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Physical organogenesis of the gut

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Abstract

The gut has been a central subject of organogenesis since Caspar Friedrich Wolf 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 in a way where 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 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 like Short Bowel Syndrome, dysmotility, interstitial cells of Cajal related disorders and Hirschsprung disease.

Summary Statement

The embryonic gut is subject to mechanical forces from differential growth, from neighboring tissues, and from its own musculature, that play determining roles in structuring the developing intestine.

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), worms, insects, squids, snails 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, the endoderm, the mesoderm and the ectoderm. The endoderm contributes 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, that longitudinally compresses the intestine,

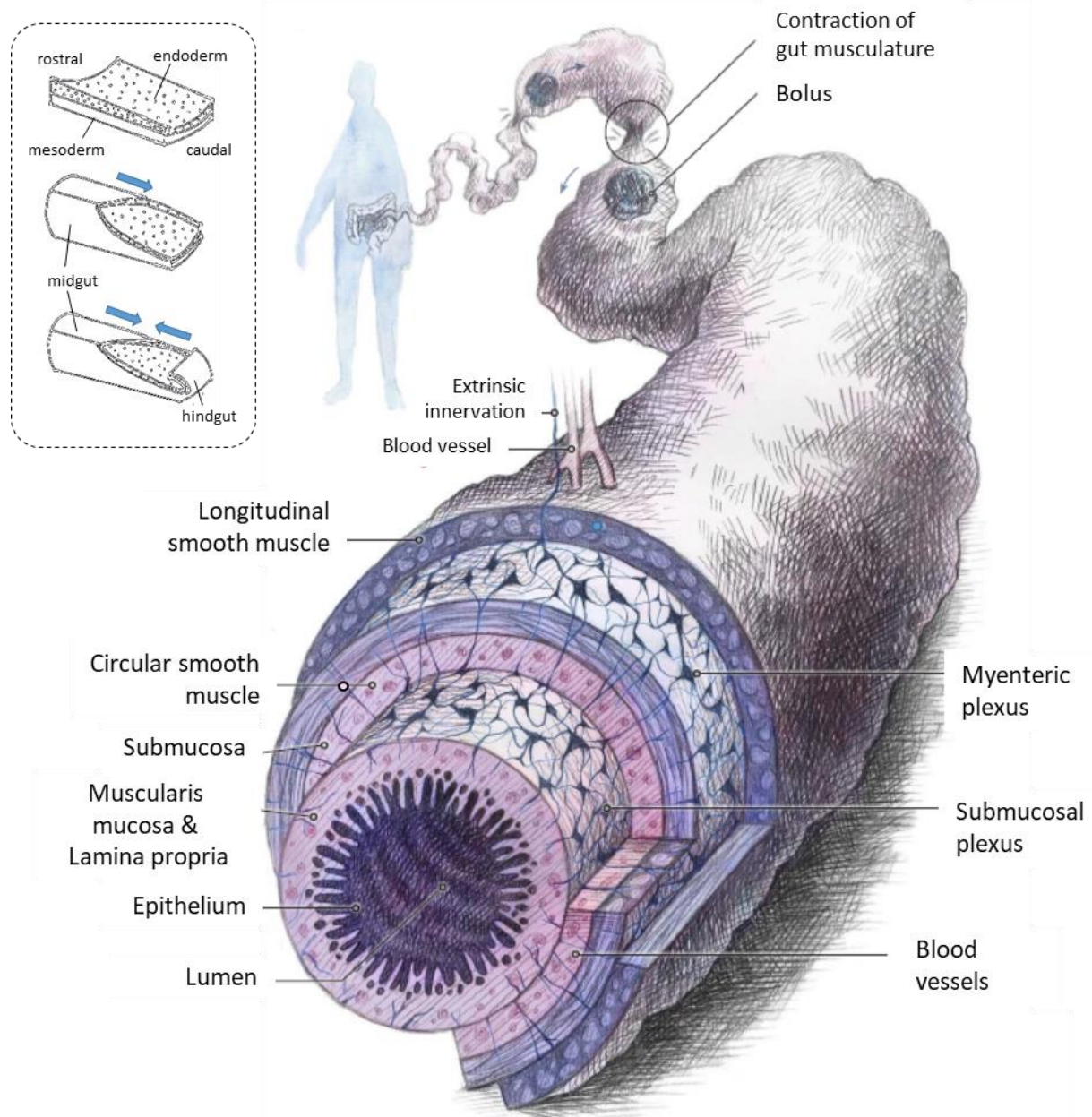


Figure 1. Gut anatomy (courtesy of « Pour la Science » (Chevalier, 2018a), illustration by Marie Marty). Dimensions are not to scale: gut wall thickness is only 3-5 mm in humans, while its diameter is 30-50 mm, i.e. the tube is essentially hollow when not filled with bolus. In humans, the muscularis, 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 cecal appendix, the colon and the rectum. The small intestine is further subdivided in duodenum, jejunum and the 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 is engulfed by right-left folding of the midgut (propagating caudally), and by dorso-ventral folding at the hindgut (propagating rostrally) (Nerurkar et al., 2019).

3°) the interstitial cells of Cajal (ICCs), that are specialized in generating periodic, rhythmic electric depolarization signals to excite the smooth muscle; these anastomosing cells form a fine mesh just above the circular smooth muscle, but also in other locations (Sanders et al., 2014), 4°) the circular smooth muscle (CSM), which can constrict the gut and locally reduce its diameter; together, the LSM-ICC-CSM

layer is known as the muscularis propria = muscularis externa, 5°) the highly vascularized submucosa, which transports nutrients from the gut to the organism, 6°) the muscularis mucosa, which is a thinner, downsized version of the muscularis externa (i.e., outer longitudinal & inner circular layer) whose contractions trigger movements of the epithelial villi but not of the whole gut wall, 7°) the lamina propria which extends smooth muscle fibers into the finger-like protrusions of the epithelium known as villi, and also serves as a support for 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 is comprised of neurons, glial cells and progenitor ENCCs. **The ENS is divided in two plexuses (Fig. 1): the outer myenteric plexus** is sandwiched between the longitudinal and circular smooth muscle layers of the muscularis propria, while 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 in 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 protecting role for neurons and axons and display neurotransmitter, immune and homeostatic functions (Bassotti et al., 2007).

It is a remarkable fact that this complex assembly of the three germ layers which constitutes the gut wall is just 3 to 5 mm thick in humans, i.e. 10 times smaller than the diameter of the gut and a 100 times smaller than the rudimentary description you just read. Whereas the general radial organization of the gut is conserved across species, its lengthwise arrangement varies considerably between animals (Bloch, 1904). At very early embryonic stages, the presumptive **amniote** gut is a flat piece of tissue composed of a layer of endoderm in contact with the yolk, and an overlying layer of mesoderm. This bilayer invaginates at the rostral end of the embryo and this fold progresses rostro-caudally towards the umbilicus (Fig. 1 inset, middle panel). **The mechanism of gut closure at the hindgut is different, as it involves sliding of the whole ventral part of the endoderm over the dorsal ectoderm, rather than left-right closure (Fig. 1 inset, bottom panel).** This morphogenetic movement of the whole tissue is driven by a gradient of tensile contractile forces and of fibroblast growth factor (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. Soon after its formation, the gut tube is invaded by ectodermal ENCCs that migrate rostro-caudally in the mesenchyme and gives rise to the future ENS.

This review aims at providing 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 will 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 technics in embryonic gut organ culture, which are central to research in the field. I finally survey recent advances in development of gut motility. I conclude by suggesting some promising lines of research in the field of gut ontogenesis.

Mechanical properties of the forming gut

In first approximation, the gut can be considered as a visco-elastic material with a bulk elastic modulus E (also called stiffness, elasticity or Young modulus), with units Pascal. 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 the elastic modulus 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 E5 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 was found to account 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 longitudinal smooth muscle (LSM) differentiation, which happens precisely during this time period. The mesentery is stiffer than the gut by a factor 1000 (Savin et al., 2011). Elastic moduli are 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 , which is measured by inflating the lumen with a controlled pressure (Khalipina et al., 2019). The circumferential modulus E_θ of the chicken embryonic intestine at E7-E10 is 3-4 times higher than its longitudinal modulus, consistent with the circumferential orientation of ECM components like collagen I, and with that of the smooth muscle layer.

Elasticity measurements at the tissue level can be performed by surgically separating the layers of the gut (Shyer et al., 2013), and can be further down-scaled at the cell level by atomic force microscopy (Chevalier et al., 2016b). These approaches have revealed that the epithelium is stiffer by a factor ~10 than the mesenchyme in the chicken; in the mouse, this difference is only a factor 1.5.

Forces acting on the embryonic gut

Having presented the essential biomechanical features of the embryonic gut, I now list the forces acting on a macroscopic level on the developing gastrointestinal tract, and compare their magnitude at different stages of development.

Circumferential internal stress

For axisymmetric tissues like the gut or blood vessels, circumferential internal residual stress can be revealed by performing a longitudinal cut of one wall of a ring preparation (Fung and Liu, 1989). After the cut is performed, 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 present in the ring 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 has been formulated (Rachev and Greenwald, 2003) to yield the circumferential strain distribution in the organ as a function of the radius. This analysis shows that the internal layers (mucosa) are under compression whereas the more 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 at E12.5 in mice (Walton et al., 2016), i.e., before differentiation of the mesenchyme (the undifferentiated mesodermal tissue surrounding the epithelium). Faster proliferation of the stiff epithelium compared to the relatively slow proliferation of the soft mesenchyme has been put forward as a major cause of circumferential residual stress at these early stages. In chicken, cell cycle length of the epithelium is ~11 h whereas it is ~25 h for the mesenchyme (Huycke et al., 2019). Reducing proliferation rates of the epithelium by genetic or chemical means reduced 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 extracellular matrix (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 cells ablation (Yang et al., 2021) during development decreases the opening angle of mouse embryonic gut rings from 120° (wildtype) to 80° (smooth-muscle ablated).

Hernia and mesentery tension

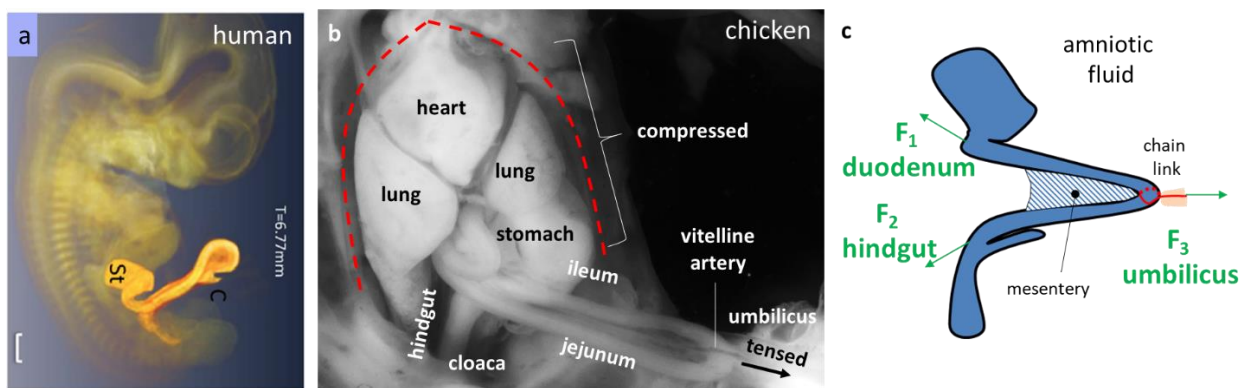


Figure 2. Physiological intestinal hernia in (a) the human embryo at CS 16 (Ueda et al., 2016), (b) the chicken embryo at E8, (c) simplified scheme showing the chain link between the omphalomesenteric artery (red) and the gut apex (blue), and the balance of forces.

In humans, chicken and mice the midgut takes on a hairpin shape (Fig.2) as 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 a Pitx2 and Is11 regulated left-right asymmetry of cell density in the dorsal mesentery (Kurpios et al., 2008). The omphalomesenteric artery (also called the vitelline artery, Fig.2b), which supplies the whole embryo with blood from the placenta (the yolk sac), forms a loop around the apex of the midgut (the tip of the hairpin, Fig.2b,c): the gut and the artery thus form 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 $\sim 20 \mu\text{N}$ in the chicken at stage E8 (Chevalier et al., 2018). As a result, the gut is the only visceral organ in the embryo which 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. This herniation had 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). These explanations are not satisfactory as they 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 just a day prior to hatching, at E19, when the looping midgut is suddenly pulled back inside the body of the embryo. Defective looping morphogenesis of the gut induced by viral modification of the bone morphogenetic protein (BMP) pathway in the chick induced a failure of resorption (Nerurkar et al., 2017; Sun et al., 2007), resulting in a phenotype resembling gastroschisis. In human and mice, the simple hairpin geometry lasts for ~ 10 days (~ 2.5 days in mice), the gut then starts rotating and looping and hernia resorption occurs at 10-11 weeks gestation (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 extracellular matrix and fibroblasts which 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 to 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) –this topic has been previously reviewed (Huycke and Tabin, 2018).

Smooth muscle generated forces: tone and contractions

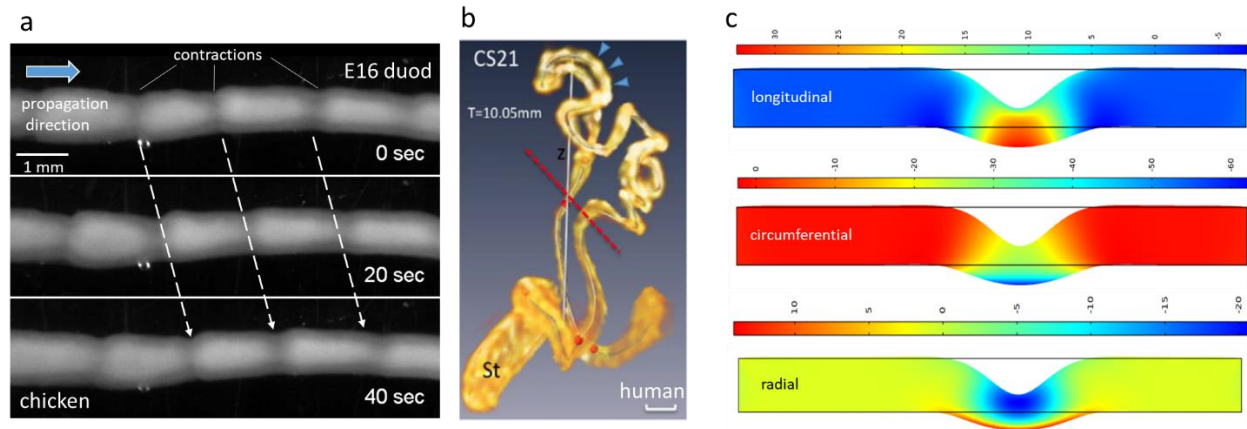


Figure 2. Spontaneous smooth muscle contraction waves. (a) Frames from a time-lapse movie showing the left-to-right propagation of CSM contractile waves along an E16 chicken duodenum. (b) Undulations of the gut tract by CSM contractions (blue arrowheads) are distinctly visible on this IRM image of a human embryo at CS 21 (Ueda et al., 2016). (c) FEM simulation of strains for isotropic tube with 15% cross-section lumen, Poisson ratio $\nu=0.35$, under the effect of a CSM contraction, showing circumferential compression, and longitudinal tension and radial compression cross-strains of similar magnitude (Chevalier et al., 2021b).

Circumferentially oriented α -SMA fibers characteristic of contractile smooth muscle start appearing in the chicken, mouse and human at respectively E5 (Chevalier et al., 2017), E13.5 (McHugh, 1995) and before 7 weeks (Beaulieu et al., 1993; Romanska et al., 1996). This differentiation proceeds rostro-caudally in the mouse lower digestive tract, with colonic SM differentiating at E14.5 (Chevalier et al., 2021a; Roberts et al., 2010). The two most important biochemical factors involved in SM differentiation are the Sonic hedgehog (Shh) and the BMP pathways (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 the chicken, differentiation starts from both the rostral and caudal ends of the GI tract simultaneously. In chicken and mice, CSM differentiates in the cecum 2-3 days after. 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).

As soon as it differentiates, 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, dashed lines), in either the rostro-caudal or caudo-rostral direction, with a speed in the range 20-500 $\mu\text{m}/\text{sec}$. The frequency of contractions increases with developmental time from 0.5 to 3 cycles per minute (Chevalier et al., 2017; Chevalier et al., 2019; Chevalier et al., 2021a; Roberts et al., 2010) between E5 and E15 in chickens (E13.5 and E18.5 in mice). The only direct evidence we have of those contractions in human development come from MRI images of fixed embryos (Fig.3b) (Ueda et al., 2016). With recent developments in ultrasonic imaging, it may soon be possible to see them in living human embryos (Sicard et al., 2022). While the first waves are barely visible, their amplitude gradually increases as further smooth muscle differentiates, aligns and matures. Finite element mechanical simulations have recently revealed the strain field in the tissue associated to a circular smooth

muscle contraction. While CSM contraction obviously gives rise to circumferential compression that reduces the local diameter of the GI tract, recent studies (Chevalier et al., 2021b; Khalipina et al., 2019) have shown that they also result in cross-directional longitudinal tensile and radial compressive strain of the same order of magnitude as the circumferential strain (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. In laymen's terms, 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 $P \sim 1000$ Pa in the embryonic gut lumen, i.e., of the same order of magnitude as the elastic modulus of the embryonic gut: this explains why CSM contractions considerably deform the whole intestine.

Embryonic gut culture methods

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 (the placenta for example). Organ culture allows for direct control of physical (mechanical, electrical), chemical (pH, O₂, metabolism, pharmacology) and biochemical growth conditions. Two to three day cultures recapitulate key developmental events like 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), elongational growth (Khalipina et al., 2019). 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 special 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 like BrdU, EdU, phospho-histone H3 and cleaved caspase-3 immunohistochemistry. Cell viability can be quantified post-culture by dissociation and Trypan blue or acridine orange - propidium iodide staining. We review here the important parameters that should be controlled when performing ex-vivo embryonic gut culture and how these have been implemented by various investigators in the last 20 years. Organotypic culture makes a lot of room for creativity (Hewes et al., 2020; Yissachar et al., 2017) and we hope this brief survey can help other scientists perform such experiments, improve upon these protocols and imagine new ones.

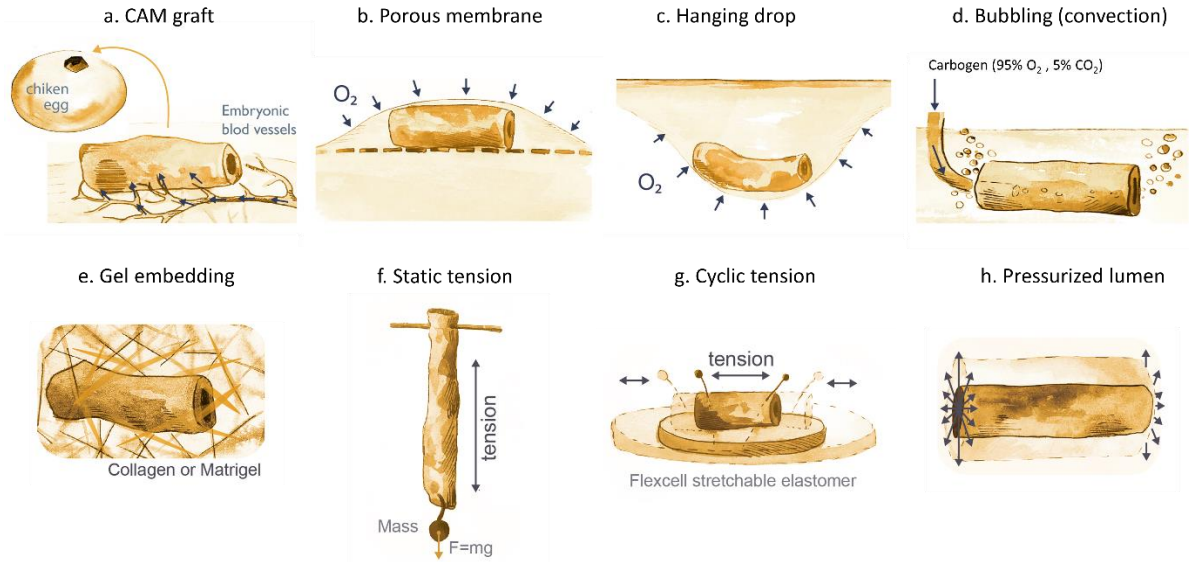


Figure 3. Ex-vivo embryonic gut culture methods. Illustrations by Olga Markova. For mouse embryonic gut, growth has been reported using DMEM with 10% fetal bovine serum (FBS) (Kurahashi et al., 2008), Jackson BGJb with 0.1% ascorbic acid (Duh et al., 2000) or the latter with additional Matrigel (Yang 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).

Oxygenation

Vascularization is ruptured during dissection, stopping blood oxygen supply. The only method that allows for reestablishment 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 was applied to embryonic hindgut and yielded spectacular, 10-fold 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 made difficult 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 find a way to reestablish physiological oxygen (O_2) levels. One governing principle is to culture the explant as close as possible 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 of this method is that the meniscus exerts a considerable pressure on the organ and flattens it (Fig.4b), which can strongly bias morphometric assessment of growth. Embryonic mouse gut has been successfully cultured in a hanging drop (Fig.4c), so that it falls by gravity at the bottom of the drop and stays close to the medium-air interface (Kurahashi et al., 2008). Another, complementary approach, is to replace 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 to be compared to total blood O_2 (dissolved + 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 average 60%

volume & 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. In addition to saturating the liquid, the rising bubbles or the perfusion induce fluid convection, thereby reducing the effective thickness of the layer in which oxygen concentration gradients develop around the explant.

The effect of tissue O₂ concentration on cell differentiation is an active area of research. Multipotent cells are associated with an anaerobic, glycolytic metabolism, whereas differentiated cells with oxidative phosphorylation (respiration) (Agathocleous et al., 2012). It is therefore likely that, in addition to promoting growth, oxygen also influences differentiation – in what direction and how are exciting questions for future research in organogenesis.

Free-floating, held or embedded?

Before E6 (chicken) or E11 (mice), 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). In contrast, as 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 reproduce the physiological attachment of the gut when studying early embryogenesis events like ENCC migration (E4-E8 in chicken, E10.5-E14.5 in mice): catenary culture, where the gut is attached at both ends to a filter paper, has been advocated (Hearn et al., 1999; Young et al., 2001), while others (Nagy and Goldstein, 2006; Yang et al., 2021) embed early guts in a collagen gel (Fig.4e). Attachment was argued to be necessary to preserve the straight, tubular geometry of the early gut. It is known that all cells exert considerable force on the surrounding tissue when they migrate (Yamada and Sixt, 2019), and in particular ENCCs (Chevalier et al., 2016b). We 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 growth was recently successfully cultured (70% length increase in 2 days) in 3D Matrigel (Fig.4e) and growth was even more important in Matrigel richer gels (Yang et al., 2021). On first thought, gel embedding would seem to act as a mechanical barrier to growth and intestinal elongation. This result can probably be explained by the very low elastic modulus of Matrigel even at higher concentrations (10-200 Pa (Slater et al., 2018)), so that stiffness does not impede growth. In addition, the increased concentration of growth factors like TGF-beta, EGF and other proteins in the richer Matrigel likely favor explant growth.

Applying forces

A simple way of applying constant tensile longitudinal stress consists in culturing guts vertically, in tubes, and attaching a small weight at its lower extremity (Fig.4f) (Chevalier et al., 2018). More recently, embryonic guts have been pinned to PDMS membranes and cyclically stretched using the StrexCell system (Fig.4g) (Huycke et al., 2019). Pins need to 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 would involve using a magnet-bead system (Savin et al., 2011) and applying time-dependent magnetic fields. A microfluidic chamber has been developed to control pressure in growing

embryonic lung (Nelson et al., 2017), but this technic has not yet to our knowledge been applied to the developing ex-vivo embryonic gut (Fig.4h). Pressure can also be generated in a controlled way by osmosis, for instance by introducing polyethylene glycol (PEG) 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 will curl up in culture. This can be prevented by using the porous membrane culture method, or by pinning the tissue sheet. This flat preparation is very 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 significant degradation of the enteric nervous system and a failure of smooth muscle differentiation were observed following culture of the tissue sheets.

Oxygenation	Boundary conditions	Applying forces
CAM graft	Free-floating (deposited)	Tension
Physiological O ₂ supply by vasculature. Allows long-term culture (>3 days), but difficult live imaging, pharmacological or physical intervention.	Allows for unconstrained growth and contractions.	Static tension in vertical culture or cyclic tension with Strexcell system or magnetic beads.
O₂ convection	Pinned	Pressure
Bubble carbogen directly in medium or perfuse with gas-saturated medium. Good O ₂ supply used in physiological experiments, difficult to multiplex.	Preserves longitudinal aspect of gut, minimal constraints for imaging, necessary for ENCC migration studies.	Hydrostatic medium column or osmotic PEG pressure in gut lumen.
O₂ diffusion	Gel-embedded	Relax internal circumferential stress
Reduce organ-liquid interface distance (porous membrane, hanging drop, shallow medium layer), use carbogen atmosphere. Decent O ₂ supply compatible with multi-well experiments.	Allows growth and contractions in soft Matrigel, ENCC migration, optimal for live imaging.	Longitudinal cut, flat tissue culture (chicken only).

Table 1. Summary of essential culture parameters and of common ways to implement them

Effect of forces on gut morphogenesis

We now have all the necessary theoretical and experimental tools to understand how the forces described previously affect morphogenesis of the different tissues within the gut. We start by considering the effects of mechanical forces on smooth muscle differentiation and orientation.

Orientation of smooth muscle layers

Huycke et al. have shown (Huycke et al., 2019) that the internal residual circumferential stress drives the circumferential orientation of the differentiating smooth muscle in chicken (Fig.5). 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 resulted in a misoriented smooth muscle layer. Interestingly, when a 20% static longitudinal strain was applied to gut segments during culture, the smooth muscle layer oriented longitudinally instead of circularly, demonstrating the sensitivity of SM orientation to external mechanical stress.

Do forces also affect the later differentiating LSM layer ? Omphalomesenteric artery tension appeared not to be necessary for LSM orientation, because it aligned properly in the absence of any external longitudinal strain. LSM orientation was however lost when CSM contractions were 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 et al. suggested this mechanism is responsible for the alignment of the LSM perpendicular to the circumferential contractions of the CSM. I am skeptic 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 show that cell orients parallel rather than perpendicular to the stretch force below 100 mHz (Jungbauer et al., 2008). Second, while 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; K 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 like 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 (Poisson effect, Fig.3c, Fig.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 mice of the thin inner muscularis mucosae that differentiates later on.

Biomechanical genesis of the gut

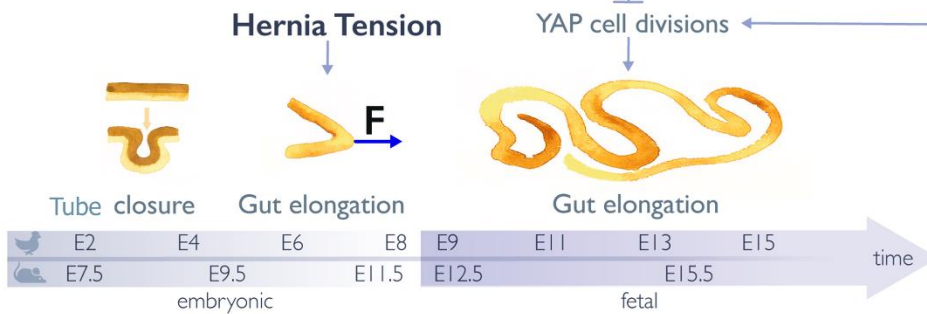
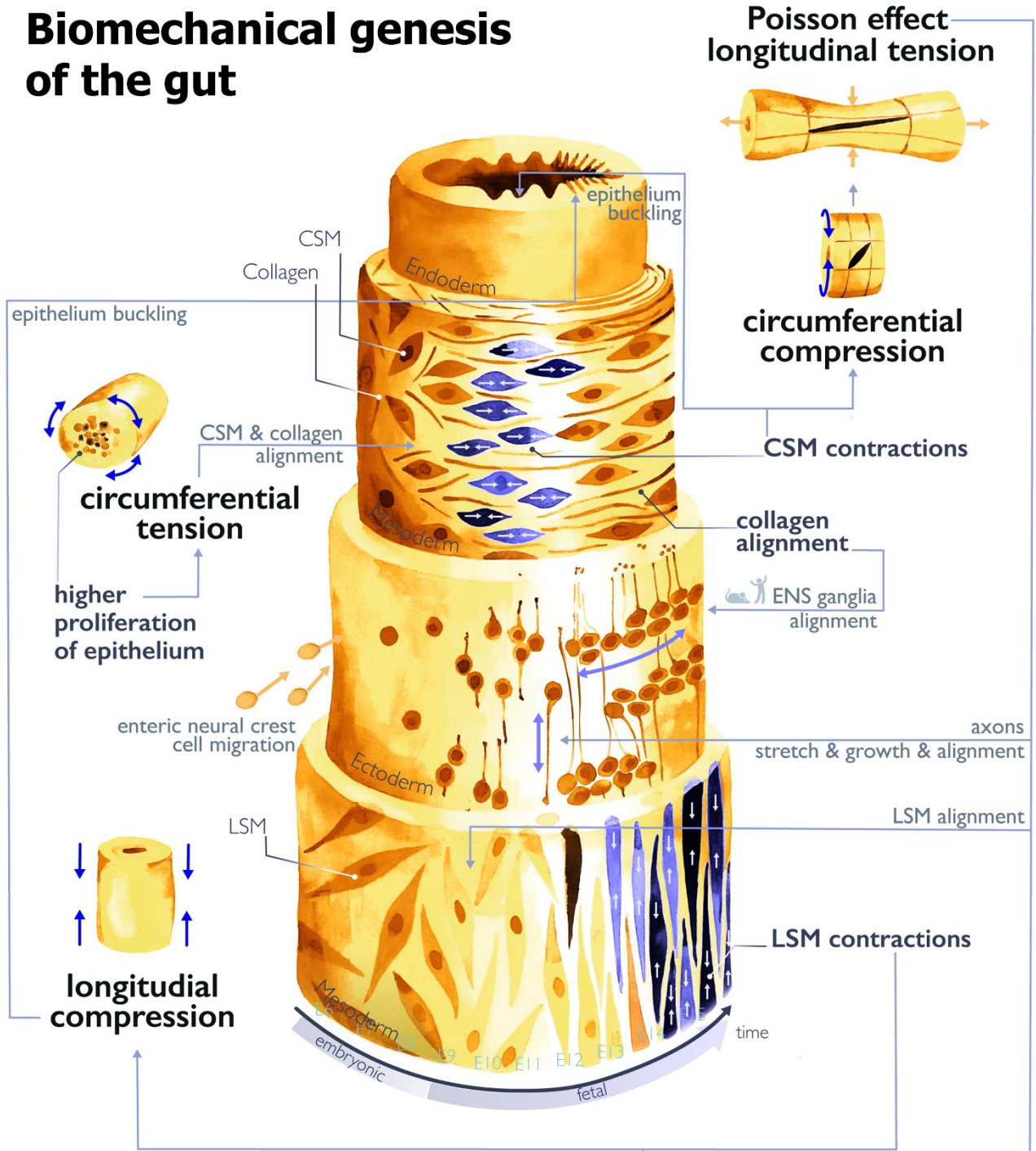


Figure 5. Biomechanical gut morphogenesis. Hernia tension exerts a longitudinal strain on the gut and is implied in early intestinal elongation, before smooth muscle contractility sets in. Fast proliferation of epithelium at early stages generates circumferential stress that orients the first CSM layer. 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, that 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.

Anisotropic gut growth

Many organs in the human body display remarkably high aspect ratio: the seminal tube, with a length of 6-7 m and a diameter of ~0.5 mm ranks first on this list and the intestine second with 7 m length and ~3 cm diameter (Bloch, 1904). What physico-biological characteristics cause these organs to grow anisotropically? This question has recently attracted the attention of many investigators.

Striving to mimic the physiologic tension exerted by the vitelline duct on the early gut loop (Fig.3,5), our group showed that applying a constant, static longitudinal stretch force to the embryonic (E8) chicken gut led to intestinal elongation and proliferation in proportion to the applied force (Chevalier et al., 2018). The proteins involved in this early mechanobiological pathway remain to be determined. 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). Another interesting candidate could be the mechano-sensitive Wnt/ β -catenin pathway (del Río Hernández and Warboys, 2018). The YAP pathway is important at later fetal stages as we will discuss, but does not seem involved at stages of umbilical hernia in mice, because mesenchyme specific deletion of YAP-TAZ did 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) have both been shown to promote elongational growth of rat adult and juvenile intestines. These methods should soon find their way to the clinic (Hosseini and Dunn, 2020; Stark and Dunn, 2012) to restore gut length in short-bowel syndrome or following major resection of the intestine (in most cases due to cancer).

At later embryonic stages (>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 (Fig.3c, Fig.6). We have shown that the growth pattern of E10 chicken guts could be switched from elongational to isotropic when CSM contractions were pharmacologically or surgically inhibited (Khalipina et al., 2019). Importantly, contraction inhibition did not change the overall growth and proliferation rate of the explants. This study showed the determining role of CSM contractility in driving fetal gut elongation in the chicken. Consistent with this picture, LSM contractions which 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 as well (Yang et al., 2021). These investigators demonstrated that the YAP pathway is the mechanotransducer that drives proliferation in response to smooth muscle stress after E13.5.

Mutant name	Target	Species	Effect on intestine length	Effect on CSM	Reference
Shh & Ihh	Sonic & Indian Hedgehog ligand produced by endoderm affects 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, SWI/SNF ATP-dependent chromatin-remodeling complexes	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 % & 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 %, adult	-70% reduction of spontaneous contractile force	(Herrmann et al., 2020)
Fgf9 Fgfr1&Fgfr2	Fibroblast growth factor 9 and associated receptors	Mouse	-40% at E18.5	No desmin staining	(Geske et al., 2008)
LMOD1	Leiomodin1 - nucleates actin monomers in association with tropomyosin	Mouse	-20% adult	-30% thickness, -40% contractility	(Halim et al., 2017)
CLMP	Coxsackie- and adenovirusreceptor-like membrane protein, co-localizes with tight junction proteins , acts as adhesion molecule	Mouse Human	Small intestine 30-54 cm in 7 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, interacts with Ror2(Nishita et al., 2006)	Human	Small intestine 55-235 cm in 5 human patients	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% Sfrp1,2 -/- -65% Sfrp1,2 -/- 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; -87 % & -70% contractility in response to KCl, respectively Ach	(He et al., 2008)
Hlx	homeo box 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 P0	~ -60% thickness, reduced SMA staining	(Kim et al., 2011)
Talpid³	TALPID3/KIAA0586 plays an essential role in protein trafficking	Chicken	-60% (chicken)	Misaligned, scattered fibers	(Delalande et al., 2021)

Table 2. Mouse and rat mutants displaying intestinal elongation defects and concomitant affection of the circular smooth muscle. We strived to be as exhaustive as possible, but apologize for any that we may have overlooked.

In Table 1, we compiled mouse or rat mutants that were reported to display shortened intestines. It is enlightening to see that 13 out of the 18 mutants that present elongation defects also feature abnormal CSM, either via a 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 circular smooth muscle. Of the remaining 5 mutants, one did not cause a loss CSM contractile force and also did not lead to gut shortening (CLMP mouse), 3 led to gut shortening but no data on CSM was reported or assessed (FLNA, Sfrp, Hlk), and 1 (YAP) led 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 reintercalation in the epithelium at E10.5 in the mouse, well before CSM differentiation. The pathways leading to proliferation or intercalation defects have been reviewed elsewhere (Chin et al., 2017).**

We 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 1) on the elongation of the embryonic intestine (Fig.6). 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, 2°) in addition to mechanical stretching (Stark and Dunn, 2012), stimulation of the CSM, by chemical (laxatives), mechanical or electrical (Soffer, 2012) means should be considered as a promising way of regenerating intestinal length . A solution might consist in pushing gaseous oxygen down the gut to both activate motility via the pressure exerted by bubbles on the gut wall, and by increasing oxygen delivery to the smooth muscle.

Enteric nervous system

Enteric neural crest cell colonization

ENCCs colonize the gut mesenchyme between E4 and E8 in the chicken, 4 and 8 weeks in the human, and from E10.5 to E14.5 in the mouse. The migration of these cells occurs mostly rostro-caudally, from vagal 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 provides 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 penetrating inside a stiffer tissue (think of inserting a needle in a material) requires more energy. Stiffer tissues also present more and thicker ECM fibers like collagen, which must be degraded by metalloprotease enzymes secreted by the cell in order to progress – we showed that inhibiting these enzymes halts ENCC migration in a collagen gel model (Chevalier et al., 2016b). Since tissues become increasingly stiff and ECM-rich as development proceeds (see section 3), the average migration speed of ENCCs decreases by about 30-50

% in the hindgut compared to the midgut (Allan and Newgreen, 1980; Druckenbrod and Epstein, 2005). Hirschsprung disease, also called colonic aganglionosis, is due to 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 fact that endothelin-3 acts both as a crucial signal for hindgut ENCC colonization (Baynash et al., 1994; Nagy and Goldstein, 2006) and as a modulator of smooth muscle contraction (Yanagisawa et al., 1988) appears in this respect to be a mere coincidence.

Gangliogenesis

CSM does however play an essential role in structuring ENS plexuses post-colonization. In the chicken, blocking CSM differentiation in the midgut with the Pdgf inhibitor AG 1295 led 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), most probably collagen I (Chevalier et al., 2021a). This orientation transition also occurs in humans, most likely between week 7 and 9 (Belle et al., 2014; <https://transparent-human-embryo.com>). It however does not occur in the chicken myenteric plexus, which adopts early on (~E7) a roughly hexagonal honeycomb geometry which it preserves to adulthood. The origin of this hexagonal pattern may be due to the progressive aggregation of the initially scattered ENCCs into condensed ganglia under the effect of cadherin-mediated cell adhesions (Newgreen et al., 2013; Rollo et al., 2015). Repulsive interactions between ganglia may also contribute to generation / stabilization of this hexagonal geometry (Merminod et al., 2014). More detailed observations of ganglia formation with fluorescently labeled ENCCs in the chicken (Delalande et al., 2014) could reveal which of these mechanisms is or are at play.

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 nifedipine abolished 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 to (Wang et al., 2015), and sense to adequately 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. Future investigations of ENS deformations in response to various contractility patterns of the smooth musculature (both circular and longitudinal) and to bolus deformations may provide

essential clues to fathom which stimuli give rise to a mechanosensitive response of neurons in normal and pathological motility.

Epithelium

The interior surface of a cylinder 7m long and 2.5 cm inner diameter – the approximate dimensions of the human small intestine – is 0.55 m². The surface amplifying factor due to the various folds of the epithelium has been estimated from light and electron microscopy picture (Helander and Fändriks, 2014): x1.6 for the plicae circulares, x6.5 for villi and x13 for microvilli (the membrane folds of each individual epithelial cell), i.e. a total x135, meaning that the real absorptive surface of this “small” intestine is actually 74 m²! In this respect, intestinal length is only a minor contributor to the overall absorptive surface of the epithelium compared to villification. The physico-biological buckling phenomena underlying villification in the chicken embryo are by now well understood (Fig.5) (Ben Amar and Jia, 2013; Coulombres and Coulombres, 1958; Shyer et al., 2013). The epithelium is flat before CSM differentiates. In the period E6-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 in finger-like villi organized in a closed-packed hexagonal pattern. In the mouse, finger-like villi directly emerge in the period E15-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) also show 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. Contrasting with this mechanical view of villi morphogenesis, Walton et al. showed that releasing CSM tension by performing a longitudinal cut still resulted in villus emergence (Walton et al., 2016). Based on this and further observations, these investigators concluded that villi in the mouse emerge from a Turing biochemical dynamic field where Shh is a villi activator and Bmp its inhibitor (Walton et al., 2016; Walton et al., 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 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 has been found to induce 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 occurring when a pressurized low-viscosity fluid moves in an immiscible, higher-viscosity fluid to form finger-like projections (Saffman and Taylor, 1958). Biophysical approaches reveal that actin polymerization induces an effective pressure at the tip and that microvilli 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 go back to its primary function in the intestine: motility. Motility is by definition a dynamic process, and studying its ontogenesis 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 waves propagating in the smooth muscle syncytium; these have been imaged in embryonic gut on transverse slice preparations using Fluo4-AM intracellular Ca^{2+} reporter (Chevalier, 2018b) (Fig.6a) and more recently on whole-mount guts after electroporating a GCamp reporter in the lateral plate of early embryos (Fig.6b from (Huycke et al., 2019), with author permission). Excitability of the smooth muscle presents a refractory period, which limits the maximum frequency at which calcium waves can be generated. It also explains why two waves traveling in opposite directions annihilate 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 travelling in opposite directions, along the gut. As from E10, nucleation sites distribute 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 calcium channels (Khalipina et al., 2019; Roberts et al., 2010) and antagonists of these channels like nifedipine, nifedipine or papaverine have been successfully used to halt them. Other compounds like ML-7, an inhibitor of the MLCK enzyme of the contractile apparatus, or gap junction blockers carbenoxolone (Huycke et al., 2019), enoxonol or heptanol (Chevalier, 2018b), have been shown to drastically diminish contractile waves. Calcium and contractile waves can be stimulated mechanically (Chevalier, 2018b), for example by applying local pressure with a pipette (pinching): this gives rise to two contractile waves traveling in opposite directions from the point where pressure was applied (Fig.6c). Mechanosensitive cationic, ryanodine or InsP_3 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. Calcium or contractile wave propagation is not dependent on the transmission of mechanical force between neighboring smooth muscle cells (Chevalier, 2018b), unlike what has been found for embryonic chicken heart contraction (Chiou et al., 2016).

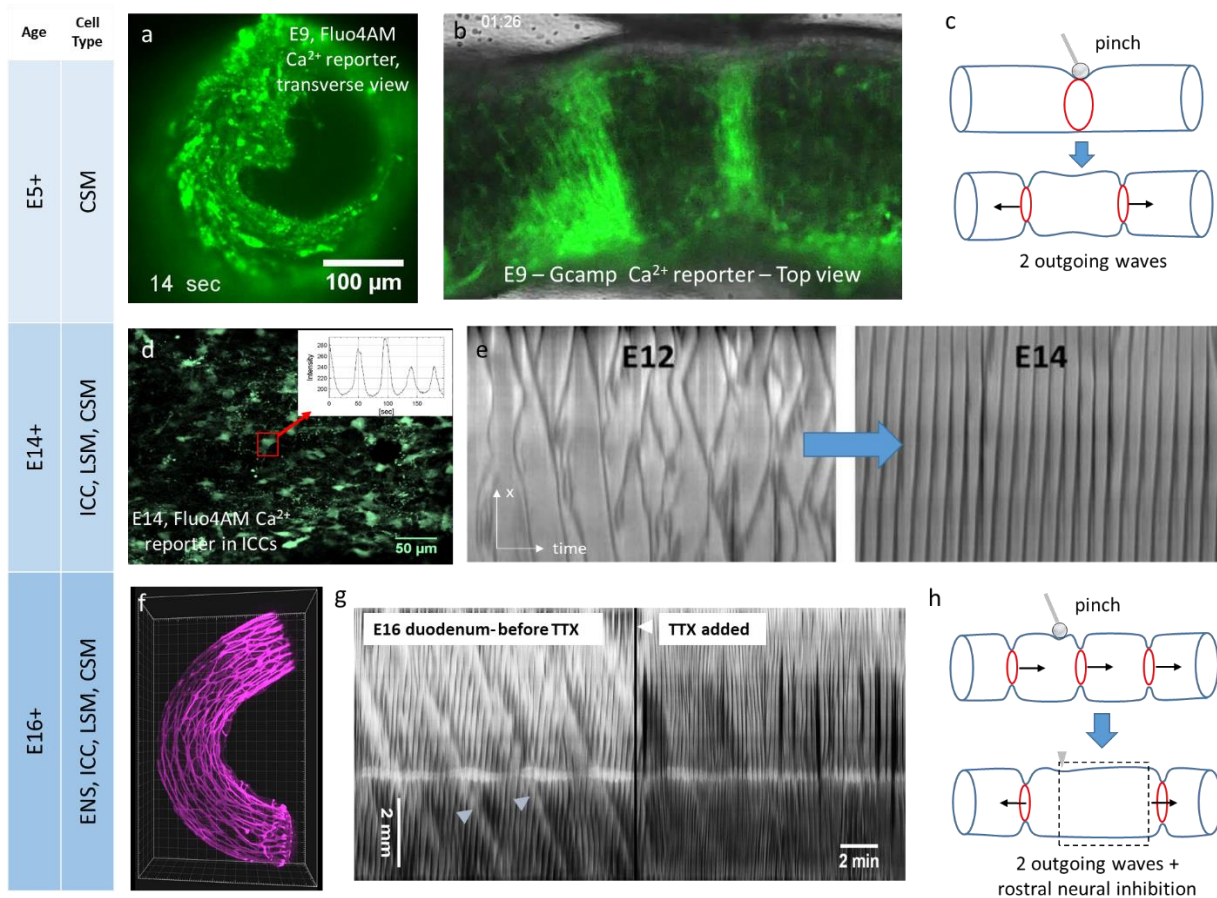


Figure 4. Ontogenesis of motility. (a-c) CSM activity, (d-e) with ICC and LSM activity, (f-h) with ENS activity. (a) Transverse view of calcium wave with Fluo4-AM reporter (Chevalier, 2018b). (b) Longitudinal view of calcium waves in the smooth muscle obtained by electroporating a calcium GCamp reporter in the early lateral plate mesoderm, from (Huycke et al., 2019). (c) Early, symmetric embryonic gut reaction to mechanical stimulation. (d) First rhythmic calcium activity can be detected in ICCs at E14. (e) ICC activation is responsible for the clock-like regularity of contractile waves starting at E14 (Chevalier et al., 2020). (f) The ENS visualized in E16 chicken duodenum by Tuj immuno-staining, tissue clearing and 3D reconstruction. (g) Tetrodotoxin sensitive (= neurally-driven) migrating motor complexes (white diagonal streaks pointed at with blue arrowheads) can be detected on a background of higher frequency CSM myogenic waves (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” = ascending contraction and descending relaxation.

Contractile waves are triggered physiologically by the ICCs as from E14, the first stage as from which periodic calcium 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 (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^o 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 of development. Future investigations should identify the physical

and biochemical factors that regulate the differentiation of intestinal mesenchymal cells to a smooth muscle contractile, myofibroblastic (=ECM-secreting, proliferative) or ICC cell-fate. The stem cell factor – KIT pathway obviously plays an important role (Wu et al., 2000). Inhibition of the Pdgf pathway seems to transform the outer LSM layer in c-kit positive, potential ICC cells (Kurahashi et al., 2008). Electrical stimulation of adult rat intestine was found to regenerate ICCs in a rat model of diabetes (Chen et al., 2018). A potential role of bioelectricity or mechanics in the physiologic, embryonic development of ICCs remains however, as far we know, unexplored.

The ENS (Fig.6f) is present in the whole GI tract from E8 in chicken (8 weeks human, E14.5 mice). It reorganizes and extends projections to the CSM between E8 and E16, but does not exert control on motility yet. The first impact of tetrodotoxin (an inhibitor of neural Na⁺ channels) on motility can be detected at E16 in chickens (Fig.6g) and E18.5 in mice (Chevalier et al., 2019). Calcium transients in ENCCs have been detected before this stage, but do not influence motility, and disappear by E16.5 in mice (Hao et al., 2017). 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, raising the possibility that the LSM acts as a mechanical trigger that switches on the ENS. The first active neurons of the ENS in the chicken are nitrinergic they relax the CSM (Chevalier et al., 2019). This neural inhibition is mechanosensitive and descending: when an E16 duodenum is pinched (local pressure application), the area 1-2 mm distal to the point where pressure was applied does not present circular contractility anymore (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) that 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 neural crest cells along the gut tract (Young et al., 2002).

At E14, 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 like the biceps and triceps are antagonistic because of their anatomy: a bone separates them. In a soft tissue like 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 travelling “bulge” (Chevalier et al., 2019), called the migrating motor complex (MMC, Fig.6g). MMCs are clearly neurogenic as they are abolished by tetrodotoxin (Fig.6g), and are 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 Wolf, who initiated research in gut morphogenesis in his 1769 “De Formatione Intestinorum” 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 hammered home, 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, that 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 was recently found that extracellular electric signals direct the apico-basal polarity of enterocytes (Pu et al., 2015). We 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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