to avoid these local minima, and this function could potentially be filled by smooth muscle contraction [4]. Notably, the model is executed in phases that apply a circumferential constraint and then a longitudinal constraint on the epithelium to correspond to each muscle layer as it differentiates. Smooth muscle is critical for the morphogenesis of the gut in chicken and may contribute in other organisms [4]. In mice, however, it seems to play, at most, a secondary role to cellular

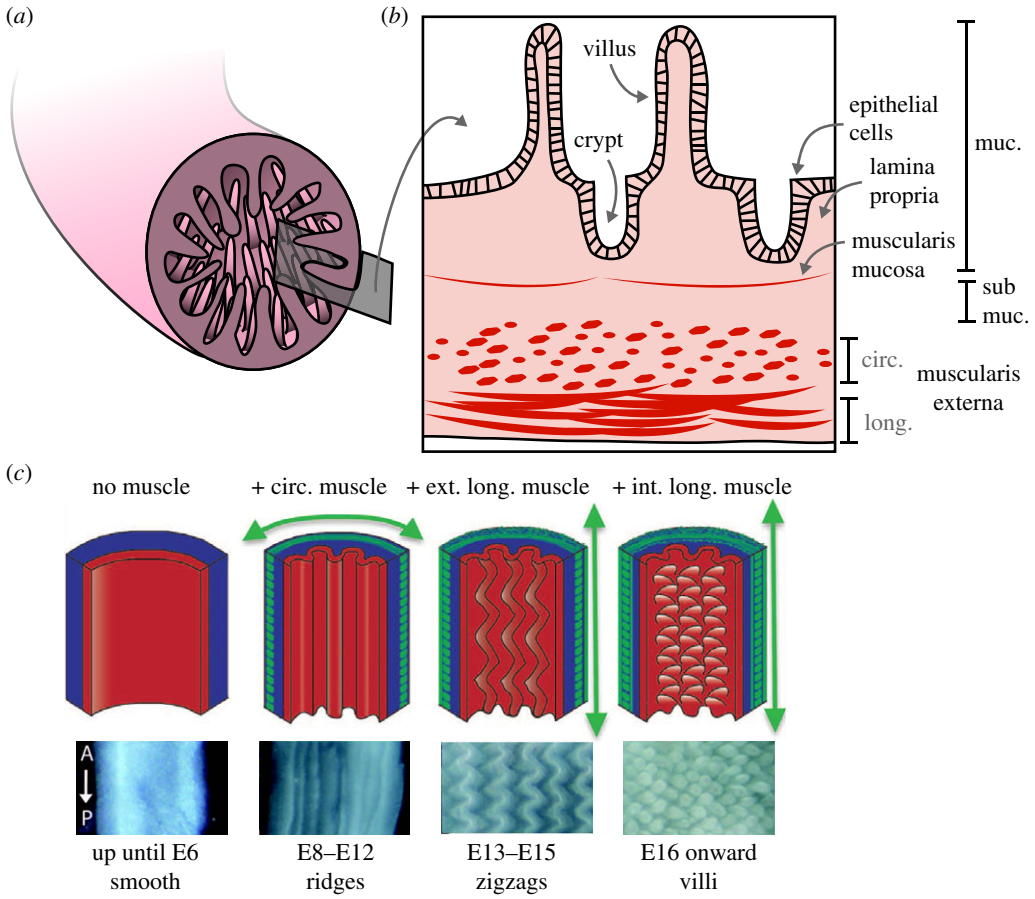

Figure 2. (*a*) Three-dimensional structure of villi in the chick intestine. Grey plane indicates the location of the slice shown in (*b*). (*b*) Cross-section of the mature chick gut tube showing the mucosa and villi and their relationship to the smooth muscle layers in the gut. The thin muscularis mucosa separates the lamina propria (connective tissue just below the mucosal epithelium) from the submucosal connective tissue. The muscularis externa (also known as the muscularis propria) is the main muscle sheath and consists of an inner layer of fibres oriented circumferentially around the gut tube and an outer layer of fibres oriented along the length of the tube. (*c*) Progression of the structure of the gut epithelium as each of these muscle layers differentiates from the mesenchyme. Top: schematics, bottom: corresponding whole-mount microscopy images. Panel (*c*) is modified from [4]. Reprinted with permission from AAAS. muc, mucosa; sub muc., submucosa; circ, circular; long., longitudinal; ext., external; int., internal.

migration under the influence of a pattern of morphogens [47,48]. A recent review [49] of the comparative development of intestinal villi in chicken versus mouse gives an illuminating case study of how two similar tissues might be constructed using different mechanisms (buckling morphogenesis versus reaction–diffusion-controlled cell migration).

(b) Oesophagus

The structure of the mammalian oesophagus is similar to that of the small intestine, except that the oesophagus has a much thicker epithelial layer and no villi [22]. In the proximal oesophagus, the muscle lining comprises skeletal muscle that transitions to smooth muscle distally. The precise location of this transition along the oesophagus varies by species [50]. The mucosa in the human, bovine and porcine oesophagus is folded longitudinally, in a geometric pattern similar to the first step of gut folding in the chick [5,22,51]. Longitudinal folding is a common structural motif throughout the body and several studies have investigated folding in the oesophagus as a simple, accessible model for longitudinal folding of biological tubes in general [11,52].

The oesophagus retains its longitudinal folds postmortem and the folds disappear after dissecting the mucosa and submucosa from the surrounding musculature [51]. Taken together, these data suggest that mucosal folding is driven by passive constriction of the soft mucosa and submucosa by the surrounding muscle. Mechanical modelling has been able to recapitulate the oesophageal folding pattern by considering the mucosa and submucosa as hyperelastic solids, with an external pressure applied by the smooth muscle [51]. During development, however, the mucosa is likely proliferating and growing. Other models have more closely approximated the developmental case by modelling the mucosa as a tube growing radially and circumferentially that is bound by a static muscle layer, without constriction from smooth muscle. In this case, the number of folds can be computationally tuned by modifying the combined thickness of the mucosal and submucosal layers in the model, and the model correctly predicts the number of folds in the bovine oesophagus when the mucosal and submucosal thicknesses of the model are matched to their thicknesses in the bovine oesophagus [5]. These investigations suggest that, during development, constraining the proliferating mucosal epithelium inside a stiff, static layer of smooth muscle is sufficient to form longitudinal folds in the oesophageal mucosa.

4. Respiratory system

In the adult mouse lung, smooth muscle wraps the airway epithelium, progressing from a thick layer around the bronchi

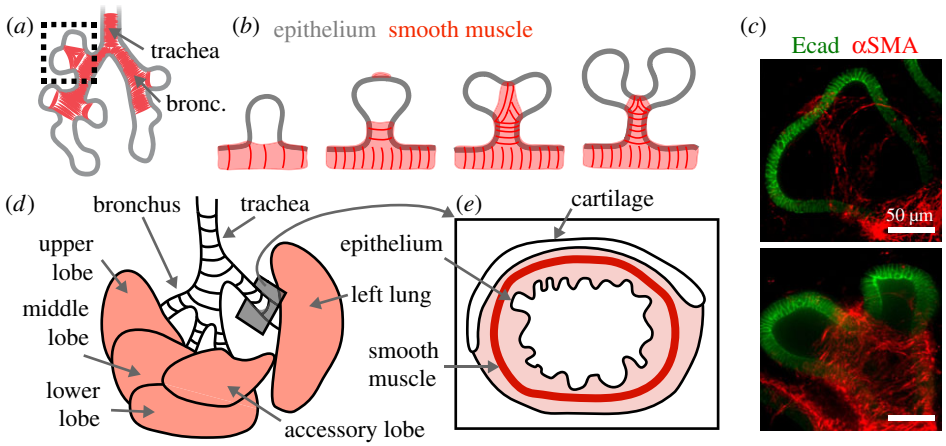

Figure 3. (*a*) Schematic of an E12.5 mouse lung. Dashed box indicates one instance of epithelial bifurcation, detailed in (*b*). (*b*) Bifurcation of an epithelial bud as smooth muscle differentiates and mechanically drives the process. (*c*) Microscopy images of a bifurcating bud of lung epithelium corresponding to the schematics in (*b*). Ecad: green; α-SMA: red. (*d*) Structure of the mature mouse lung. Grey plane indicates the location of the cross-section shown in (*e*). (*e*) Cross-section of the adult bronchus showing the airway epithelium and its relation to the smooth muscle and connective tissue layers. Panels (*b*) and (*c*) modified from [6]. bronc., bronchus; Ecad, E-cadherin; α-SMA, α-smooth muscle actin.

to thinner, sparser bundles around the small conducting airways [53] (figure 3*d*,*e*). During development, the trachea buds off the gut tube at E9.5 and two bronchial buds soon branch off of the developing trachea [54]. At this point, each side of the embryonic lung comprises an epithelial tube surrounded by mesenchyme. The epithelium forms the architecture of the mature lung through repeated use of a few branching motifs: domain branching, where one epithelial tube buds off of the side of another, and terminal bifurcation, where the end of an epithelial tube splits into two new tubes [55]. Cells located within the mesenchyme adjacent to the leading edge of the growing branch differentiate into smooth muscle and begin to wrap around the tube as it extends [56,57]. Therefore, smooth muscle appears to differentiate along the branch, always lagging somewhat behind the tip of the epithelium (figure 3*a*). At branches that are about to bifurcate, the smooth muscle cells differentiate ahead of the epithelial tip, causing it to flatten out and eventually form a cleft [6]. Additional smooth muscle cells differentiate and form a continuous layer that envelops the cleft site and wraps around the original branch; as this happens, the epithelium fully bifurcates into two daughter branches (figure 3*b*,*c*). Inhibiting smooth muscle differentiation either pharmacologically or genetically causes the proliferating epithelium to buckle into complex, uncontrolled geometries. Enhancing smooth muscle differentiation abrogates epithelial bifurcation as smooth muscle wraps fully around the bud [6]. Interactions between the epithelium and smooth muscle are likely largely mechanical because dissecting the smooth muscle away from the bifurcating epithelium causes the epithelium to revert to an unbifurcated geometry. Inhibiting smooth muscle contraction with a calcium channel blocker also inhibits smooth muscle differentiation in this system, making it difficult to isolate the effects of smooth muscle contraction from tissue stiffness [6]. This fundamental role for airway smooth muscle in shaping the lung is particularly revealing because this tissue has no clear physiological function in the adult [58,59].

Some evidence suggests that smooth muscle can also affect domain branching. When buds form in a region of the epithelium that is already wrapped in smooth muscle, the smooth muscle appears to reorganize to allow a new bud to form in that location [56]. It remains unclear how smooth muscle in this system affects branching; future work is needed to determine if mechanics plays a role in this process.

Finally, the adult respiratory mucosa has longitudinal folds similar to the adult oesophagus (figure 3*e*). Studies of oesophageal folding through smooth muscle-induced instabilities have proposed that this mechanism may also extend to airway folding [5,60].

5. Reproductive system

(a) Oviduct

The mouse oviduct (also known as the fallopian tube or uterine tube) serves as a passageway from the ovary to the uterus (figure 4*a*). It has deeper and more complex longitudinal folds than the oesophagus [53], but experimental and computational evidence suggests that the longitudinal folds in both organs arise through a common mechanism: mechanical instabilities in a proliferating epithelium constrained by a static sheath of smooth muscle [7]. The structure of the oviduct is relatively simple: a folded epithelium and a thin layer of mucosal connective tissue are directly surrounded by a circular and a longitudinal smooth muscle layer (figure 4*b*) [22]. These folds appear to be important for proper transport of eggs and sperm along the oviduct because the ciliated cells of the oviduct are primarily found at fold peaks [61] and the loss of folds is associated with human infertility [62].

The adult mouse oviduct changes its shape and folding pattern during ovulation. Since the smooth muscle layer does not fold along with the epithelium, the circumferential length of the epithelium is greater than that of the smooth muscle, suggesting that the epithelial folds form through a buckling mechanism induced by the surrounding smooth muscle [7]. Knocking out the planar cell polarity gene *Celsr1* leads to disordered alignment of epithelial cells, disordered folding in the longitudinal direction and the development of some circumferential folds. In these mutants, the longitudinal length of the epithelium is greater than that of the surrounding smooth muscle, while in wild-type mice the length of the epithelium and smooth muscle are nearly

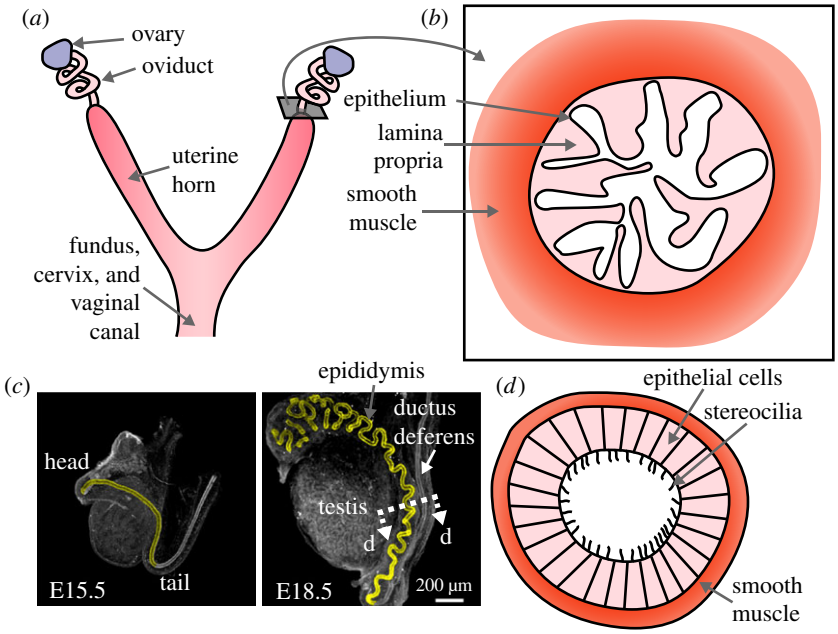

Figure 4. (*a*) Structure of the mature mouse uterus showing the location of the oviducts (also known as the fallopian tubes or uterine tubes) leading from the ovaries to the uterus. Grey plane indicates the location of the cross-section shown in (*b*). (*b*) Cross-section of the mature oviduct showing the relationship between the epithelium and smooth muscle. The undulating pattern of the epithelium develops through a process very similar to the first step of villus morphogenesis in the chicken. (*c*) The tortuous pattern of the mouse epididymis is created by compression of a growing epithelial tube by the surrounding stiff mesenchymal tissue between E15.5 and E18.5. Dashed arrows indicate the location of the cross-section shown in (*d*). (*d*) Cross-section of the mouse epididymis showing the relationship of the smooth muscle tissue to the epithelium. Panel (*c*) is modified from [8] under the CC BY licence (http://creativecommons.org/licenses/by/3.0/).

equal. These results raised the possibility that the morphology of the oviduct could arise from smooth muscle constraining the epithelium in the circumferential and longitudinal directions. A mathematical model treating the thin epithelium as a grid of springs within a rigid cylinder of smooth muscle was able to recapitulate the epithelial folding pattern observed in wild-type oviducts. This model could also recapitulate the folding pattern in *Celsr1*-knockout mice by increasing the longitudinal length of the epithelium relative to its constraining cylinder. Adding support for this mechanical viewpoint, laser ablation experiments confirmed that the wild-type epithelium retracts more than the mutant in the longitudinal direction, indicating that the mutant is under lower longitudinal tension, as would be expected if the complex folds of the knockout were due to longitudinal compression of the epithelium [7]. This simple model can further recapitulate diverse and complex folding patterns by modifying the circumference and length of the epithelium relative to its constraining cylinder [7]. The mechanical investigations in this system again consider the smooth muscle as a stiff boundary constraint, making it unclear if contraction of the muscle is necessary for oviduct folding.

(b) Epididymis

The epididymis is a muscular tube that transmits sperm from the efferent ductules of the testis to the ductus deferens (or vas deferens) [63]. It is composed of a layer of pseudostratified epithelial cells closely surrounded by smooth muscle [22]. Unlike the gut and uterine tubes, there are no folds in the lumen of the epididymis (figure 4*d*); however, the tube itself is highly convoluted, forming several loops and waves (figure 4*c*) [8]. Still, this morphology seems to also result from smooth muscle-mediated restriction of the growing epithelium. In the mouse, the epididymis starts as a straight tube at E15.5. The epididymis begins to fold into its tortuous pattern, starting at the head, until the whole tube is twisted by E18.5. During this same time period, smooth muscle differentiates along the length of the epididymis [8]. Dissecting the mesenchyme away from parts of the tortuous epithelium causes epithelial uncoiling. Furthermore, removing the end of the mesenchyme causes the epithelial tube to extend out, like a spring released from a constraining box. These observations suggest that epididymal epithelial coiling may result from the mesenchyme mechanically constraining the growing epithelium longitudinally. This mechanism is further supported by the observation that there is no circumferentially biased pattern of proliferation that could account for the coiling, but the head of the epididymis, which is the first part to fold, has a higher proliferation rate than the body or tail [8].

This folding pattern is recapitulated by a mechanical model representing the epididymal epithelium as a chain of spheres embedded in a viscous mesenchyme, with growth modelled by randomly inserting additional spheres into the chain. The temporal pattern of folding, with the head region folding first, is mimicked by this model when growth is restricted to the head region [8].

In the embryo, inhibiting smooth muscle differentiation increases the wavelength of the epididymal folds. The model produces a corresponding increase in wavelength when the viscosity of the mesenchyme is decreased, suggesting that smooth muscle plays a mechanical role in epididymal folding by increasing the effective viscosity of the mesenchyme [8]. Although smooth muscle was not explicitly modelled as a static tissue in this system, it again seems to play a passive, stiffness-increasing role in epididymal development, rather than actively contracting on the epithelium.

6. Urinary and vascular systems

Even in systems where the mechanical morphogenetic effects of smooth muscle have not been directly investigated, some experimental evidence hints at a possible mechanical role for smooth muscle in determining epithelial architecture.

(a) Urinary system

The ureter is an epithelial tube surrounded by thick smooth muscle layers that propel urine from the kidneys to the bladder [22,24]. Several studies investigating the pathways upstream of smooth muscle differentiation in the ureter have generated knockouts of ureteric smooth muscle in mice [9,64]. Some of these models do not develop smooth muscle throughout the whole ureter [9], while others lack smooth muscle only proximally [64]. These lead, in turn, to dilation of the whole ureter [9] or just the proximal ureter [64]. This dilation is thought to result from a lack of peristalsis in the absence of smooth muscle. In light of the structural role of smooth muscle in other tissues, however, it is possible that smooth muscle physically supports the ureter, keeping it from dilating in wild-type mice.

(b) Vascular system

The vascular endothelium is lined by a coat of smooth muscle that regulates blood pressure and blood flow. Larger blood vessels such as the aorta have elastic and smooth muscle tissue in their walls, while medium-sized arteries have more prominent smooth muscle coats. Veins have thin layers of smooth muscle in their walls, which function to modulate their fluid capacitance [22,24]. In capillaries, pericytes sparsely wrap the thin endothelia and appear to serve similar functions to the smooth muscle cells of larger blood vessels [65].

Vascular smooth muscle cells have many different developmental origins. For example, the smooth muscle cells of the abdominal aorta arise directly from the local mesenchyme, while the smooth muscle cells which line the aortic arch are derived from neural crest cells that migrate from the folding neural tube [66]. In adults, smooth muscle plays a mechanical role in shaping blood vessels: smooth muscle constriction causes the vascular endothelium to fold similarly to the oesophagus [67], and smooth muscle contraction can support blood vessels against buckling into twisted patterns [68], the opposite of its role in the developing epididymis.

Several studies of vascular development have generated transgenic mice with either decreased differentiation [69] or recruitment [70] of vascular smooth muscle. In these models, the blood vessels develop tortuously, and aneurysms and haemorrhages appear diffusely in the embryo, suggesting that smooth muscle mechanically sculpts the structure of blood vessels by supporting the walls against dilation under the pressure of the blood [69,70].

Similarly, in models of disordered pericyte development, aneurysms develop in the microcirculation throughout the body, suggesting that the capillary analogue of smooth muscle plays a similar mechanical role [10,71].

7. The next step: studying smooth muscle differentiation

A further study of smooth muscle in these systems requires being able to identify a specific smooth muscle tissue of interest in histological sections, whole-mount organs, or cultured explants. These studies are a critical next step for discovering how epithelial architecture is directed downstream of instructions by smooth muscle, or for discovering additional organs that are mechanically shaped by smooth muscle. For example, smooth muscle in the lung differentiates in a precise pattern to drive epithelial bifurcation, and understanding how that pattern is created is a key missing piece of our understanding of lung morphogenesis. To support these investigations, we here review markers and assays that are commonly used to distinguish among smooth muscle-related tissues both *in vivo* and in culture.

On histology, smooth muscle typically appears as a sheet of interconnected, elongated cells within connective tissue. It is called 'smooth' muscle because it lacks the characteristic striations observed in histological sections of skeletal and cardiac muscle [22]. This is because the contractile apparatus of smooth muscle consists of a meshwork of contracting actin and myosin filaments instead of the highly regular, regimented arrangement of skeletal and cardiac muscle sarcomeres [24]. As the locations of smooth muscle tissues in the adult are well known, observing a sheet of cells that express α-SMA in an anatomical area known to contain smooth muscle (such as around the airway epithelium or in the wall of the bladder) is often sufficient to classify it as smooth muscle. However, several more specific markers have been identified for distinguishing smooth muscle cells from similar cell types, especially during embryonic development when the final tissue architecture has not yet been established, or in differentiation or cell sorting experiments. Most smooth muscle markers are proteins associated with the cytoskeleton or contractile machinery, including α-SMA [23], smooth muscle myosin heavy chain (smMHC) [72], transgelin [73], calponin [74] or meta-vinculin [75], while others are signalling molecules or transcription factors, for example, serum response factor (SRF) [76] (tables 1 and 2).

There are, however, other cell types that express classic smooth muscle markers, including myofibroblasts and myoepithelial cells [108,109]. Myofibroblasts reside in the stroma of many organs and synthesize extracellular matrix, similar to fibroblasts. When epithelia are damaged, myofibroblasts proliferate and synthesize matrix, but unlike fibroblasts they also use their contractile properties to pull on the damaged site and shrink its area [110]. Myofibroblasts also play roles in organ development such as laying down matrix to guide the development of pulmonary alveoli [111]. Myofibroblasts are typically found as single cells within stromal tissue while smooth muscle usually forms larger tissues of many cells in the stroma. Myoepithelial cells are found as part of the epithelium in secretory organs such as the salivary gland and mammary gland [22,108]. There, they reside between the secretory epithelial layer and the basement membrane (which separates the epithelium from the underlying stroma) and express both epithelial and smooth muscle markers [108]. By contracting, they help squeeze out the exocrine products of the gland [22]. These cells are distinct from smooth muscle because they reside above the epithelial basement membrane and express E-cadherin and other epithelial markers [108].

There is significant heterogeneity even among smooth muscle: that which lines internal organs such as the gut (visceral smooth muscle) differs from the smooth muscle which lines blood vessels (vascular smooth muscle). There are

Table 1. Cellular functions of common smooth muscle markers. SMA, smooth muscle actin; smMHC, smooth muscle myosin heavy chain; APEG-1, aortic preferentially expressed gene-1; LPP, lipoma-preferred partner; SRF, serum response factor; MRTF, myocardin-related transcription factor; HEYL, hairy/enhancer-of-split related with YRPW motif-like protein; NG-2, neural/glial antigen 2; Foxf1, forkhead box F1; GATA-5, GATA family zinc finger transcription factor-5.

marker	function	refs
α-SMA	cytoskeletal and contractile fibre protein	[77,78]
γ-SMA	cytoskeletal and contractile fibre protein	[77–79]
smMHC	contractile protein interfacing with smooth muscle actin	[72]
transgelin	filamentous actin-binding and -stabilizing protein (SM22α)	[73]
calponin	calcium-binding protein for activation of contraction	[74]
caldesmon	links calponin activation to smooth muscle actomyosin contraction	[80]
actinin	cross-linking protein for actin and other cytoskeletal filaments and adhesions. Present at dense bodies and adhesion plaques (intercellular junctions); isoforms 2 and 3 are specific to muscle, but not isoforms 1 and 4	[81,82]
APEG-1	unclear function, but has homology to other smooth muscle proteins like smooth muscle myosin light chain kinase	[83]
LPP	associated with focal adhesions and is implicated in cellular motility	[84,85]
SRF	master transcription factor for smooth muscle genetic programme	[12,76]
myocardin	SRF coactivator	[86]
MRTF-A	SRF coactivator	[76,86]
MRTF-B	SRF coactivator	[76,86]
leiomodin-1	nucleates actin polymerization in both smooth and striated muscle cells; isoform 1 is specific to smooth muscle	[30,87,88]
desmin	intermediate filament in all muscle cell types	[75,89]
vimentin	intermediate filament in all mesenchymal cells (including muscle cells)	[75,89,90]
meta-vinculin	actin-binding protein that may modulate vinculin binding to actin at cell–cell and cell–matrix junctions	[91]
telokin	protein with the same sequence as the C-terminal domain of myosin light chain kinase; binds to myosin and contributes to smooth muscle relaxation	[92]
smoothelin A	smooth muscle cytoskeletal protein that binds filamentous actin; short isoform found in visceral smooth muscle	[93]
smoothelin B	long smoothelin isoform found in vascular smooth muscle	[93]
HEYL	Notch responsive transcription factor	[94]
noggin	binds to and inhibits bone morphogenic protein (BMP) family members	[95]
NG-2	transmembrane proteoglycan in vascular smooth muscle	[96]
Foxf1	forkhead box family transcription factor necessary for proper development of the lung and the foregut	[97,98]
GATA-5	transcription factor found in muscle during cardiovascular and airway development	[99]

further phenotypic differences even among vascular smooth muscle from different vascular beds including the coronary arteries, aorta, and pulmonary artery [100,112]. In addition, the smooth muscle that surrounds the epithelium of the larger airways of the lung (airway smooth muscle, ASM) is considered separately from visceral smooth muscle because it is heavily studied in the pathophysiology of asthma [113]. Given this heterogeneity, several markers have been identified to distinguish among these cell types (table 2). For example, smoothelin is expressed exclusively in smooth muscle cells (and not in myofibroblasts) and has two isoforms, A and B [23]. Smoothelin A is the shorter isoform and is found in visceral smooth muscle and ASM, while smoothelin B (the longer isoform) is found in vascular smooth muscle [114–117]. Additionally, hairy/enhancer-of-split related with YRPW motif-like protein (HEYL) is expressed in vascular smooth muscle while noggin is expressed in ASM. Reporters for these genes have been used to distinguish among these tissues during lung development [105]. These markers are used alongside cellular morphology, tissue structure and contractile function to identify cells that have differentiated into smooth muscle during embryonic development.

8. Conclusion

In each of the systems described above, it appears that the stiffness of the smooth muscle tissue rather than cell shortening is sufficient to drive the morphogenesis of an underlying proliferating epithelium or endothelium. This property makes each developing system strikingly simple: once the location of smooth muscle differentiation is specified, the system falls into the correct morphology simply by assuming its lowest energy state.

Many of these organs have similar morphologies, for example the lumina of the gut, airway and oviduct are all folded longitudinally at one point in time. This seems to be because the circumferential length of the epithelium is longer than the stiff surrounding smooth muscle layer. However, smooth muscle shapes the overall structure of the lung and epididymis into quite different structures. In the airway, this is achieved through precise control over where smooth muscle differentiates. In the epididymis, smooth muscle has the unique effect of causing the epithelial tube to coil. Future work is needed to determine if this unique behaviour is due to the thickness of the epididymal epithelial cells relative to

Table 2. Smooth muscle markers and their expression patterns in smooth muscle cells and myofibroblasts. Blanks indicate that definitive information of the expression of the protein in that cell type has not been found. SM, smooth muscle. For other abbreviations, see the caption for table 1.

marker	vascular SM	visceral SM	airway SM	myofibroblast	refs
α-SMA	more	less	intermediate	yes	[23,77,100]
γ-SMA	less	more	intermediate	yes	[23,77,80]
smMHC	SM-1, SM-2, SM-A	SM-1, SM-2, SM-B	SM-1, SM-2, SM-B	low	[12,101,102]
transgelin	yes	yes	yes	yes	[6,100]
calponin	yes	yes	yes	yes	[12]
caldesmon	yes	yes	yes	yes	[12]
actinin	yes	yes	yes	yes	[82,103]
APEG-1	more	less	less		[12]
LPP	more	less	less		[12]
SRF	yes	yes	yes	yes	[12]
myocardin	yes	yes	yes	yes	[86]
MRTF-A	yes	yes	yes	yes	[104]
MRTF-B	yes	yes	yes	yes	[104]
leiomodin-1	yes	yes	yes	yes	[12,30]
desmin	yes	yes	yes	yes	[75,89]
vimentin	yes	yes	yes	yes	[75,89,90]
meta-vinculin	yes	yes	yes	yes	[12]
telokin	less	more			[12,23]
smoothelin A	no	yes	yes	no	[23,100]
smoothelin B	yes	no	no	no	[23,100]
HEYL	during development		no		[94,105]
noggin	no		during development		[105,106]
NG-2	yes		no		[56,96,107]
Foxf1	no		yes		[12]
GATA-5	no		yes		[12]

the lumen diameter or to a looser connection between the epithelium and smooth muscle in this system. Fully understanding the mechanics of these developing systems will require more detailed measurements of the *in vivo* stiffness and stiffness anisotropy of intact, embryonic smooth muscle tissues.

The next step towards fully understanding the genetic control of these systems is investigating how differentiation of smooth muscle is controlled. This is particularly important in the lung, where smooth muscle differentiates in a highly stereotyped asymmetric pattern. Future studies will need to focus on carefully understanding what controls smooth muscle differentiation, and *in vitro* assays will need to clearly define whether a progenitor cell has differentiated into smooth muscle based on its expression of specific markers and its functional phenotype.

Smooth muscle is a widespread tool for directing epithelial morphogenesis. Therefore, understanding these systems may produce broadly applicable insights into both normal development and developmental disorders of the lung, gut, reproductive system, or any other epithelial tissue ensheathed by smooth muscle. Furthermore, investigating how nature uses smooth muscle as a morphogenetic tool may help us understand how to repurpose it for engineering organs in clinical applications. For example, artificial tissues may be built with a predefined pattern of smooth muscle and cultured in a homogeneous field of growth factors, which we would predict would cause the epithelium to proliferate uniformly but buckle into the desired morphology under the spatial and mechanical constraint of the smooth muscle. Inducing mechanical instabilities in epithelia is an effective and efficient mechanism for forming tissues. The above discoveries illustrate that smooth muscle is a frequent driver of mechanical instabilities, a powerful sculptor of organs during embryonic development, and a promising area for future study in both developmental biology and tissue engineering.

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