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REVIEW

Physical organogenesis of the gut

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ABSTRACT

The gut has been a central subject of organogenesis since Caspar Friedrich Wolff's seminal 1769 work 'De Formatione Intestinorum'. Today, we are moving from a purely genetic understanding of cell specification to a model in which genetics codes for layers of physical–mechanical and electrical properties that drive organogenesis such that organ function and morphogenesis are deeply intertwined. This Review provides an up-to-date survey of the extrinsic and intrinsic mechanical forces acting on the embryonic vertebrate gut during development and of their role in all aspects of intestinal morphogenesis: enteric nervous system formation, epithelium structuring, muscle orientation and differentiation, anisotropic growth and the development of myogenic and neurogenic motility. I outline numerous implications of this biomechanical perspective in the etiology and treatment of pathologies, such as short bowel syndrome, dysmotility, interstitial cells of Cajal-related disorders and Hirschsprung disease.

KEY WORDS: Biomechanics, Intestine, Embryo, Mouse, Chicken, Human

Introduction

The gut is one of the most ancient organs: hydra (Furness and Stebbing, 2018; Mueller, 1950; Shimizu et al., 2004), sponges (Renard et al., 2013), protostomes and all vertebrates present an interior, epithelium-lined, axially symmetric, contractile tube that is topologically continuous with their outer skin and permits nutrient absorption essential for life. The amniote gastrointestinal tract (Fig. 1) comprises cells from all three embryonic germ layers: endoderm, mesoderm and ectoderm. The endoderm contributes to the inner epithelial layer, which includes proliferative crypt cells, transit-amplifying cells, enterocytes, goblet cells, enteroendocrine cells, tuft cells, microfold cells and cup cells (Hewes et al., 2020).

The mesoderm contributes by far the most to the intestine in terms of cell number. As we penetrate radially inside the intestine from outside, mesodermal derivatives constitute: (1) the serosa, the outer lining of the gut, which is composed of a single layer of mesothelial cells that rest on a thin layer of connective tissue; (2) the longitudinal smooth muscle (LSM) at the outer periphery, which longitudinally compresses the intestine; (3) the interstitial cells of Cajal (ICCs), which generate periodic, rhythmic electric depolarization signals to excite the smooth muscle [these anastomosing cells form a fine mesh just above the circular smooth muscle (CSM), but also in other locations; Sanders et al., 2014]; (4) the CSM, which can constrict the gut and locally reduce its diameter (together, the LSM-ICC-CSM layers are known as the 'muscularis propria' or 'muscularis externa'); (5) the highly vascularized submucosa, which transports nutrients from the gut to the organism; (6) the muscularis mucosa, which is a thinner, scaled-down version of the muscularis externa (i.e. outer longitudinal and inner circular layer), contractions of which trigger movements of the epithelial villi but not the whole gut wall; and (7) the lamina propria, which extends smooth muscle fibers into the villi and supports their vascularization and innervation.

The ectoderm in the gut derives from the enteric neural crest cells (ENCCs), which form the enteric nervous system (ENS). The ENS comprises neurons, glial cells and progenitor ENCCs. The ENS is divided into two plexuses (Fig. 1): the outer myenteric plexus is sandwiched between the LSM and CSM layers of the muscularis propria, and the inner submucosal plexus is located between the submucosa and the mucosa. The myenteric plexus, the 'mechanical plexus', coordinates muscle activity and bowel wall movement in response to bolus pressure and inputs from the submucosal plexus. The submucosal plexus, the 'biochemical plexus', is connected to the myenteric plexus, but also extends dendrites into individual villi that relay information on the biochemical composition of the contents of the gut lumen (e.g. the bolus, which includes water, nutrients, toxins and potential pathogens). Morphologically, each plexus is a mesh composed of ganglia (the nodes of the mesh) and interganglionic fibers (the inter-nodes). Neuronal cell bodies occupy the central part of each ganglion and extend their axons to form the interganglionic fibers. Glial cells are present both inside the ganglion, at its outer periphery, and to a lesser extent in the interganglionic fibers (Rollo et al., 2015). The exact function of glial cells remains a matter of research, but they are believed to play a mechanical, protective role for neurons and axons, and display neurotransmitter, immune and homeostatic functions (Bassotti et al., 2007).

Remarkably, this complex assembly of the three germ layers that constitute the gut wall is just 3-5 mm thick in humans, i.e. ten times smaller than the diameter of the gut and 100 times smaller than the rudimentary description above. Whereas the general radial organization of the gut is conserved across species, its lengthwise arrangement varies considerably between animals (Bloch, 1904). In early development, the presumptive amniote gut is flat tissue composed of an endoderm layer contacting the yolk, and an overlying mesoderm layer. This bilayer invaginates at the rostral end of the embryo and folds rostro-caudally towards the umbilicus (Fig. 1). The mechanism of hindgut closure is different; the whole ventral part of the endoderm slides over the dorsal ectoderm, rather than left-right closure (Fig. 1). This morphogenetic movement of the whole tissue is driven by a gradient of tensile contractile forces and fibroblast growth factor (FGF) (Durel and Nerurkar, 2020; Nerurkar et al., 2019). The rostral and caudal ends then fuse at Meckel's diverticulum (the presumptive umbilicus), forming a closed tube of endoderm ensheathed in mesenchymal tissue from the mesoderm. Next, the gut tube is invaded by ectodermal ENCCs that migrate rostro-caudally in the mesenchyme and gives rise to the ENS.

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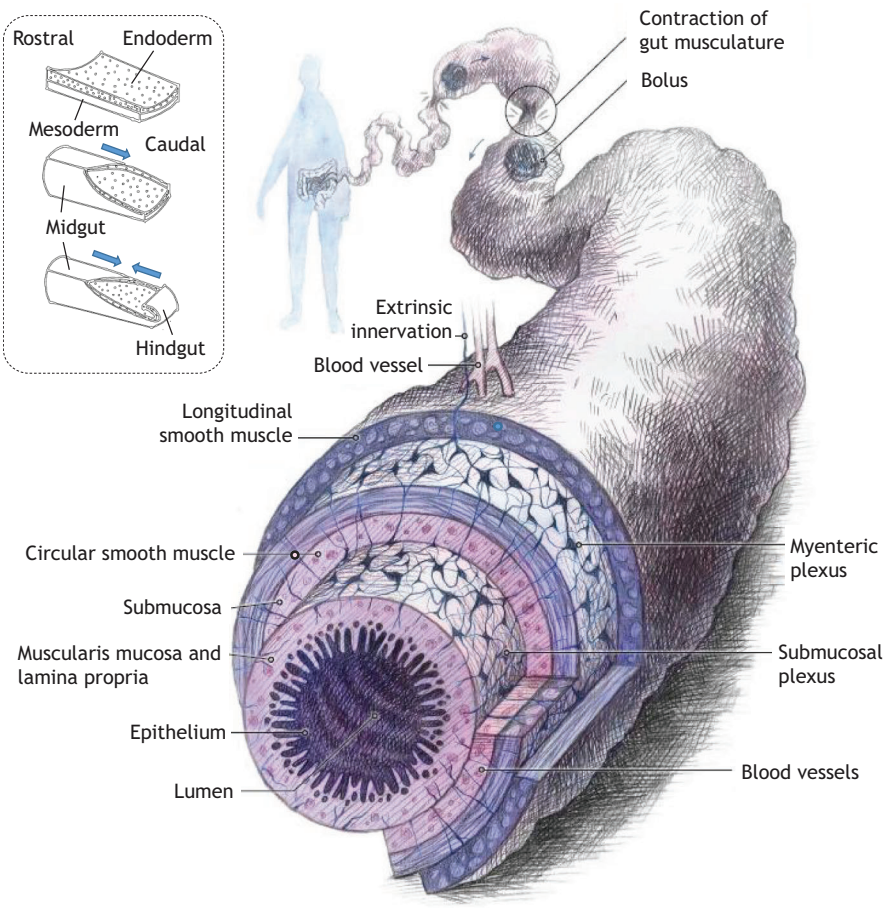

Fig. 1. Gut anatomy. Dimensions are not to scale: gut wall thickness is only 3-5 mm in humans, with a diameter of 30-50 mm, i.e. the tube is essentially hollow when not filled with bolus. In humans, the muscularis, submucosa and mucosa including villi are typically 1.5 mm, 1 mm and 1.5 mm thick, respectively. As we travel down the GI tract from the mouth, we find the esophagus, the stomach, the small intestine, the ceca appendix, the colon and the rectum. The small intestine is further subdivided into duodenum, jejunum and ileum, the frontier between the latter two being Meckel's diverticulum – the point where the mother's placental (omphalomesenteric) artery attaches to the small intestine, connecting it to the vasculature of the developing embryo. Inset: During early gut tube formation in the chicken embryo, the endodermal layer in engulfed by right-left folding of the midgut (propagating caudally), and by dorsoventral folding at the hindgut (propagating rostrally) (Nerurkar et al., 2019). Courtesy of 'Pour la Science' (Chevalier, 2018); illustration by Marie Marty.

This Review provides a biomechanical perspective on the dynamic sequence of events that gives rise to the lower gastrointestinal (GI) tract, from the early 'closed-tube' to the late fetal stage. Because most studies in biomechanics of gut morphogenesis have been performed in chicken and mice, I focus on these two species and discuss, where relevant, how these findings apply to human gut ontogenesis. I first describe the mechanical properties of the gut and the macroscopic forces acting on the gut at its various stages of development. Before turning to the effects of these forces on morphogenesis, I summarize experimental techniques in embryonic gut organ culture, which are central to research in the field. Finally, I survey recent advances in understanding the development of gut motility and conclude by suggesting some promising lines of research in the field of gut ontogenesis.

Mechanical properties of the forming gut

The gut can be considered as a visco-elastic material with a bulk elastic modulus E (also called stiffness, elasticity or Young's modulus). The elastic modulus relates the amount of deformation (strain) that a tissue will experience to the amount of force (stress) that is acting upon it. The simplest experiments to evaluate E involve using glass cantilevers (Chevalier et al., 2016a) or magnetic beads (Savin et al., 2011) to apply a controlled longitudinal stretch force and measure the resulting deformation optically. These measurements have revealed that the gut becomes stiffer as it develops, with E increasing from 400 Pa (Chevalier et al., 2016b) at embryonic day (E) 5 to 1000-5000 Pa (Chevalier et al., 2016b; Khalipina et al., 2019; Savin et al., 2011) at E10. This stiffening is due to a large extent to increased extracellular matrix (ECM), in particular collagen, which accounts for 50% of the elasticity (Chevalier et al., 2016b). Intriguingly, E decreases from E10 to E16 by about 25% (Savin et al., 2011). This may be correlated to changes in mechanical properties due to LSM differentiation, which happens precisely during this period. The mesentery is stiffer than the gut by a factor of 1000 (Savin et al., 2011). E is dependent on the buffer composition and temperature at which the measurement is performed, because calcium-dependent intercellular adhesions (cadherins) and smooth muscle contractility contribute to the elastic properties of the organ; this likely explains the considerable scatter of elasticity values measured by different investigators.

A refined biomechanical model takes into account the fact that the elasticity depends on the direction along which the force is exerted. In the case of the gut, one distinguishes the longitudinal elastic modulus E_z, which is measured by stretch experiments, from the circumferential modulus E_θ, measured by inflating the lumen with a controlled pressure (Khalipina et al., 2019). E_θ of the chicken embryonic intestine between E7 and E10 is three to four times higher than its E_z, consistent with the circumferential orientation of ECM components, such as collagen I, and the smooth muscle layer.

Tissue-level elasticity measurements can be performed by surgically separating the gut layers (Shyer et al., 2013), and at the cellular level by atomic force microscopy (Chevalier et al., 2016b). These approaches have revealed that the epithelium is approximately ten times stiffer than the mesenchyme in the chicken; in the mouse, this difference is only a factor of 1.5.

Forces acting on the embryonic gut

Here, I list the forces acting on a macroscopic level on the developing gastrointestinal tract and compare their magnitude at different stages of development.

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Circumferential internal stress

For axisymmetric tissues, such as the gut, circumferential internal residual stress can be revealed by performing a longitudinal cut of a ring preparation (Fung and Liu, 1989). The ring springs open forming a measurable opening angle. The total perimeter of the preparation decreases and the thickness of the wall increases, further indicating that circumferential stress was relaxed by cutting. Most studies (Huycke et al., 2019; Yang et al., 2021) only present the magnitude of the opening angle as a proxy for the amplitude of the residual stress. An accessible, more quantitative interpretation (Rachev and Greenwald, 2003) yields the circumferential strain distribution in the organ as a function of the radius, showing that the internal layers (mucosa) are under compression, whereas the outer layers (tunica muscularis) are under circumferential tension.

Circumferential internal stress is present immediately after the gut closes to form a tube at E4.5 in chickens (Huycke et al., 2019) and E12.5 in mice (Walton et al., 2016), i.e. before mesenchyme differentiation. Faster proliferation of the stiff epithelium compared with the relatively slow proliferation of the soft mesenchyme has been proposed as a major cause of circumferential residual stress at these early stages (Huycke et al., 2019). Reducing epithelial proliferation rates by genetic or chemical means reduces the opening angles from 80° to 25-60° (Yang et al., 2021). As an opening angle is still present after these manipulations, early embryonic gut residual stress has other components; the acto-myosin machinery that is implicated in circumferential closure of the gut tube at E3-E5 (in chickens) also contributes to residual stress, as well as ECM fibers: collagen (Chevalier et al., 2016b), elastin (Gao et al., 2009) or fibronectin (Nagy et al., 2015). After it differentiates, CSM also contributes to circumferential internal stress: smooth muscle cell ablation (Yang et al., 2021) during development decreases the opening angle of mouse embryonic gut rings from 120° (wild type) to 80° (smooth muscle ablated).

Hernia and mesentery tension

In humans, chickens and mice, the midgut takes on a hairpin shape (Fig. 2) from Carnegie stage 14 (~5 weeks) (Ueda et al., 2016), E5 (Chevalier et al., 2018) and E10 (Cervantes et al., 2009), respectively. This hairpin has a characteristic leftward tilt (Fig. 2B), which is induced by Pitx2- and Isl1-regulated left-right asymmetrical dorsal mesentery cell density (Kurpios et al., 2008). The omphalomesenteric artery (also called the vitelline artery; Fig. 2B), which supplies the whole embryo with blood from the placenta/yolk sac, forms a loop around the apex of the midgut (the tip of the hairpin), thus forming a 'chain link' floating in the amniotic fluid filling the umbilical cord (Fig. 2B,C).

This chain link is under tension: the omphalomesenteric artery pulls on the gut with a tension of ~20 µN in the chicken at stage E8 (Chevalier et al., 2018). As a result, the gut is the only visceral organ in the embryo that grows under macroscopic tension, all other viscera being compressed inside the body cavity. This tension is sufficient to cause the hairpin geometry (i.e. the first 'gut loop') and the herniation of the midgut outside of the embryo's body, which has been previously attributed to a lack of space in the visceral cavity due to rapid growth of the liver (Kaufman and Bard, 1999; Timor-Tritsch et al., 1989), or of the intestine itself (Grzymkowski et al., 2020). However, these would result in a gut dangling in the amniotic fluid, rather than being in a tensile state. Interestingly, gut rotation causes increased umbilical tension (think of how a braid of hair pulls on the scalp) and, conversely, umbilical tension accelerates the rotation of the gut. In the chicken, herniation continues until E19, when the looping midgut is suddenly pulled back inside the body of the embryo. Defective looping morphogenesis of the chick gut induced by viral modification of the bone morphogenetic protein (BMP) pathway induces a failure of resorption (Nerurkar et al., 2017; Sun et al., 2007) and a gastroschisis-like phenotype. In humans and mice, the simple hairpin geometry lasts for ~10 or ~2.5 days, respectively. The gut then starts rotating and looping, with hernia resorption occurring at 10-11 weeks of gestation or E15.5 in mice (Kaufman and Bard, 1999; Ueda et al., 2016). Although the kinematics of gut rotation and resorption are well described (Grzymkowski et al., 2020), we do not currently know what molecular and biomechanical mechanisms induce the rapid re-entry of the gut inside the body cavity.

The omphalomesenteric artery branches out in the mesentery, a thin membrane of collagenous ECM and fibroblasts that supports the vasculature and innervation going to and out of the intestine. This membrane is attached to the gut along a line (the mesenteric border) and wraps itself around the whole serosa. The higher elongation (growth) rate of the intestine compared with the mesentery is driven by BMP (Nerurkar et al., 2017) and results in the buildup of tension inside the mesentery. This tension gives rise to the stereotypical looping pattern of the intestine by a buckling mechanism (Nerurkar et al., 2017; Savin et al., 2011).

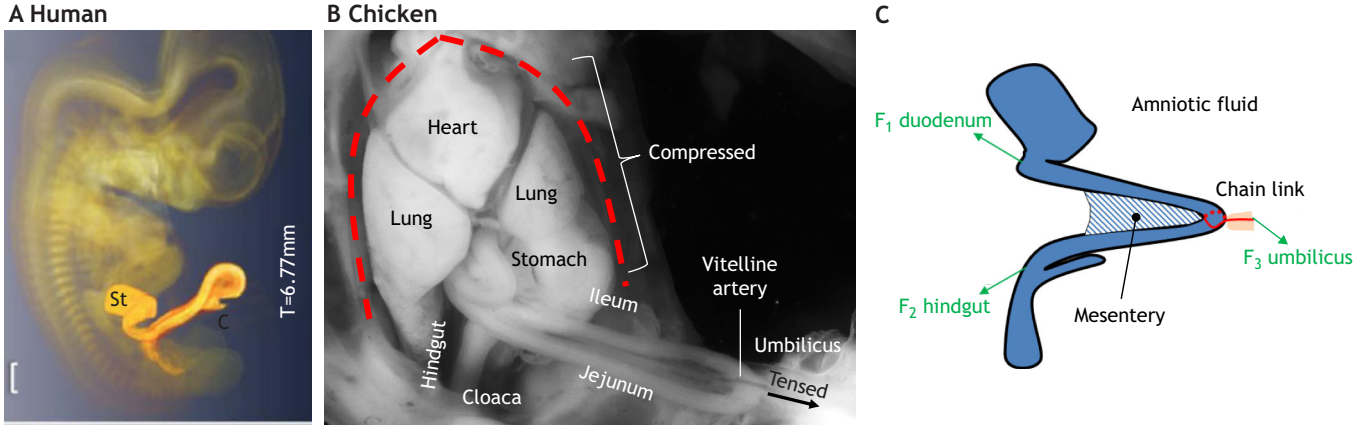

Fig. 2. Physiological intestinal hernia. (A-C) Physiological intestinal hernia in (A) a human embryo at Carnegie stage 16 (reproduced with permission from Ueda et al., 2016), (B) a chicken embryo at E8 (reproduced with permission from Chevalier et al., 2018) and (C) a simplified scheme showing the chain link between the omphalomesenteric artery (red) and the gut apex (blue), and the balance of forces (F_1-F_3). C, cecum; St, stomach.

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Smooth muscle-generated forces: tone and contractions

The characteristic circumferentially oriented α-SMA (ACTA2) fibers of contractile smooth muscle start appearing in the chicken, mouse and human at E5 (Chevalier et al., 2017), E13.5 (McHugh, 1995) and <7 weeks (Beaulieu et al., 1993; Romanska et al., 1996), respectively. Differentiation proceeds rostro-caudally in the mouse lower digestive tract, with colonic CSM differentiating at E14.5 (Chevalier et al., 2021a; Roberts et al., 2010), whereas in chicken CSM appears at both the rostral and caudal ends of the GI tract simultaneously. Sonic hedgehog (Shh) and BMP signaling are important for smooth muscle differentiation (Huycke et al., 2019). Manipulations of both result in strong alterations of CSM genesis, from complete disappearance (Mao et al., 2010) to hypertrophy (Huycke et al., 2019) and misalignment (Yang et al., 2021). In chicken and mouse, CSM differentiates in the cecum 2-3 days later. This likely results from the particular nature of the cecum in terms of morphogen secretion: high levels of Bmp4, an inhibitor of smooth muscle differentiation (Barbara et al., 2005), are detected specifically in the chicken cecum at E6 (Nielsen et al., 2001).

Immediately upon differentiation, CSM generates both tone (static force) and transient, spontaneous contractions. Contractions occur spontaneously in the embryonic GI tract, and propagate as constant-velocity waves along the gut (Fig. 3A), in either the rostro-caudal or caudo-rostral direction, with a speed in the range 20-500 µm/s. The frequency of contractions increases with developmental time from 0.5 to 3 cycles/minute between E5 and E15 in chickens and between E13.5 and E18.5 in mice (Chevalier et al., 2017, 2019; Roberts et al., 2010). The only direct evidence of those contractions in human development come from magnetic resonance images of fixed embryos (Fig. 3B) (Ueda et al., 2016). With recent developments in ultrasonic imaging, it may soon be possible to see contractions in living human embryos (Sicard et al., 2022). Although the first waves are barely visible, their amplitude gradually increases as smooth muscle differentiates, aligns and matures. CSM contractions circumferentially compress the GI tract, but also result in cross-directional longitudinal tensile and radial compressive strain of the same order of magnitude as the circumferential strain (Chevalier et al., 2021b; Khalipina et al., 2019) (Fig. 3C). This behavior, called the Poisson effect, results from the relative incompressibility of the tissue and from the very small size of the lumen at embryonic stages (i.e. when the gut is locally compressed by the CSM, it 'oozes out' through its extremities, just as when squeezing a tube of toothpaste).

The isometric force generated per area of embryonic CSM is ∼10 mN/mm², an order of magnitude lower than reported values for adult smooth muscle (70-200 mN/mm²) (Gabella, 1976). This discrepancy is due to the immature state of the embryonic smooth muscle, which has not yet reached optimal actin-myosin levels, alignment and inter-cellular junction development. Notwithstanding, CSM generates pressure of ~1000Pa in the embryonic gut lumen (i.e. of the same order of magnitude as the elastic modulus of the embryonic gut), explaining why CSM contractions considerably deform the whole intestine.

Embryonic gut culture methods

I review here the important parameters that should be controlled when performing *ex vivo* embryonic gut culture (Box 1) and how these have been implemented in the most creative ways (Hewes et al., 2020; Yissachar et al., 2017) by various investigators in the last 20 years. I hope this brief survey helps others perform such experiments, improve upon these protocols and imagine new ones.

Oxygenation

Vascularization is ruptured during dissection, stopping blood oxygen supply. The only method that allows for re-establishment of a functional, blood-perfused vasculature is explant growth on the chorio-allantoic membrane (CAM; Fig. 4A). This method is the only one that can be used for long-term culture (>3 days). It has been applied to embryonic hindgut and yielded spectacular, tenfold growth after 7 days (Nagy and Goldstein, 2006). It can, however, be difficult to control drug concentration and forces applied during CAM growth. Imaging, although possible, is hindered by the poor contrast offered by the yolk, by spontaneous movements of the whole embryo, and by the sheer size of the egg. All other 'Petri-dish' methods must re-establish physiological oxygen (O_2) levels. One governing principle is to culture the explant as close as possible

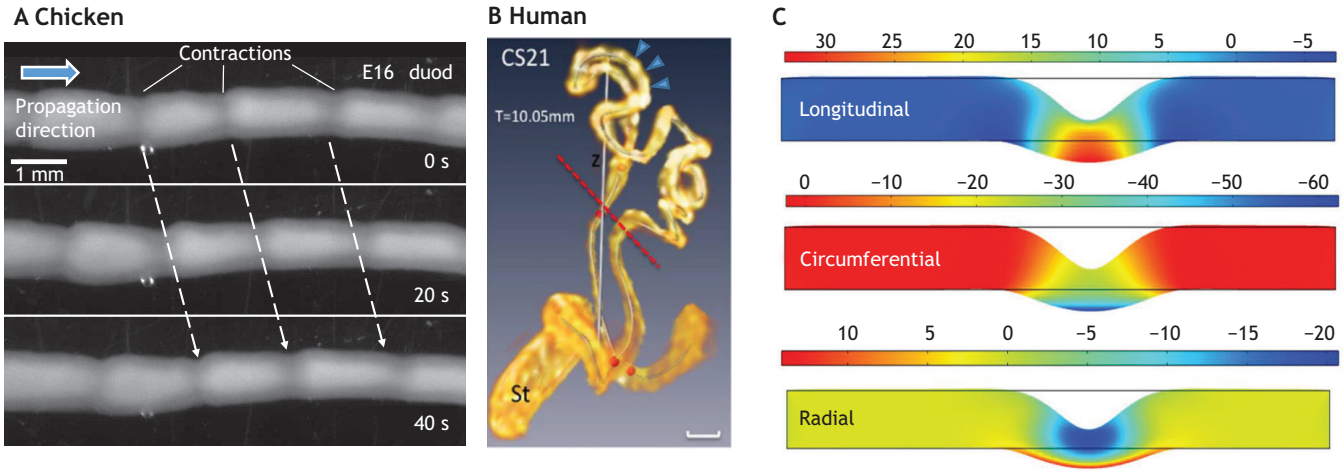

Fig. 3. Spontaneous smooth muscle contraction waves. (A) Frames from a time-lapse movie showing the left-to-right propagation of CSM contractile waves along an E16 chicken duodenum. Dashed arrows follow three contractile waves as they propagate from left to right (reproduced with permission from Chevalier, 2018). (B) Undulations of the gut tract by CSM contractions (blue arrowheads) are distinctly visible on this magnetic resonance imaging image of a human embryo at Carnegie stage 21 (reproduced with permission from Ueda et al., 2016). (C) Finite element simulation of strains for an isotropic tube with 15% cross-section lumen, Poisson ratio ν=0.35, under the effect of a CSM contraction, showing circumferential compression, and longitudinal tension and radial compression cross-strains of similar magnitude (reproduced with permission from Chevalier et al., 2021b).

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Box 1. *Ex vivo* culture versus *in vivo* approaches

Ex vivo culture is a powerful method that complements *in vivo* gene-editing approaches. *Ex vivo* cultures allow for direct, live imaging, which is generally not feasible for the gut *in vivo*. They permit direct pharmacological intervention, which is more difficult *in vivo* due to drug absorption by other organs and membranes (e.g. the placenta). Organ culture allows for direct control of physical (mechanical, electrical), chemical (pH, oxygen, metabolism, pharmacology) and biochemical growth conditions. Two to three day cultures recapitulate key developmental events, such as smooth muscle differentiation (Huycke et al., 2019), ENCC migration (Young et al., 2001), enteric ganglia reorientation (Chevalier et al., 2021a), epithelial structuring (Shyer et al., 2013) and elongational growth (Khalipina et al., 2019), although growth rates are ∼×5 lower than *in vivo* (Chevalier et al., 2018). Longer culture periods can result in straying from physiological development and should be examined carefully. Growth can be quantified based on morphometric changes of the organ pre- and post-culture. Our group has developed software (Khalipina et al., 2019) to precisely extract the volume, length, diameter of tubular organs with non-uniform diameters. These macroscopic measures can further be correlated to cell proliferation and apoptosis using standard techniques (e.g. BrdU, etc.). Cell viability can be quantified post-culture by dissociation and Trypan blue or acridine orange–propidium iodide staining.

to the surface of the medium, because the flux of O_2 is inversely proportional to the distance between the organ (O_2 sink) and the atmosphere-saturated medium surface. Practically, this has been implemented by depositing organ explants on porous membranes (Fig. 4B), such as plastic filter wells (Huycke et al., 2019), Anodisc aluminium oxide membranes (Chevalier et al., 2017), Millipore filter discs (Duh et al., 2000) or metal grids. In these setups, the explant is wetted only by a micrometer-thin meniscus of medium. One drawback is that the meniscus exerts considerable pressure on the organ and flattens it (Fig. 4B), which can strongly bias morphometric assessment of growth. Culturing the organ in a hanging drop (Fig. 4C) circumvents this issue (Kurahashi et al., 2008). Another complementary approach, replaces the atmosphere with carbogen (95% O_2, 5% CO_2), which increases oxygen saturation levels at 37°C from 5.1 ml/l to 24.1 ml/l. These values are comparable to total blood O_2 (dissolved O_2 as well as O_2 carried by hemoglobin) concentrations in the chicken embryo: 13.5 ml/l in the vitelline artery at E6 (Baumann and Meuer, 1992), 89 ml/l in the chorioallantoic vein at E10 (Tazawa, 1980). Carbogen culture in a shallow layer of medium (∼1 mm) has yielded an average of 60% volume and dry mass increase of E10 chicken guts after 2 days (Khalipina et al., 2019). The highest oxygenation rates can be achieved by bubbling carbogen directly in the culture medium (Fig. 4D) (Khalipina et al., 2019) or by perfusing carbogen-saturated medium, as has traditionally been done by physiologists for over a century. Perfusion induces fluid convection, reducing the effective thickness of the layer in which oxygen concentration gradients develop around the explant.

The effect of tissue O_2 concentration on cell differentiation is an active area of research. Multipotent cells are associated with an anaerobic, glycolytic metabolism, whereas differentiated cells undergo oxidative phosphorylation (respiration) (Agathocleous et al., 2012). In addition to promoting growth, O_2 likely also influences differentiation – in what direction, and how, are exciting questions for future research.

Free-floating, held or embedded?

Before E6 (chicken) or E11 (mouse), the gut is physiologically locked in place by its attachments to the back of the embryo via the stomach, the mesentery and the nerve of Remak, which runs along the hindgut in chicken. Conversely, from E6, the midgut develops in a free-floating condition inside the amniotic fluid-filled umbilical cord. Interestingly, most investigators have found it necessary to

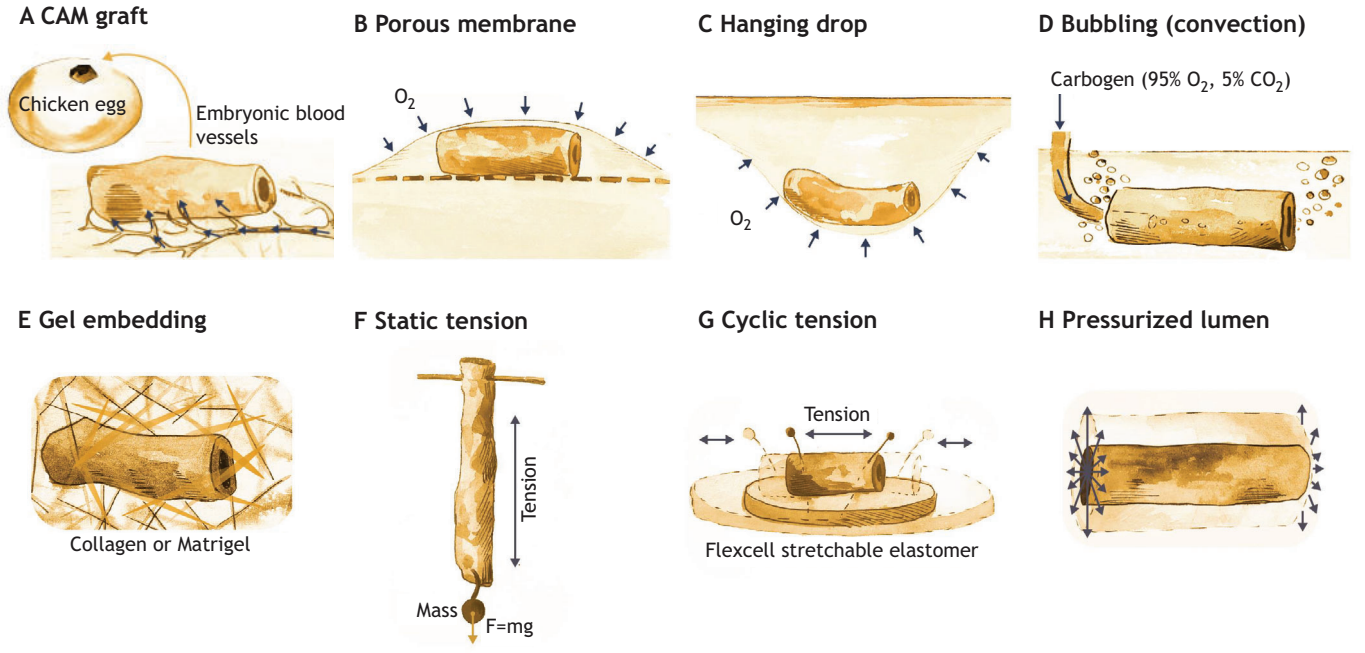

Fig. 4. *Ex vivo* embryonic gut culture methods. (A) Chorio-allantoic membrane graft. (B-D) Methods to improve explant oxygenation. (E) 3D scaffold. (F-H) Methods of applying forces. For mouse embryonic gut, growth has been reported using DMEM with 10% fetal bovine serum (FBS) (Kurahashi et al., 2008), Jackson BGJb with 0.1% ascorbic acid (Duh et al., 2000) or the latter with additional Matrigel (Yang et al., 2021). For chicken embryonic gut, DMEM or DMEM:F12 both allow for growth and proliferation (Chevalier et al., 2018; Khalipina et al., 2019) without supplemented FBS or chicken embryonic extract. ENCC migration studies are often performed in DMEM for chicken (Nagy and Goldstein, 2006), or DMEM:F12 supplemented with FBS in mice (Young et al., 2014). Illustrations by Olga Markova.

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reproduce the physiological attachment of the gut when studying early embryogenesis events, such as ENCC migration (E4-E8 in chicken, E10.5-E14.5 in mouse): some employ catenary culture, whereby the gut is attached at both ends to a filter paper (Hearn et al., 1999; Young et al., 2001), whereas others embed early guts in a collagen gel (Nagy and Goldstein, 2006; Yang et al., 2021) (Fig. 4E). Attachment is necessary to preserve the straight, tubular geometry of the early gut. All cells exert considerable force on the surrounding tissue when they migrate (Yamada and Sixt, 2019), and in particular ENCCs (Chevalier et al., 2016b). I surmise that if the tissue is not attached, the traction force exerted by ENCCs may lead to tissue deformation instead of driving their migration down the gut.

Embryonic mouse gut has recently been successfully cultured (70% length increase in 2 days) in 3D Matrigel (Fig. 4E) and growth is greater in Matrigel-rich gels (Yang et al., 2021). Initially, gel embedding seems to act as a mechanical barrier to growth and intestinal elongation, which can probably be explained by the very low elastic modulus of Matrigel, even at higher concentrations (10-200 Pa; see https://www.corning.com/catalog/cls/documents/application-notes/CLS-AC-AN-449.pdf), so that stiffness does not impede growth. Furthermore, the increased concentration of growth factors [e.g. TGFβ, epidermal growth factor (EGF) and other proteins] in the richer Matrigel likely favor explant growth.

Applying forces

A simple way of applying constant tensile longitudinal stress consists of culturing guts vertically, in tubes, and attaching weight at its lower extremity (Fig. 4F) (Chevalier et al., 2018). More recently, embryonic guts have been pinned to polydimethylsiloxane (PDMS) membranes and cyclically stretched using the StrexCell system (Fig. 4G) (Huycke et al., 2019). Pins must be repositioned regularly in this system because tension is lost as the intestine grows or elongates visco-elastically. Another interesting way of applying periodic stress involves using a magnet-bead system (Savin et al., 2011) and applying time-dependent magnetic fields. Microfluidic chambers have been developed to control pressure in growing embryonic lung (Nelson et al., 2017), but this technique has not yet been applied to the developing *ex vivo* embryonic gut (Fig. 4H). Pressure can also be generated in a controlled way by osmosis (e.g. by introducing polyethylene glycol in the gut lumen) (Sueyoshi et al., 2013). The only known way of relaxing internal residual stress is by performing a longitudinal cut along one gut wall. A flat sheet of tissue is thereby obtained, which curls up in culture, but this can be prevented by using the porous membrane culture method or by pinning the tissue sheet. The flat preparation is amenable to imaging. In our experience, however, this microsurgical operation could only be applied to chicken gut. It seems too invasive in early mouse embryonic gut, where we observed significant degradation of the ENS and smooth muscle following this procedure.

Effect of forces on gut morphogenesis

We now have the theoretical and experimental tools to understand how these forces affect gut morphogenesis. Here, I consider the effects of mechanical forces on smooth muscle differentiation and orientation.

Orientation of smooth muscle layers

The internal residual circumferential stress drives the circumferential orientation of the differentiating smooth muscle in chicken (Fig. 5) (Huycke et al., 2019). When this tensile stress is relaxed by culturing the gut as a flat explant, the smooth muscle layer fails to orient properly. Reducing the residual stress by diminishing epithelial proliferation results in a misoriented smooth muscle layer. Interestingly, when a 20% static longitudinal strain is applied to gut segments during culture, the smooth muscle layer orients longitudinally instead of circularly, demonstrating the sensitivity of smooth muscle orientation to external mechanical stress.

Do forces also affect the later-differentiating LSM layer? Omphalomesenteric artery tension appears to be dispensable for LSM orientation, because LSM aligns properly in the absence of any external longitudinal strain. LSM orientation is, however, lost when CSM contractions are inhibited with nicardipine, ML-7 or carbenoxolone (Huycke et al., 2019). Cultured cells submitted to cyclic stretch on 2D elastic substrates orient at an angle relative to the stretch direction (Livne et al., 2014). Huycke and colleagues suggest that this mechanism is responsible for the alignment of the LSM perpendicular to the circumferential contractions of the CSM. I am skeptical of this explanation for several reasons. First, the 1 Hz stretch frequency that is applied in most 2D cyclic stretch experiments to reproduce the periodic pulsations of blood pressure is one to two orders of magnitude higher than the physiological contraction frequency of CSM in the gut (∼10-100 mHz). Experiments on fibroblasts have shown that cells orient parallel, rather than perpendicular, to the stretch force below 100 mHz (Jungbauer et al., 2008). Second, although some experiments have found perpendicular alignment relative to the stretch direction (Huycke et al., 2019; Standley et al., 2002), many others have found intermediate angles in the range 45-70° for smooth muscle (Hayakawa et al., 2000; Kanda et al., 1992; Kim et al., 1999; Yang et al., 2021) and fibroblasts (Livne et al., 2014). Finally, and most importantly, the physical situation of cells embedded in a 3D tissue, such as the presumptive LSM, is different from that of cells deposited on a 2D elastomer. Cyclic stretch experiments on smooth muscle embedded in a 3D hydrogel leads to an alignment parallel to the stress direction (Asano et al., 2018). Instead, I believe that the orientation of the LSM is due to the cross-directional longitudinal tension induced by circular contractions (Figs 3C and 5). This effect is substantial even when the gut is cut flat open and cultured on a porous membrane, which is consistent with the observations that LSM aligns properly in this situation (Huycke et al., 2019). The Poisson effect may also explain the longitudinal orientation in chicken and mouse of the thin inner muscularis mucosae that differentiates later.

Anisotropic gut growth

Many human organs display a remarkably high aspect ratio: the seminal tube, with a length of 6-7 m and a diameter of ∼0.5 mm ranks highest and the intestine, with 7 m length and ∼3 cm diameter, second (Bloch, 1904). What physico-biological characteristics cause these organs to grow anisotropically?

Striving to mimic the physiological tension exerted by the vitelline duct on the early gut loop (Figs 3 and 5), our group has shown that applying a constant, static longitudinal stretch force to the embryonic (E8) chicken gut leads to intestinal elongation and proliferation in proportion to the applied force (Chevalier et al., 2018), but the underlying molecular mechanism remains unknown. The involvement of the p-focal adhesion kinase (FAK)-actinin complex has been demonstrated in stretch-activated growth of juvenile rat intestine, suggesting the participation of the Rac1-P38MAPK and Erk-MEK pathways (Sueyoshi et al., 2013). The mechano-sensitive Wnt/β-catenin pathway is another candidate (del

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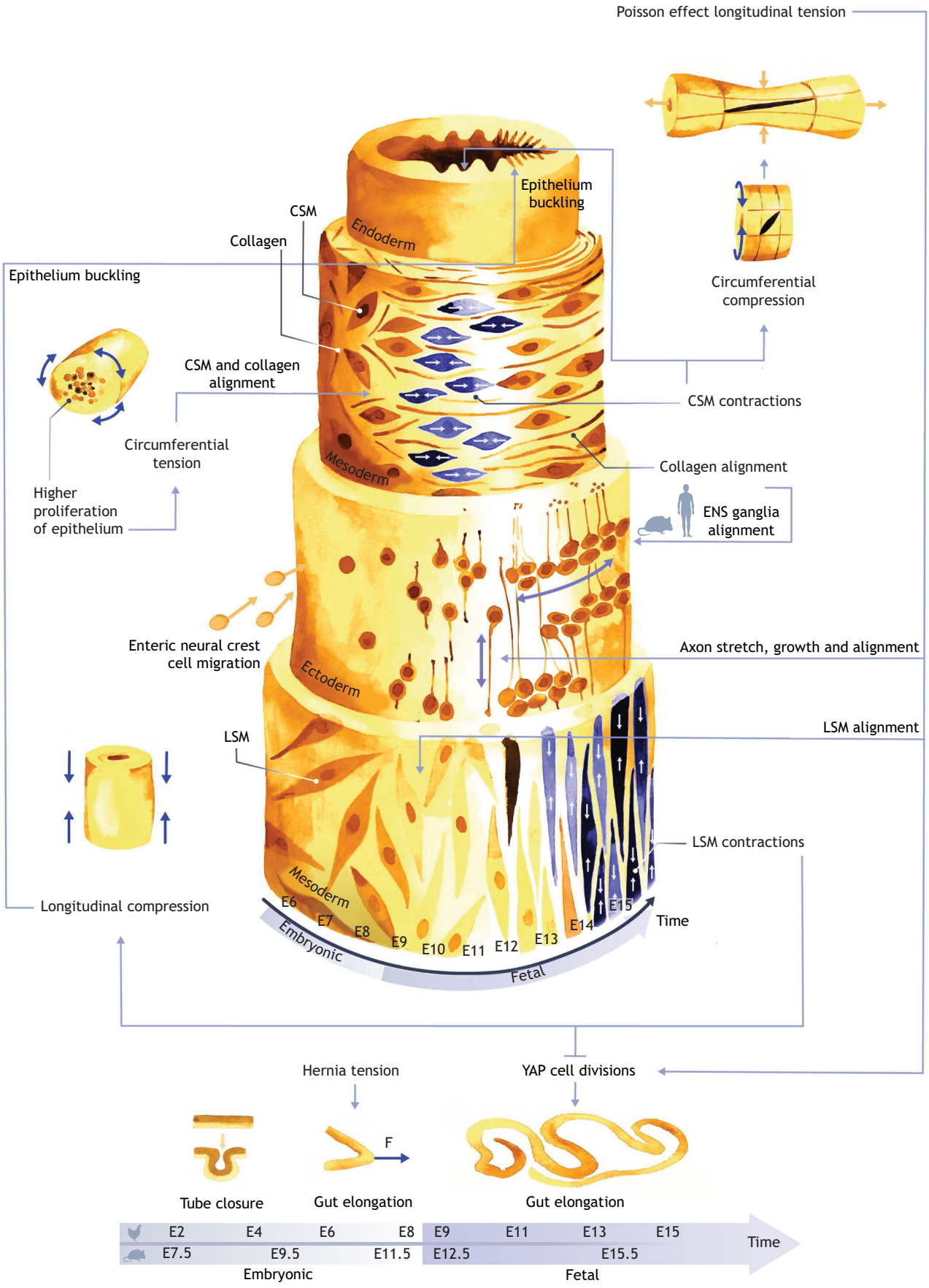

Fig. 5. See next page for legend.

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Fig. 5. Biomechanical gut morphogenesis. Hernia tension exerts a longitudinal strain on the gut and is implied in early intestinal elongation, before smooth muscle contractility sets in. Fast proliferation of epithelium at early stages generates circumferential stress that orients the first CSM layer. CSM secretes ECM fibers that serve as a scaffold for circumferential ENS ganglia in mammals; the circumferential compression of the smooth muscle induces longitudinal tension because of the Poisson effect, which orients the future longitudinal smooth muscle layer and makes the whole gut elongate via the YAP pathway. The action of the two muscle layers makes the epithelium buckle progressively to form villi and increase epithelial absorptive surface. Illustration by Olga Markova.

Río Hernández and Warboys, 2018). The YAP pathway is important at later fetal stages, but does not seem to be involved at stages of umbilical hernia in mice; mesenchyme-specific deletion of YAP-TAZ does not affect the length of the intestine prior to E12.5 (Yang et al., 2021). Longitudinal stretch (Safford et al., 2005) and lumen pressurization (Sueyoshi et al., 2013) both promote elongational growth of rat adult and juvenile intestines, with clinical applications (Hosseini and Dunn, 2020; Stark and Dunn, 2012) to restore gut length in short bowel syndrome or following major resection of the intestine (e.g. due to cancer).

At later embryonic stages (e.g. >E9-E10 chicken, >E12.5 mouse), the prevalent forces acting on the gut are CSM static and phasic circumferential compression, and the associated cross-directional longitudinal tension (Figs 3C and 5). The growth pattern of E10 chicken guts could be switched from elongational to isotropic when CSM contractions are pharmacologically or surgically inhibited (Khalipina et al., 2019). Importantly, contraction inhibition does not change the overall growth and proliferation rate of the explants, revealing a determining role of CSM contractility in driving fetal gut elongation in the chicken. Supporting this, LSM contractions that start at E14 compress the organ longitudinally and initiate a period of relatively slower elongation and faster diameter growth (Coulombres and Coulombres, 1958; Khalipina et al., 2019). The essential role of the CSM in driving elongation of the fetal gut has recently been confirmed in the mouse (Yang et al., 2021), demonstrating that the YAP pathway is the mechanotransducer driving proliferation in response to smooth muscle stress after E13.5.

Table 1 shows that 13 out of 18 mutants reported in the literature to present intestinal elongation defects also feature abnormal CSM, either reduced/absent differentiation, fiber misalignment, or a defect of its contractile apparatus. The most crucial elongation defects (Shh, Cilk1, Wnt5a) correspond to the most severe affections of the CSM. Of the remaining five mutants, one (CLMP mouse) does not cause a loss of CSM contractile force and does not cause gut shortening, three (FLNA, Sfrp, Hlx) lead to gut shortening but no data on CSM has been reported or assessed, and one (YAP) leads to shortening without affecting the CSM because this pathway is downstream of the contraction-induced proliferation. These considerations do not imply that CSM discontractility is the only cause of elongation defects: Wnt5a, for example, affects mesenchymal cell proliferation and cell re-intercalation in the epithelium at E10.5 in the mouse, before CSM differentiation (Chin et al., 2017).

I conclude that gut morphological development is intimately linked to the contractility of the CSM. The biophysical longitudinal stretch (Poisson effect) caused by both tonic and phasic CSM contractility provides, together with YAP mechano-mediated proliferation, a unifying framework to understand the effects of all these mutations (Table 2) on the elongation of the embryonic intestine (Fig. 5). This has two important medical consequences: (1) short bowel syndrome in humans may be caused by a variety of mutations that affect the visceral smooth muscle, and (2) in addition to mechanical stretching (Stark and Dunn, 2012), stimulation of the CSM by chemical (laxatives), mechanical or electrical (Soffer, 2012) means is a promising way of regenerating intestinal length. One solution might be to push gaseous oxygen down the gut to both activate motility via the pressure exerted by bubbles on the gut wall and to increase O_2 delivery to the smooth muscle.

Enteric nervous system

Enteric neural crest cell colonization

ENCCs colonize the gut mesenchyme at E4-E8 in the chicken, 4-8 weeks gestation in the human and E10.5-E14.5 in the mouse. The migration of these cells occurs mostly rostro-caudally, from vagal neural crest cells (NCCs) (Sasselli et al., 2012); a small caudo-rostral contribution by sacral NCCs has been documented (Burns and Douarin, 1998; Kapur, 2000). ENCCs, like other migrating cells, move more readily on stiff 2D substrates (Chevalier et al., 2016b), which provide more resistance to the focal adhesion–integrin–actin cytoskeleton chain and, therefore, allow for greater cell force development (Espina et al., 2021; Hill, 1938; Mitrossilis et al., 2009). However, in the physiological 3D environment of a tissue, migration speed is inversely proportional to stiffness (Chang et al., 2020; Chevalier et al., 2016b; Ehrbar et al., 2011). This is not surprising from a physical point of view, because penetration of a stiffer tissue (think of inserting a needle in a material) requires more energy. Stiffer tissues also present more, and thicker, ECM fibers (e.g. collagen), which must be degraded by metalloprotease enzymes secreted by the cell for migration to progress; we have shown that inhibiting these enzymes halts ENCC migration in a collagen-gel model (Chevalier et al., 2016b). Because tissues become increasingly stiff and ECM-rich as development proceeds, the average migration speed of ENCCs decreases by about 30-50% in the hindgut compared with the midgut (Allan and Newgreen, 1980; Druckenbrod and Epstein, 2005). Hirschsprung disease is caused by incomplete migration of ENCCs in the hindgut. These material considerations may play a role in the Hirschsprung phenotype of the Holstein mouse (Soret et al., 2015), in which collagen VI is over-abundantly secreted by ENCCs. In humans and mice, differentiation of CSM occurs after ENCC migration in the midgut, and concomitantly with ENCC migration at E14.5 in the hindgut (Chevalier et al., 2021a). CSM is therefore not required for ENCC migration. The moonlighting action of endothelin 3, which is crucial for hindgut ENCC colonization (Baynash et al., 1994; Nagy and Goldstein, 2006) and modulates smooth muscle contraction (Yanagisawa et al., 1988), appears in this respect to be coincidental.

Gangliogenesis

CSM does, however, play an essential role in structuring ENS plexuses post-colonization. In chickens, blocking CSM differentiation in the midgut with the Pdgf inhibitor AG 1295 leads to abnormal morphological development of the ENS plexuses, with the presence of a disorganized ENCC network deeper in the mesenchyme (Graham et al., 2017). The physiological circumferential reorientation of ENCC ganglia occurring in the mouse mid- and hindgut between E14.5 and E19.5 is driven by circumferentially spun ECM fibers secreted by the CSM (Fig. 5), probably collagen I (Chevalier et al., 2021a). This orientation transition also occurs in humans, probably between weeks 7 and 9 of gestation (Belle et al., 2014; https://transparent-human-embryo.com). However, this does not occur in the chicken myenteric plexus, which adopts a roughly hexagonal honeycomb geometry early on (~E7), which it preserves

Table 1. Mouse and rat mutants displaying intestinal elongation defects and concomitant affection of the circular smooth muscle

Mutant name	Target	Species	Effect on intestine length	Effect on CSM	Reference
Shh and Ihh	Sonic and indian hedgehog ligand produced by endoderm and acts on mesenchyme	Mouse	No growth after E12.5	No CSM present	Mao et al., 2010
Cilk1 (mesenchymal)	Ciliogenesis associated kinase 1; transduces hedgehog signaling	Mouse	−60% at E17.5	Misaligned, scattered fibers	Yang et al., 2021
Smoothened (mesenchymal)	Effector downstream of Cilk1	Mouse	−50% at E13.5	−50% CSM thickness	Yang et al., 2021
Myh11Cre-EGFP	Diphtheria toxin expression in gut smooth muscle	Mouse	−20% at E15.5	−20% CSM thickness	Yang et al., 2021
YAP (mesenchymal)	Yes-associated protein; mechanosensitive proliferation effector	Mouse	−50% at E13.5	None	Yang et al., 2021
SmBrg1 (smooth muscle)	Brahma related gene 1 (also known as Smarca4); SWI/SNF ATP-dependent chromatin-remodeling complex	Mouse	−20% length at embryonic and early postnatal	Disorganized CSM −50% contractility	Zhang et al., 2011
Wnt5a/Ror2	Non-canonical ligand signaling in gut mesenchyme to receptor Ror2	Mouse	−70% and gut bifurcation (Wnt5a) −60% (Ror2)	−75% CSM thickness in Wnt5a No data for Ror2	Cervantes et al., 2009; Yamada et al., 2010
Laminin α5	Heterotrimeric glycoproteins integral to all basement membranes	Mouse	−45% at E16	Significantly reduced α-actin and desmin staining	Bolcato-Bellemin et al., 2003
R406W-desmin	Intermediate filament of smooth muscle contractile apparatus	Rat	−25% in adult	−70% reduction of spontaneous contractile force	Herrmann et al., 2020
Fgf9, Fgfr1 and Fgfr2	Fibroblast growth factor 9 and associated receptors	Mouse	−40% at E18.5	No desmin staining	Geske et al., 2008
LMOD1	Leiomodin 1 nucleates actin monomers in association with tropomyosin	Mouse	−20% in adult	−30% thickness −40% contractility	Halim et al., 2017
CLMP	Coxsackie- and adenovirus receptor-like membrane protein; colocalizes with tight junction proteins; acts as adhesion molecule	Mouse; human	Small intestine 30-54 cm in seven human patients No effect in mice	Not known in humans +20% thickness in mice, no effect on circular contractile force Loss of connexin 43 and 45 results in dismotility	Langhorst et al., 2018; van der Werf et al., 2015
FLNA	Filamin A; cytoskeletal protein that binds to actin filaments and interacts with ROR2 (Nishita et al., 2006)	Human	Small intestine 55-235 cm in five human patient	Vascular defects No data on GI tract reported/assessed	Hart et al., 2006; Feng et al., 2006
Sfrp1, 2, 5	Secreted frizzled-related protein; modulates Wnt5a signaling	Mouse	−50% in *Sfrp1,2*$^{-/-}$ −65% in *Sfrp5*$^{+/-}$	Not reported/assessed	Matsuyama et al., 2009
MLCK	Myosin light chain kinase; catalyzes myosin cross-bridge cycling on actin filaments	Mouse	−20%	Smooth muscle hypertrophy but −87% and −70% contractility in response to KCl and carbachol, respectively	
Hlx	Homeobox gene Hlx; expressed in mesenchyme	Mouse	−75% at E13.5, death at E15	Not assessed	Hentsch et al., 1996
Notch	Morphogen controlled by Shh activity in gut mesenchyme	Mouse	−30% at postnatal day 0	∼−60% thickness Reduced SMA staining	Kim et al., 2011
TALPID3	TALPID3 (also known as KIAA0586) plays an essential role in protein trafficking	Chicken	−60%	Misaligned, scattered fibers	Delalande et al., 2021

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Table 2. Summary of essential culture parameters and of common ways to implement them

Oxygenation	Boundary conditions	Applying forces
CAM graft		
Physiological O_2 supply by vasculature. Allows long-term culture (>3 days), but live imaging and pharmacological or physical intervention are difficult.	Free-floating (deposited); allows for unconstrained growth and contractions.	Static tension in vertical culture or cyclic tension with StrexCell system or magnetic beads.
O_2 convection		
Bubble carbogen directly in medium or perfuse with gas-saturated medium. Good O_2 supply used in physiological experiments, but difficult to multiplex.	Pinned; preserves longitudinal aspect of gut; minimal constraints for imaging; necessary for ENCC migration studies.	Hydrostatic medium column or osmotic pressure in gut lumen induced by polyethylene glycol.
O_2 diffusion		
Reduce organ-liquid interface distance (porous membrane, hanging drop, shallow medium layer); use carbogen atmosphere. Decent O_2 supply compatible with multi-well experiments.	Gel-embedded; allows growth and contractions in soft Matrigel, ENCC migration; optimal for live imaging.	Relax internal circumferential stress; longitudinal cut, flat tissue culture (chicken only).

to adulthood. The origin of this hexagonal pattern may be the progressive aggregation of ENCCs into condensed ganglia under the effect of cadherin-mediated cell adhesions (Newgreen et al., 2013; Rollo et al., 2015).

Effect of contractions on ENS morphogenesis

How do smooth muscle contractions affect the developing ENS? We have shown in chicken and mice that the longitudinal strain associated to CSM contractions stretches the interganglionic fibers (axons), driving their elongational growth (Fig. 5) (Chevalier et al., 2021b). Inhibition of contractions with nicardipine abolishes this anisotropic axon elongation. The brain and spinal cord are protected by the skull and the spine to limit any potentially devastating influence of external mechanical forces. The ENS is in a drastically different situation: it is embedded in a viscoelastic, highly deformable, self-contractile tissue, and is continuously subject to mechanical strain, which it must both resist (Wang et al., 2015), and sense to transport bolus (Dinning et al., 2014). Many investigators have outlined the astonishing response of axons to mechanical stress in cell culture (Anava et al., 2009; Bray, 1979; Franze, 2013; Pfister, 2004): these findings obviously pertain to the physiological situation of the developing and homeostatic ENS (Kulkarni et al., 2017). Future investigations of ENS deformations in response to various contractility patterns of CSM and LSM, and to bolus deformations, may reveal which stimuli give rise to a mechanosensitive response of neurons in normal and pathological motility.

Epithelium

The interior surface of a villi-less human gut would be ∼0.5 m², but because of the surface amplifying factor due to the various folds of the epithelium (1.6 for plicae circulares, 6.5 for villi, 13 for microvilli) (Helander and Fändriks, 2014), the real absorptive surface of this 'small' intestine is actually 74 m²! In this respect, intestinal length is only a minor contributor to the absorptive surface of the epithelium compared with villification. The physico-biological buckling phenomena underlying villification in the chicken embryo are well understood (Fig. 5) (Ben Amar and Jia, 2013; Coulombres and Coulombres, 1958; Shyer et al., 2013). The epithelium is flat before CSM differentiates. Between E6 and E12, constriction of epithelial expansion by the stiff and contractile CSM makes the tissue buckle to form longitudinal ridges. After LSM differentiates (E12) and starts contracting (E14), the epithelium buckles in the other direction to form zigzags. Finally, differentiation of the third inner longitudinal muscle layer (muscularis mucosa) at E16 transforms the zigzags into finger-like villi organized in a closed-packed hexagonal pattern. In the mouse, finger-like villi directly emerge between E15 and E16, after CSM differentiation, but before LSM is present. Blocking smooth muscle differentiation leads to a lack of villi (Shyer et al., 2013). The lack of an intermediate ridge geometry may be due to the shorter time between CSM and LSM differentiation in the mouse. Mechanical simulations (Shyer et al., 2013) have also shown that finger-like villi can emerge spontaneously, without transitional ridge geometry, when the low epithelium to mesenchyme contrast stiffness (1.5) of the mouse is factored in. Conversely, Walton and colleagues have shown that releasing CSM tension by performing a longitudinal cut still results in villus emergence (Walton et al., 2016). Together, these investigators have concluded that villi in the mouse emerge from a Turing biochemical dynamic field, wherein Shh is a villi activator and Bmp its inhibitor (Walton et al., 2016, 2018). It is likely that a complete understanding of villus emergence in the mouse will incorporate both mechanical and biochemical aspects.

Recently, the genesis of individual microvilli and the underlying actin bundle dynamics has been imaged by live microscopy in porcine epithelial cells (Gaeta et al., 2021). Physical factors also play a role in their emergence: fluid shear stress induces microvilli formation in human placental trophoblastic cells (Miura et al., 2015); applying pressure to intestinal epithelium can erase microvilli and the process is reversible as releasing pressure makes them reappear within minutes (Tilney and Cardell, 1970). The morphology of microvilli is reminiscent of the hydrodynamic Saffman–Taylor instability, which occurs when a pressurized low-viscosity fluid moves in an immiscible, higher-viscosity fluid to form finger-like projections (Saffman and Taylor, 1958). Biophysical approaches have revealed that actin polymerization induces an effective pressure at the tip and that microvillus shape is determined by the actin depolymerization rate at the base (Prost et al., 2007).

Motility

We have seen that smooth muscle impacts virtually all aspects of gut morphogenesis, from its overall anisotropic growth to the shape of the epithelium or of the ENS. Let us return to its primary function in the intestine: motility, a dynamic process, the study of which brings us to reflect upon the emergence of autonomous, self-organized reflexes in the embryo.

CSM contractile waves are the earliest, most primitive form of motility. They are mediated by underlying calcium (Ca^{2+}) waves propagating in the smooth muscle syncytium; these have been imaged in embryonic gut on transverse slice preparations using the intracellular Ca^{2+} reporter Fluo-4 AM (Chevalier, 2018) (Fig. 6A) and on whole-mount guts after electroporating a GCaMP reporter in the lateral plate of early embryos (Fig. 6B). Excitability of the

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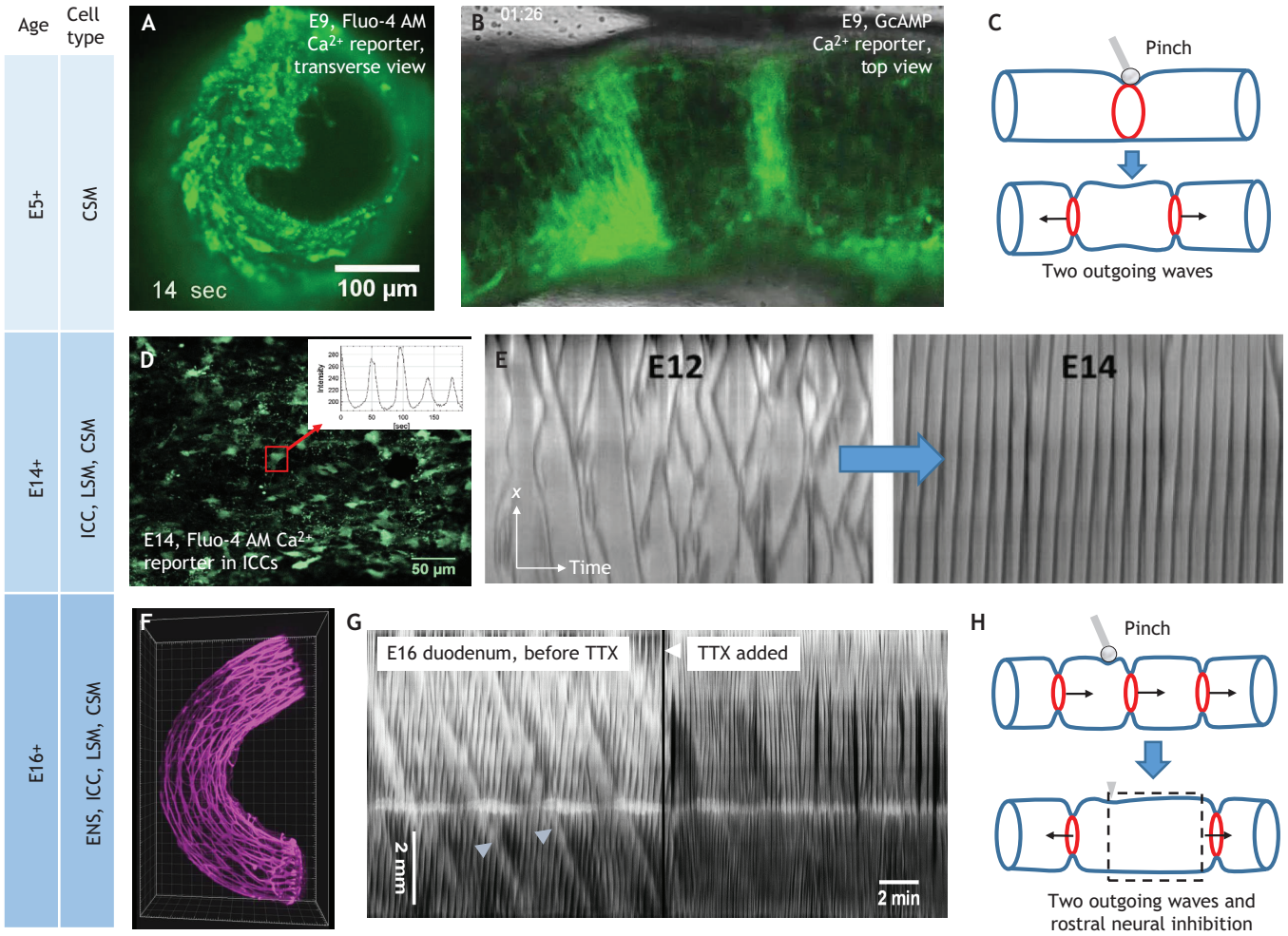

Fig. 6. Ontogenesis of motility. (A-C) Circular smooth muscle (CSM) activity (A-C), with ICC and LSM activity (D,E) and with ENS activity (F-H). (A) Transverse view of a calcium wave visualized with Fluo-4 AM reporter (reproduced with permission from Chevalier, 2018). (B) Longitudinal view of calcium waves in smooth muscle obtained by electroporating a calcium GcAMP reporter in the early lateral plate mesoderm (reproduced with permission from Huycke et al., 2019). (C) Early, symmetric embryonic gut reaction to mechanical stimulation. (D) First rhythmic calcium activity (inset) can be detected in ICCs at E14 (reproduced with permission from Chevalier, 2018). (E) ICC activation is responsible for the clock-like regularity of contractile waves starting at E14 (reproduced with permission from Chevalier et al., 2020). (F) The ENS visualized in E16 chicken duodenum by Tuj1 immunostaining, tissue clearing and 3D reconstruction (reproduced with permission from Chevalier et al., 2019). (G) Tetrodotoxin (TTX)-sensitive (i.e. neurally driven) migrating motor complexes (white diagonal streaks indicated by blue arrowheads) can be detected on a background of higher frequency CSM myogenic waves (reproduced with permission from Chevalier et al., 2019). (H) Fetal (mature), asymmetric response to mechanical stimulation is due to superposition of the nervous response on myogenic response. It is called the 'law of the intestine' (i.e. ascending contraction and descending relaxation).

smooth muscle presents a refractory period, which limits the maximum frequency at which Ca^{2+} waves can be generated. It also explains why two waves traveling in opposite directions become annihilated when they meet, as the CSM on either side of the point of encounter have just contracted and are no longer excitable. At early stages (E5-E7), contractions initiate mainly at the oral and anal end of the gut, which corresponds to the sites where CSM differentiates first. In the period E7-E10, contractions occur at apparently random locations along the gut, giving rise to two waves traveling in opposite directions. From E10, nucleation sites are distributed at a fairly regular interval along the gut tract (Shikaya et al., 2022); the origin of these preferential sites in the *a priori* longitudinally uniform intestine remains to be elucidated. CSM contractions require L-type Ca^{2+} channels (Khalipina et al., 2019; Roberts et al., 2010) and antagonists of these channels (e.g. nicardipine, nifedipine or papaverine) have been successfully used to halt them. Gap-junction blockers and ML-7, an inhibitor of the MLCK enzyme of the contractile apparatus, drastically diminish contractile waves (Chevalier, 2018; Huycke et al., 2019). Ca^{2+} and contractile waves can be stimulated mechanically (Chevalier, 2018), by pinching, which gives rise to two contractile waves traveling in opposite directions from the point where pressure was applied (Fig. 6C). Mechanosensitive cationic, ryanodine or inositol 1,4,5-trisphosphate channels (Ji et al., 2002) may be involved in this response to pressure. This mechanosensitive myogenic reflex can be viewed as an immature (because unpolarized, i.e. symmetric) form of the 'law of the intestine' (Bayliss and Starling, 1899), also called the barometric or peristaltic reflex, a hallmark of gut motility.

Contractile waves are triggered physiologically by the ICCs from E14, the first stage at which periodic Ca^{2+} activity could be recorded in these cells (Fig. 6D) (Chevalier et al., 2020). It remains unclear what triggers contractions at earlier stages (E5-E13): I hypothesize that smooth muscle cells present both a contractile and self-depolarizing phenotype intermediate between that of mature smooth muscle cells and ICCs. The onset of ICC activity marks a spectacular change in regularity and directionality of contractions

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(Fig. 6E). Pacing and active propagation of the action potential in the ICC network makes the contractile waves travel over longer distances without colliding with other waves. Interestingly, ICC-depleted W/W^{v} mutants retain the characteristic multidirectional, pre-ICC contractile pattern (Malysz et al., 1996). Intramuscular electrophysiological recordings have shown that ICC activity starts at E18.5 in mice (Beckett et al., 2007; Roberts et al., 2010). Applying the day-week equivalence between chickens and humans (Chevalier et al., 2019) predicts that a pacemaking transition occurs in the human embryo around 12-14 weeks gestation. Future investigations should identify the factors that regulate the differentiation of intestinal mesenchymal cells to a smooth muscle contractile, myofibroblastic (i.e. ECM-secreting, proliferative) or ICC cell fate. The stem cell factor KIT pathway plays an important role (Wu et al., 2000), as well as the Pdgf pathway (Kurahashi et al., 2008). Electrical stimulation of adult rat intestine regenerates ICCs in a rat model of diabetes (Chen et al., 2018). A potential role of bioelectricity or mechanics in the physiological, embryonic development of ICCs remains, however, as far we know, unexplored.

The ENS is present in the whole GI tract from E8 in chicken (8 weeks gestation in human; E14.5 in mice). It reorganizes and extends projections to the CSM between E8 and E16 (Fig. 6F), but does not exert control on motility yet. The first impact on motility of tetrodotoxin, an inhibitor of neural sodium (Na^{+}) channels, can be detected at E16 in chickens (Fig. 6G) and E18.5 in mice (Chevalier et al., 2019). Interestingly, ENS activation occurs in all species examined [chicken (Chevalier et al., 2019), human (McCann et al., 2019), mouse (Roberts et al., 2010) and zebrafish (Holmberg et al., 2004)] soon after LSM contractions become prominent, suggesting that the LSM could act as a mechanical trigger that 'switches on' neural motility. The first active ENS neurons in the chicken are nitrinergic, i.e. they relax the CSM (Chevalier et al., 2019). This neural inhibition is mechanosensitive and descending: when an E16 duodenum is pinched, the area 1-2 mm distal to the point where pressure has been applied no longer presents circular contractility (Fig. 6H). This asymmetric response to mechanical stimulation (ascending contraction and descending relaxation) is the well-known 'law of the intestine' (Bayliss and Starling, 1899), which permits directional, rostro-caudal bolus movement. Applying tetrodotoxin reverts the reaction to pinching to what it was at earlier myogenic stages: two waves traveling symmetrically away from the point of pinching. This reveals the embryonic make-up of the law of the intestine: the myogenic reflex is responsible for a symmetric (ascending and descending) contraction (wave propagation), half of which is inhibited by neural circuitry (descending inhibition). The neural asymmetry is likely caused by rostro-caudally projecting neurons, a topological feature that can be traced back to the rostro-caudal migration of ENCCs along the gut tract (Young et al., 2002).

At E14 in chicken, when neurons are not yet active, circular and longitudinal contractions occur independently, each with their own distinct frequency. In contrast, at E16, when the ENS becomes active, circular contraction amplitude almost vanishes during longitudinal contractions, so that the two muscle layers work antagonistically (Chevalier et al., 2019). Skeletal muscles (e.g. biceps and triceps) are antagonistic because of their anatomy: a bone separates them. In a soft tissue such as the gut, antagonism is driven by the myenteric ENS plexus separating the longitudinal and circular muscle layers. LSM contractions can also propagate along the gut, and because they locally inhibit the CSM, they give rise to a traveling 'bulge' (Chevalier et al., 2019) called the migrating motor complex, which is neurogenic because it is abolished by tetrodotoxin (Fig. 6G), and is the first rostro-caudally directed motor pattern in the developing embryonic gut (Chevalier et al., 2019). Future studies will examine whether similar dynamic mechanisms are at play in other species as well.

Conclusion

Caspar Friedrich Wolff, who initiated research in gut morphogenesis in his 1769 'De Formatione Intestinorum' (Wolff, 1769), would no doubt have been thrilled by the developments of this field. The importance of a single event, the increased proliferation of the early epithelium causing circumferential internal stress, needs to be emphasized, because it is the source of a cascade of events without which the gut would not be the gut (Fig. 5). The circumferential stress orients the first smooth muscle layer, orienting collagen fibers that serve as a scaffold for circumferential ENS ganglia in mammals; the circumferential compression of the smooth muscle induces longitudinal tension because of the Poisson effect, which orients the future longitudinal smooth muscle layer and makes the whole gut elongate via the YAP pathway. The action of the two muscle layers makes the epithelium buckle progressively to form villi and increase epithelial absorptive surface. Spontaneous contractility of the smooth muscle layers then comes under the yoke of the enteric nervous system, which is itself mechanosensitive, to drive motility and digestion.

Together with biomechanics, bioelectricity is emerging as a major coordinator of embryonic development (Levin et al., 2017). The gut epithelium is, for example, characterized by a transepithelial electric potential, and it has been shown that extracellular electric signals direct the apico-basal polarity of enterocytes (Pu et al., 2015). I believe that many discoveries on the role of bioelectricity in the intestine and how it couples to biomechanics lie ahead of us. In addition to addressing fundamental issues in organogenesis, they will have important implications for gastrointestinal pathophysiology.

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Competing interests

The authors declare no competing or financial interests.

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References

Agathocleous, M., Love, N. K., Randlett, O., Harris, J. J., Liu, J., Murray, A. J. and Harris, W. A. (2012). Metabolic differentiation in the embryonic retina. *Nat. Cell Biol.* **14**, 859-864. doi:10.1038/ncb2531

Allan, I. J. and Newgreen, D. F. (1980). The origin and differentiation of enteric neurons of the intestine of the fowl embryo. *Am. J. Anat.* **157**, 137-154. doi:10.1002/aja.1001570203

Anava, S., Greenbaum, A., Jacob, E. B., Hanein, Y. and Ayali, A. (2009). The regulative role of neurite mechanical tension in network development. *Biophys. J.* **96**, 1661-1670. doi:10.1016/j.bpj.2008.10.058

Asano, S., Ito, S., Morosawa, M., Furuya, K., Naruse, K., Sokabe, M., Yamaguchi, E. and Hasegawa, Y. (2018). Cyclic stretch enhances reorientation and differentiation of 3-D culture model of human airway smooth muscle. *Biochem. Biophys. Reports* **16**, 32-38. doi:10.1016/j.bbrep.2018.09.003

Barbara, P. D. S., Williams, J., Goldstein, A. M., Doyle, A. M., Nielsen, C., Winfield, S., Faure, S. and Roberts, D. J. (2005). Bone morphogenetic protein

signaling pathway plays multiple roles during gastrointestinal tract development. *Dev. Dyn.* **234**, 312-322. doi:10.1002/dvdy.20554
Bassotti, G., Villanacci, V., Antonelli, E., Morelli, A. and Salerni, B. (2007). Enteric glial cells: new players in gastrointestinal motility? *Lab. Investig.* **87**, 628-632. doi:10.1038/labinvest.3700564
Baumann, R. and Meuer, H. J. (1992). Blood oxygen transport in the early avian embryo. *Physiol. Rev.* **72**, 941-965. doi:10.1152/physrev.1992.72.4.941
Bayliss, W. M. and Starling, E. H. (1899). The movements and innervation of the small intestine. *J. Physiol.* **24**, 99-143. doi:10.1113/jphysiol.1899.sp000752
Baynash, A. G., Hosoda, K., Giaid, A., Richardson, J. A., Emoto, N., Hammer, R. E. and Yanagisawa, M. (1994). Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. *Cell* **79**, 1277-1285. doi:10.1016/0092-8674(94)90018-3
Beaulieu, J., Jutras, S., Durand, J., Vachon, P. H. and Perreault, N. (1993). Relationship between tenascin and t-smooth muscle actin expression in the developing human small intestinal mucosa. *Anat. Embryol.* **188**, 149-158. doi:10.1007/BF00186248
Beckett, E. A. H., Ro, S., Bayguinov, Y., Sanders, K. M. and Ward, S. M. (2007). Kit signaling is essential for development and maintenance of interstitial cells of Cajal and electrical rhythmicity in the embryonic gastrointestinal tract. *Dev. Dyn.* **236**, 60-72. doi:10.1002/dvdy.20929
Belle, M., Godefroy, D., Dominici, C., Heitz-Marchaland, C., Zelina, P., Hellal, F., Bradke, F. and Chédotal, A. (2014). A simple method for 3D analysis of immunolabeled axonal tracts in a transparent nervous system. *Cell Rep.* **9**, 1191-1201. doi:10.1016/j.celrep.2014.10.037
Ben Amar, M. and Jia, F. (2013). Anisotropic growth shapes intestinal tissues during embryogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 10525-10530. doi:10.1073/pnas.1217391110
Bloch, A. (1904). Des variations de longueur de l'intestin. *Bull. Mem. Soc. Anthropol. Paris* **5**, 160-197. doi:10.3406/bmsap.1904.7864
Bolcato-Bellemin, A. L., Lefebvre, O., Arnold, C., Sorokin, L., Miner, J. H., Kedinger, M. and Simon-Assmann, P. (2003). Laminin α5 chain is required for intestinal smooth muscle development. *Dev. Biol.* **260**, 376-390. doi:10.1016/S0012-1606(03)00254-9
Bray, D. (1979). Mechanical tension produced by nerve cells in tissue culture. *J. Cell Sci.* **37**, 391-410. doi:10.1242/jcs.37.1.391
Burns, A. J. and Douarin, N. M. (1998). The sacral neural crest contributes neurons and glia to the post-umbilical gut: spatiotemporal analysis of the development of the enteric nervous system. *Development* **125**, 4335-4347. doi:10.1242/dev.125.21.4335
Cervantes, S., Yamaguchi, T. P. and Hebrok, M. (2009). Wnt5a is essential for intestinal elongation in mice. *Dev. Biol.* **326**, 285-294. doi:10.1016/j.ydbio.2008.11.020
Chang, J., Pang, E. M., Adebowale, K., Wisdom, K. M. and Chaudhuri, O. (2020). increased stiffness inhibits invadopodia formation and cell migration in 3D. *Biophys. J.* **119**, 726-736. doi:10.1016/j.bpj.2020.07.003
Chen, Y., Wang, H., Li, H. and Liu, S. (2018). Long-pulse gastric electrical stimulation repairs interstitial cells of cajal and smooth muscle cells in the gastric antrum of diabetic rats. *Gastroenterol. Res. Pract.* **2018**, 6309157. doi:10.1155/2018/6309157
Chevalier, N. R. (2018). The first digestive movements in the embryo are mediated by mechanosensitive smooth muscle calcium waves. *Philos. Trans. R. Soc. B Biol. Sci.* **373**, 1759. doi:10.1098/rstb.2017.0322
Chevalier, N. R., Gazguez, E., Dufour, S. and Fleury, V. (2016a). Measuring the micromechanical properties of embryonic tissues. *Methods* **94**, 120-128. doi:10.1016/j.ymeth.2015.08.001
Chevalier, N. R., Gazguez, E., Bidault, L., Guilbert, T., Vias, C., Vian, E., Watanabe, Y., Muller, L., Germain, S., Bondurand, N. et al. (2016b). How tissue mechanical properties affect enteric neural crest cell migration. *Sci. Rep.* **6**, 20927. doi:10.1038/srep20927
Chevalier, N. R., Fleury, V., Dufour, S., Proux-Gillardeaux, V. and Asnacios, A. (2017). Emergence and development of gut motility in the chicken embryo. *PLoS One* **12**, e0172511. doi:10.1371/journal.pone.0172511
Chevalier, N. R., de Witte, T.-M., Cornelissen, A. J. M., Dufour, S., Proux-Gillardeaux, V. and Asnacios, A. (2018). Mechanical tension drives elongational growth of the embryonic gut. *Sci. Reports* **8**, 5995. doi:10.1038/s41598-018-24368-1
Chevalier, N. R., Dacher, N., Jacques, C., Langlois, L., Guedj, C. and Faklaris, O. (2019). Embryogenesis of the peristaltic reflex. *J. Physiol.* **597**, 2785. doi:10.1113/JP277746
Chevalier, N. R., Ammouche, Y., Gomis, A., Teyssaire, C., de Santa Barbara, P. and Faure, S. (2020). Shifting into high gear: how interstitial cells of Cajal change the motility pattern of the developing intestine. *Am. J. Physiol. Liver Physiol.* **319**, G519-G528. doi:10.1152/ajpgi.00112.2020
Chevalier, N. R., Ammouche, Y., Gomis, A., Langlois, L., Guilbert, T., Bourdoncle, P. and Dufour, S. (2021a). A neural crest cell isotropic-to-nematic phase transition in the developing mammalian gut. *Commun. Biol.* **4**, 770. doi:10.1038/s42003-021-02333-5
Chevalier, N. R., Agbesi, R. J. A., Ammouche, Y. and Dufour, S. (2021b). How smooth muscle contractions shape the developing enteric nervous system. *Front. Cell Dev. Biol.* **9**, 678975. doi:10.3389/fcell.2021.678975
Chin, A. M., Hill, D. R., Aurora, M. and Spence, J. R. (2017). Morphogenesis and maturation of the embryonic and postnatal intestine. *Semin. Cell Dev. Biol.* **66**, 81-93. doi:10.1016/j.semcdb.2017.01.011
Coulombres, A. and Coulombres, J. (1958). Intestinal development: morphogenesis of the villi and musculature. *J. Embryol. Exp. Morph.* **3**, 403-411. doi:10.1242/dev.6.3.403
Delalande, J. M., Nagy, N., McCann, C. J., Natarajan, D., Cooper, J. E., Carreno, G., Dora, D., Campbell, A., Laurent, N., Kemos, P. et al. (2021). TALPID3/KIAA0586 Regulates multiple aspects of neuromuscular patterning during gastrointestinal development in animal models and human. *Front. Mol. Neurosci.* **14**, 757646. doi:10.3389/fnmol.2021.757646
del Río Hernández, A. and Warboys, C. M. (2018). Mechanoactivation of Wnt/β-catenin pathways in health and disease. *Emerg. Top. Life Sci.* **2**, 701-712. doi:10.1042/ETLS20180042
Dinning, P. G., Wiklendt, L., Omari, T., Arkwright, J. W., Spencer, N. J., Brookes, S. J. H. and Costa, M. (2014). Neural mechanisms of peristalsis in the isolated rabbit distal colon: a neuromechanical loop hypothesis. *Front. Neurosci.* **8**, 1-15. doi:10.3389/fnins.2014.00075
Druckenbrod, N. R. and Epstein, M. L. (2005). The pattern of neural crest advance in the cecum and colon. *Dev. Biol.* **287**, 125-133. doi:10.1016/j.ydbio.2005.08.040
Duh, G., Mouri, N., Warburton, D. and Thomas, D. W. (2000). EGF regulates early embryonic mouse gut development in chemically defined organ culture. *Pediatr. Res.* **48**, 794-802. doi:10.1203/00006450-200012000-00016
Durel, J. F. and Nerurkar, N. L. (2020). Mechanobiology of vertebrate gut morphogenesis. *Curr. Opin. Genet. Dev.* **63**, 45-52. doi:10.1016/j.gde.2020.04.002
Ehrbar, M., Sala, A., Lienemann, P., Ranga, A., Mosiewicz, K., Bittermann, A., Rizzi, S. C., Weber, F. E. and Lutolf, M. P. (2011). Elucidating the role of matrix stiffness in 3D cell migration and remodeling. *Biophys. J.* **100**, 284-293. doi:10.1016/j.bpj.2010.11.082
Espina, J. A., Marchant, C. L. and Barriga, E. H. (2021). Durotaxis: the mechanical control of directed cell migration. *FEBS J.* **289**, 2736-2754. doi:10.1111/febs.15862
Feng, Y., Chen, M. H., Moskowitz, I. P., Mendonza, A. M., Vidali, L., Nakamura, F., Kwiatkowski, D. J. and Walsh, C. A. (2006). Filamin a (FLNA) is required for cell-cell contact in vascular development and cardiac morphogenesis. *Proc. Natl. Acad. Sci. USA* **103**, 19836-19841. doi:10.1073/pnas.0609628104
Franze, K. (2013). The mechanical control of nervous system development. *Development* **140**, 3069-3077. doi:10.1242/dev.079145
Fung, Y. C. and Liu, S. Q. (1989). Change of residual strains in arteries due to hypertrophy caused by aortic constriction. *Circ. Res.* **65**, 1340-1349. doi:10.1161/01.RES.65.5.1340
Furness, J. B. and Stebbing, M. J. (2018). The first brain: Species comparisons and evolutionary implications for the enteric and central nervous systems. *Neurogastroenterol. Motil.* **30**, e13234. doi:10.1111/nmo.13234
Gabella, G. (1976). The force generated by a visceral smooth msucle. *J. Physiol.* **263**, 199-213. doi:10.1113/jphysiol.1976.sp011628
Gaeta, I. M., Meenderink, L. M., Postema, M. M., Cencer, C. S. and Tyska, M. J. (2021). Direct visualization of epithelial microvilli biogenesis. *Curr. Biol.* **31**, 2561-2575.e6. doi:10.1016/j.cub.2021.04.012
Gao, F., Liao, D., Drewes, A. M. and Gregersen, H. (2009). Modelling the elastin, collagen and smooth muscle contribution to the duodenal mechanical behaviour in patients with systemic sclerosis. *Neurogastroenterol. Motil.* **21**, 914-e68. doi:10.1111/j.1365-2982.2009.01314.x
Geske, M. J., Zhang, X., Patel, K. K., Ornitz, D. M. and Stappenbeck, T. S. (2008). Fgf9 signaling regulates small intestinal elongation and mesenchymal development. *Development* **135**, 2959-2968. doi:10.1242/dev.020453
Graham, H. K., Maina, I., Goldstein, A. M. and Nagy, N. (2017). Intestinal smooth muscle is required for patterning the enteric nervous system. *J. Anat.* **230**, 567-574. doi:10.1111/joa.12583
Grzymkowski, J., Wyatt, B. and Nascone-Yoder, N. (2020). The twists and turns of left-right asymmetric gut morphogenesis. *Development* **147**, dev187583. doi:10.1242/dev.187583
Halim, D., Wilson, M. P., Oliver, D., Brosens, E., Verheij, J. B. G. M., Han, Y., Nanda, V., Lyu, Q., Doukas, M., Stoop, H. et al. (2017). Loss of LMOD1 impairs smooth muscle cytocontractility and causes megacystis microcolon intestinal hypoperistalsis syndrome in humans and mice. *Proc. Natl. Acad. Sci. USA* **114**, E2739-E2747. doi:10.1073/pnas.1620507114
Hart, A. W., Morgan, J. E., Schneider, J., West, K., McKie, L., Bhattacharya, S., Jackson, I. J. and Cross, S. H. (2006). Cardiac malformations and midline skeletal defects in mice lacking filamin A. *Hum. Mol. Genet.* **15**, 2457-2467. doi:10.1093/hmg/ddl168
Hayakawa, K., Hosokawa, A., Yabusaki, K. and Obinata, T. (2000). Orientation of smooth muscle-derived A10 cells in culture by cyclic stretching: relationship between stress fiber rearrangement and cell reorientation. *Zoolog. Sci.* **17**, 617-624. doi:10.2108/zsj.17.617

He, W. Q., Peng, Y. J., Zhang, W. C., Lv, N., Tang, J., Chen, C., Zhang, C. H., Gao, S., Chen, H. Q., Zhi, G. et al. (2008). Myosin light chain kinase is central to smooth muscle contraction and required for gastrointestinal motility in mice. *Gastroenterology* **135**, 610. doi:10.1053/j.gastro.2008.05.032

Hearn, C. J., Young, H. M., Ciampoli, D., Lomax, A. E. G. and Newgreen, D. (1999). Catenary cultures of embryonic gastrointestinal tract support organ morphogenesis, motility, neural crest cell migration, and cell differentiation. *Dev. Dyn.* **214**, 239-247. doi:10.1002/(SICI)1097-0177(199903)214:3<239::AID-AJA7>3.0.CO;2-O

Helander, H. F. and Fändriks, L. (2014). Surface area of the digestive tract-revisited. *Scand. J. Gastroenterol.* **49**, 681-689. doi:10.3109/00365521.2014.898326

Hentsch, B., Lyons, I., Li, R., Hartley, L., Lints, T. J., Adams, J. M. and Harvey, R. P. (1996). Hlx homeo box gene is essential for an inductive tissue interaction that drives expansion of embryonic liver and gut. *Genes Dev.* **10**, 70-79. doi:10.1101/gad.10.1.70

Herrmann, H., Cabet, E., Chevalier, N. R., Moosmann, J., Schultheis, D., Haas, J., Schowalter, M., Berwanger, C., Weyerer, V., Agaimy, A. et al. (2020). Dual functional states of R406W-desmin assembly complexes cause cardiomyopathy with severe intercalated disc derangement in humans and in knock-in mice. *Circulation* **142**, 2155-2171. doi:10.1161/CIRCULATIONAHA.120.050218

Hewes, S. A., Wilson, R. L., Estes, M. K., Shroyer, N. F., Blutt, S. E. and Grande-Allen, K. J. (2020). In Vitro models of the small intestine: engineering challenges and engineering solutions. *Tissue Eng. Part B Rev.* **26**, 313-326. doi:10.1089/ten.teb.2019.0334

Hill, A. (1938). The heat of shortening and the dynamic constants of muscle. *Proc. R. Soc. London. Ser. B Biol. Sci.* **126**, 136-195. doi:10.1098/rspb.1938.0050

Holmberg, A., Schwerte, T., Pelster, B. and Holmgren, S. (2004). Ontogeny of the gut motility control system in zebrafish Danio rerio embryos and larvae. *J. Exp. Biol.* **207**, 4085-4094. doi:10.1242/jeb.01260

Hosseini, H. S. and Dunn, J. C. Y. (2020). Biomechanical force prediction for lengthening of small intestine during distraction enterogenesis. *Bioengineering (Basel)* **7**, 140. doi:10.3390/bioengineering7040140

Huycke, T. R., Miller, B. M., Gill, H. K., Nerurkar, N. L., Sprinzak, D., Mahadevan, L. and Tabin, C. J. (2019). Genetic and mechanical regulation of intestinal smooth muscle development. *Cell* **179**, 90-105.e21. doi:10.1016/j.cell.2019.08.041

Ji, G., Barsotti, R. J., Feldman, M. E. and Kotlikoff, M. I. (2002). Stretch-induced calcium release in smooth muscle. *J. Gen. Physiol.* **119**, 533-543. doi:10.1085/jgp.20028514

Jungbauer, S., Gao, H., Spatz, J. P. and Kemkemer, R. (2008). Two characteristic regimes in frequency-dependent dynamic reorientation of fibroblasts on cyclically stretched substrates. *Biophys. J.* **95**, 3470-3478. doi:10.1529/biophysj.107.128611

Kanda, K., Matsuda, T. and Oka, T. (1992). Two-dimensional orientational response of smooth muscle cells to cyclic stretching. *ASAIO J.* **38**, M382-5. doi:10.1097/00002480-199207000-00060

Kapur, R. P. (2000). Colonization of the murine hindgut by sacral crest-derived neural precursors: Experimental support for an evolutionarily conserved model. *Dev. Biol.* **227**, 146-155. doi:10.1006/dbio.2000.9886

Kaufman, M. H. and Bard, J. B. L. (1999). *The Anatomical Basis of Mouse Development*. Academic Press.

Khalipina, D., Kaga, Y., Dacher, N. and Chevalier, N. (2019). Smooth muscle contractility causes the gut to grow anisotropically. *J. R. Soc. Interface* **16**, 20190484. doi:10.1098/rsif.2019.0484

Kim, B.-S., Nikolovski, J., Bonadio, J. and Mooney, D. J. (1999). Cyclic mechanical strain regulates the development of engineered smooth muscle tissue. *Nat. Biotechnol.* **17**, 979-983. doi:10.1038/13671

Kim, T.-H., Kim, B.-M., Mao, J., Rowan, S. and Shivdasani, R. A. (2011). Endodermal hedgehog signals modulate Notch pathway activity in the developing digestive tract mesenchyme. *Development* **138**, 3225-3233. doi:10.1242/dev.066233

Kulkarni, S., Micci, M.-A., Leser, J., Shin, C., Tang, S.-C., Fu, Y.-Y., Liu, L., Li, Q., Saha, M., Li, C. et al. (2017). Adult enteric nervous system in health is maintained by a dynamic balance between neuronal apoptosis and neurogenesis. *Proc. Natl. Acad. Sci. USA* **114**, E3709-E3718. doi:10.1073/pnas.1619406114

Kurahashi, M., Niwa, Y., Cheng, J., Ohsaki, Y., Fujita, A., Goto, H., Fujimoto, T. and Torihashi, S. (2008). Platelet-derived growth factor signals play critical roles in differentiation of longitudinal smooth muscle cells in mouse embryonic gut. *Neurogastroenterol. Motil.* **20**, 521-531. doi:10.1111/j.1365-2982.2007.01055.x

Kurpios, N. A., Ibañes, M., Davis, N. M., Lui, W., Katz, T., Martin, J. F., Belmonte, J. C. I. and Tabin, C. J. (2008). The direction of gut looping is established by changes in the extracellular matrix and in cell: cell adhesion. *Proc. Natl. Acad. Sci. USA* **105**, 8499-8506. doi:10.1073/pnas.0803578105

Langhorst, H., Jüttner, R., Groneberg, D., Mohtashamdolatshahi, A., Pelz, L., Purfürst, B., Schmidt-Ott, K. M., Friebe, A. and Rathjen, F. G. (2018). The IgCAM CLMP regulates expression of Connexin43 and Connexin45 in intestinal and ureteral smooth muscle contraction in mice. *Dis. Model. Mech.* **11**, dmm032128. doi:10.1242/dmm.032128

Levin, M., Pezzulo, G. and Finkelstein, J. M. (2017). Endogenous bioelectric signaling networks: exploiting voltage gradients for control of growth and form. *Annu. Rev. Biomed. Eng.* **19**, 353-387. doi:10.1146/annurev-bioeng-071114-040647

Livne, A., Bouchbinder, E. and Geiger, B. (2014). Cell reorientation under cyclic stretching. *Nat. Commun.* **5**, 3938. doi:10.1038/ncomms4938

Malysz, J., Thuneberg, L., Mikkelsen, H. B. and Huizinga, J. D. (1996). Action potential generation in the small intestine of W mutant mice that lack interstitial cells of Cajal. *Am. J. Physiol.* **271**, G387-G399. doi:10.1152/ajpgi.1996.271.3.G387

Mao, J., Kim, B., Rajurkar, M., Shivdasani, R. and McMahon, A. (2010). Hedgehog signaling controls mesenchymal growth in the developing mammalian digestive tract. *Development* **137**, 1721-1729. doi:10.1242/dev.044586

Matsuyama, M., Aizawa, S. and Shimono, A. (2009). Sfrp controls apicobasal polarity and oriented cell division in developing gut epithelium. *PLoS Genet.* **5**, e1000427. doi:10.1371/journal.pgen.1000427

McCann, C. J., Alves, M. M., Brosens, E., Natarajan, D., Perin, S., Chapman, C., Hofstra, R. M., Burns, A. J. and Thapar, N. (2019). Neuronal Development and onset of electrical activity in the human enteric nervous system. *Gastroenterology* **156**, 1483-1495.e6. doi:10.1053/j.gastro.2018.12.020

McHugh, K. M. (1995). Molecular analysis of smooth muscle development in the mouse. *Dev. Dyn.* **204**, 278-290. doi:10.1002/aja.1002040306

Mitrossilis, D., Fouchard, J., Guiroy, A., Desprat, N., Rodriguez, N., Fabry, B. and Asnacios, A. (2009). Single-cell response to stiffness exhibits muscle-like behavior. *Proc. Natl. Acad. Sci. USA* **106**, 18243-18248. doi:10.1073/pnas.0903994106

Miura, S., Sato, K., Kato-Negishi, M., Teshima, T. and Takeuchi, S. (2015). Fluid shear triggers microvilli formation via mechanosensitive activation of TRPV6. *Nat. Commun.* **6**, 8871. doi:10.1038/ncomms9871

Mueller, J. F. (1950). Some observations on the structure of hydra, with particular reference to the muscular system. *Trans. Am. Microsc. Soc.* **69**, 133. doi:10.2307/3223402

Nagy, N. and Goldstein, A. M. (2006). Endothelin-3 regulates neural crest cell proliferation and differentiation in the hindgut enteric nervous system. *Dev. Biol.* **293**, 203-217. doi:10.1016/j.ydbio.2006.01.032

Nagy, N., Barad, C., Graham, H., Hotta, R., Cheng, L., Fejszak, N. and Goldstein, A. M. (2015). Sonic hedgehog controls enteric nervous system development by patterning the extracellular matrix. *Development* **143**, 264-275. doi:10.1242/dev.128132

Nelson, C. M., Gleghorn, J. P., Pang, M.-F., Jaslove, J. M., Goodwin, K., Varner, V. D., Miller, E., Radisky, D. C. and Stone, H. A. (2017). Microfluidic chest cavities reveal that transmural pressure controls the rate of lung development. *Development* **144**, 4328-4335. doi:10.1242/dev.154823

Nerurkar, N. L., Mahadevan, L. and Tabin, C. J. (2017). BMP signaling controls buckling forces to modulate looping morphogenesis of the gut. *Proc. Natl. Acad. Sci. USA* **114**, 2277-2282. doi:10.1073/pnas.1700307114

Nerurkar, N. L., Lee, C., Mahadevan, L. and Tabin, C. J. (2019). Molecular control of macroscopic forces drives formation of the vertebrate hindgut. *Nature* **565**, 480-484. doi:10.1038/s41586-018-0865-9

Newgreen, D. F., Dufour, S., Howard, M. J. and Landman, K. A. (2013). Simple rules for a 'simple' nervous system? Molecular and biomathematical approaches to enteric nervous system formation and malformation. *Dev. Biol.* **382**, 305-319. doi:10.1016/j.ydbio.2013.06.029

Nielsen, C., Murtaugh, L. C., Chyung, J. C., Lassar, A. and Roberts, D. J. (2001). Gizzard formation and the role of Bapx1. *Dev. Biol.* **231**, 164-174. doi:10.1006/dbio.2000.0151

Nishita, M., Yoo, S. K., Nomachi, A., Kani, S., Sougawa, N., Ohta, Y., Takada, S., Kikuchi, A. and Minami, Y. (2006). Filopodia formation mediated by receptor tyrosine kinase Ror2 is required for Wnt5a-induced cell migration. *J. Cell Biol.* **175**, 555-562. doi:10.1083/jcb.200607127

Pfister, B. J. (2004). Extreme stretch growth of integrated axons. *J. Neurosci.* **24**, 7978-7983. doi:10.1523/JNEUROSCI.1974-04.2004

Prost, J., Barbetta, C. and Joanny, J.-F. (2007). Dynamical control of the shape and size of stereocilia and microvilli. *Biophys. J.* **93**, 1124-1133. doi:10.1529/biophysj.106.098038

Pu, J., Cao, L. and McCaig, C. D. (2015). Physiological extracellular electrical signals guide and orient the polarity of gut epithelial cells. *Tissue Barriers* **3**, e1037417. doi:10.1080/21688370.2015.1037417

Rachev, A. and Greenwald, S. E. (2003). Residual strains in conduit arteries. *J. Biomech.* **36**, 661-670. doi:10.1016/S0021-9290(02)00444-X

Renard, E., Gazave, E., Fierro-Constain, L., Schenkelaars, Q., Ereskovsky, A., Vacelet, J. and Borchiellini, C. (2013). Porifera (sponges): recent knowledge and new perspectives. *eLS*. doi:10.1002/9780470015902.a0001582.pub2

Roberts, R. R., Ellis, M., Gwynne, R. M., Bergner, A. J., Lewis, M. D., Beckett, E. A., Bornstein, J. C. and Young, H. M. (2010). The first intestinal motility patterns in fetal mice are not mediated by neurons or interstitial cells of Cajal. *J. Physiol.* **588**, 1153-1169. doi:10.1113/jphysiol.2009.185421

Rollo, B. N., Zhang, D., Simkin, J. E., Menheniott, T. R. and Newgreen, D. F. (2015). Why are enteric ganglia so small? Role of differential adhesion of enteric

neurons and enteric neural crest cells. *F1000Res.* **4**, 113. doi:10.12688/f1000research.6370.1
Romanska, H., Moscoso, G., Polak, J. and Draeger, A. (1996). Smooth muscle differentiation during human intestinal development. *BAM* **6**, 13-19.
Saffman, P. G. and Taylor, G. (1958). The penetration of a fluid into a porous medium or Hele-Shaw cell containing a more viscous liquid. *Proc. R. Soc. London. Ser. A. Math. Phys. Sci.* **245**, 312-329. doi:10.1098/rspa.1958.0085
Safford, S. D., Freemerman, A. J., Safford, K. M., Bentley, R. and Skinner, M. A. (2005). Longitudinal mechanical tension induces growth in the small bowel of juvenile rats. *Gut* **54**, 1085-1090. doi:10.1136/gut.2004.061481
Sanders, K. M., Ward, S. M. and Koh, S. D. (2014). Interstitial cells: regulators of smooth muscle function. *Physiol. Rev.* **94**, 859-907. doi:10.1152/physrev.00037.2013
Sasselli, V., Pachnis, V. and Burns, A. J. (2012). The enteric nervous system. *Dev. Biol.* **366**, 64-73. doi:10.1016/j.ydbio.2012.01.012
Savin, T., Kurpios, N. A., Shyer, A. E., Florescu, P., Liang, H., Mahadevan, L. and Tabin, C. J. (2011). On the growth and form of the gut. *Nature* **476**, 57-62. doi:10.1038/nature10277
Shikaya, Y., Takase, Y., Tadokoro, R., Nakamura, R., Inaba, M. and Takahashi, Y. (2022). Distribution map of peristaltic waves in the chicken embryonic gut reveals importance of enteric nervous system and inter-region cross talks along the gut axis. *Front. Cell Dev. Biol.* **10**, 827079. doi:10.3389/fcell.2022.827079
Shimizu, H., Koizumi, O. and Fujisawa, T. (2004). Three digestive movements in Hydra regulated by the diffuse nerve net in the body column. *J. Comp. Physiol. A* **190**, 623-630. doi:10.1007/s00359-004-0518-3
Shyer, A. E., Tallinen, T., Nerurkar, N. L., Wei, Z., Gil, E. S., Kaplan, D. L., Tabin, C. J. and Mahadevan, L. (2013). Villification: how the gut gets its villi. *Science* **342**, 212-218. doi:10.1126/science.1238842
Sicard, P., Falco, A., Faure, S., Thireau, J., Lindsey, S. E., Chauvet, N. and de Santa Barbara, P. (2022). High-resolution ultrasound and speckle tracking: a non-invasive approach to assess in vivo gastrointestinal motility during development. *Development* dev200625. doi:10.1242/dev.200625
Soffer, E. E. (2012). Gastric electrical stimulation for gastroparesis. *J. Neurogastroenterol. Motil.* **18**, 131-137. doi:10.5056/jnm.2012.18.2.131
Soret, R., Mennetrey, M., Bergeron, K. F., Dariel, A., Neunlist, M., Grunder, F., Faure, C., Silversides, D. W. and Pilon, N. (2015). A collagen VI – dependent pathogenic mechanism for Hirschsprung's disease. *J. Clin. Invest.* **125**, 4483-4496. doi:10.1172/JCI83178
Standley, P. R., Camaratta, A., Nolan, B. P., Purgason, C. T. and Stanley, M. A. (2002). Cyclic stretch induces vascular smooth muscle cell alignment via NO signaling. *Am. J. Physiol. Heart Circ. Physiol.* **283**, H1907-H1914.
Stark, R. and Dunn, J. C. Y. (2012). Mechanical enterogenesis – a review. *J. Healthc. Eng.* **3**, 229-242. doi:10.1260/2040-2295.3.2.229
Sueyoshi, R., Woods Ignatoski, K. M., Okawada, M. and Teitelbaum, D. H. (2013). Distraction-induced intestinal growth: the role of mechanotransduction mechanisms in a mouse model of short bowel syndrome. *Tissue. Eng. Part A* **20**, 131106060201007. doi:10.1089/ten.tea.2013.0383
Sun, J., Liu, Y.-H., Chen, H., Nguyen, M. P., Mishina, Y., Upperman, J. S., Ford, H. R. and Shi, W. (2007). Deficient Alk3-mediated BMP signaling causes prenatal omphalocele-like defect. *Biochem. Biophys. Res. Commun.* **360**, 238. doi:10.1016/j.bbrc.2007.06.049
Tazawa, H. (1980). Oxygen and CO_2 exchange and acid-base regulation in the avian embryo. *Am. Zool.* **20**, 395-404. doi:10.1093/icb/20.2.395
Tilney, L. G. and Cardell, R. R. (1970). Factors controlling the reassembly of the microvillous border of the small intestine of the salamander. *J. Cell Biol.* **47**, 408-422. doi:10.1083/jcb.47.2.408
Timor-Tritsch, I. E., Warren, W. B., Peisner, D. B. and Pirrone, E. (1989). First-trimester midgut herniation: a high-frequency transvaginal sonographic study. *Am. J. Obstet. Gynecol.* **161**, 831-833. doi:10.1016/0002-9378(89)90411-0
Ueda, Y., Yamada, S., Uwabe, C., Kose, K. and Takakuwa, T. (2016). Intestinal rotation and physiological umbilical herniation during the embryonic period. *Anat. Rec.* **299**, 197-206. doi:10.1002/ar.23296
van der Werf, C. S., Halim, D., Verheij, J. B. G. M., Alves, M. M. and Hofstra, R. M. W. (2015). congenital short bowel syndrome: from clinical and genetic diagnosis to the molecular mechanisms involved in intestinal elongation. *Biochim. Biophys. Acta Mol. Basis Dis.* **1852**, 2352-2361. doi:10.1016/j.bbadis.2015.08.007
Walton, K. D., Whidden, M., Kolterud, A., Shoffner, S. K., Czerwinski, M. J., Kushwaha, J., Parmar, N., Chandhrasekhar, D., Freddo, A. M., Schnell, S. et al. (2016). Villification in the mouse: Bmp signals control intestinal villus patterning. *Development* **143**, 427-436. doi:10.1242/dev.130112
Walton, K. D., Mishkind, D., Riddle, M. R., Tabin, C. J. and Gumucio, D. L. (2018). Blueprint for an intestinal villus: species-specific assembly required. *Wiley Interdiscip. Rev. Dev. Biol.* **7**, e317. doi:10.1002/wdev.317
Wang, Y., Wang, W., Li, Z., Hao, S. and Wang, B. (2015). A novel perspective on neuron study: damaging and promoting effects in different neurons induced by mechanical stress. *Biomech. Model. Mechanobiol.* **15**, 1019-1027. doi:10.1007/s10237-015-0743-4
Wolff C. F. (1769). De formatione intestinorum: La Formation des Intestins (1768–1769). *J. St Petersburg Acad. Sci.*
Wu, J. J., Rothman, T. P. and Gershon, M. D. (2000). Development of the interstitial cell of Cajal: origin, kit dependence and neuronal and nonneuronal sources of kit ligand. *J. Neurosci. Res* **59**, 384-401. doi:10.1002/(SICI)1097-4547(20000201)59:3<384::AID-JNR13>3.0.CO;2-4
Yamada, K. M. and Sixt, M. (2019). Mechanisms of 3D cell migration. *Nat. Rev. Mol. Cell Biol.* **20**, 738-752. doi:10.1038/s41580-019-0172-9
Yamada, M., Udagawa, J., Matsumoto, A., Hashimoto, R., Hatta, T., Nishita, M., Minami, Y. and Otani, H. (2010). Ror2 is required for midgut elongation during mouse development. *Dev. Dyn.* **239**, 941-953. doi:10.1002/dvdy.22212
Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**, 411-415. doi:10.1038/332411a0
Yang, Y., Paivinen, P., Xie, C., Krup, A. L., Makela, T. P., Mostov, K. E. and Reiter, J. F. (2021). Ciliary Hedgehog signaling patterns the digestive system to generate mechanical forces driving elongation. *Nat. Commun.* **12**, 7186. doi:10.1038/s41467-021-27319-z
Yissachar, N., Zhou, Y., Ung, L., Lai, N. Y., Mohan, J. F., Ehrlicher, A., Weitz, D. A., Kasper, D. L., Chiu, I. M., Mathis, D. et al. (2017). An intestinal organ culture system uncovers a role for the nervous system in microbe-immune crosstalk. *Cell* **168**, 1135-1148.e12. doi:10.1016/j.cell.2017.02.009
Young, H. M., Hearn, C. J., Farlie, P. G., Canty, A. J., Thomas, P. Q. and Newgreen, D. F. (2001). GDNF is a chemoattractant for enteric neural cells. *Dev. Biol.* **229**, 503-516. doi:10.1006/dbio.2000.0100
Young, H. M., Jones, B. R. and McKeown, S. J. (2002). The projections of early enteric neurons are influenced by the direction of neural crest cell migration. *J. Neurosci.* **22**, 6005-6018. doi:10.1523/JNEUROSCI.22-14-06005.2002
Young, H. M., Bergner, A. J., Simpson, M. J., McKeown, S. J., Hao, M. M., Anderson, C. R. and Enomoto, H. (2014). Colonizing while migrating: how do individual enteric neural crest cells behave? *BMC Biol.* **12**, 23. doi:10.1186/1741-7007-12-23
Zhang, M., Chen, M., Kim, J.-R., Zhou, J., Jones, R. E., Tune, J. D., Kassab, G. S., Metzger, D., Ahlfeld, S., Conway, S. J. et al. (2011). SWI/SNF complexes containing Brahma or Brahma-related gene 1 play distinct roles in smooth muscle development. *Mol. Cell. Biol.* **31**, 2618-2631. doi:10.1128/MCB.01338-10