REVIEW ARTICLE

Exploiting mechanical stimuli to rescue growth of the hypoplastic lung

Edwin C. Jesudason

Abstract Impaired lung development afflicts a range of newborns cared for by paediatric surgeons. As a result the speciality has led in the development of surgical models that illustrate the biomechanical regulation of lung growth. Using transgenic mutants, biologists have similarly discovered much about the biochemical regulation of prenatal lung growth. Airway smooth muscle (ASM) and its prenatal contractility airway peristalsis (AP) represent a novel link between these areas: ASM progenitors produce an essential biochemical factor for lung morphogenesis, whilst calcium-driven biomechanical ASM activity appears to regulate the same. In this invited paper, I take the opportunity both to review our recent findings on lung growth and prenatal ASM, and also to discuss mechanisms by which ASM contractility can regulate growth. Finally, I will introduce some novel ideas for exploration: ASM contractility could help to schedule parturition (pulmonary parturition clock) and could even be a generic model for smooth muscle regulation of morphogenesis in similar organs.

Keywords Congenital diaphragmatic hernia · Pulmonary hypoplasia · Airway smooth muscle · Fetal surgery · Fibroblast growth factors · Calcium signalling · Parturition clock

Why lung development matters to paediatric surgeons

Lung development is important to paediatric surgeons. Lung hypoplasia and its lethal sequlae afflict a spectrum of our patients ranging from those with congenital diaphragmatic hernia (CDH) or prenatal uropathies to massive abdominal wall defects [1–4]. Similarly, congenital cyst-adenomatoid lung malformations and bronchopulmonary sequestrations present to paediatric surgeons as antenatally diagnosed masses or postnatally with symptomatic chest lesions [5, 6]. Moreover, several of our patients are born prematurely with incomplete development that predisposes to lung disease of prematurity [7]. Finally many of our patients with CDH and tracheo-oesophageal fistula/oesophageal atresia have chronic respiratory morbidity that affects growth and may affect decisions for further surgeries (such as fundoplication) [8, 9] (Fig. 1).

Given this wide impact of lung development and disease on paediatric surgical practice, it is perhaps no surprise that neonatal surgeons have been at the forefront of investigation: the most notable contribution has been the creation of in vivo fetal models of lung maldevelopment that have allowed us to understand the regulation of pulmonary growth and to design strategies to promote it prenatally (e.g. surgically created models of CDH and obstructive uropathies) [10, 11] (Fig. 2). Moreover, paediatric surgeons have taken a leading role in development of extracorporeal membrane oxygenation as a rescue therapy for refractory respiratory failure and have collaborated in the evaluation and adoption of perinatology approaches such as antenatal glucocorticoids, inhaled

E. C. Jesudason (✉)
Academy of Medical Sciences Clinician Scientist/
Consultant Paediatric Surgeon, Alder Hey Children's Hospital,
The Division of Child Health, University of Liverpool,
Liverpool L12 2AP, UK
e-mail: e.jesudason@liv.ac.uk

E. C. Jesudason
Association of Laparoscopic Surgeons of Great Britain
and Ireland David Dunn Scholar,
UCSF Fetal Treatment Center, Division of Pediatric Surgery,
University of California San Francisco, San Francisco, CA, USA

Fig. 1 Problems of lung development and function are not confined to CDH: **a** Contrast swallow (lateral view) after delayed primary repair of oesophageal atresia (*OA*) without distal fistula. Such children may face major respiratory problems (tracheomalacia and aspiration +/– reflux) that can present as, e.g. life-threatening episodes or later with reactive airways disease; **b** Giant gastroschisis with major liver herniation: such newborns face significant respiratory difficulties requiring, in this case, long-term ventilation after eventual abdominal closure. Both images used with parental consent and courtesy of the author
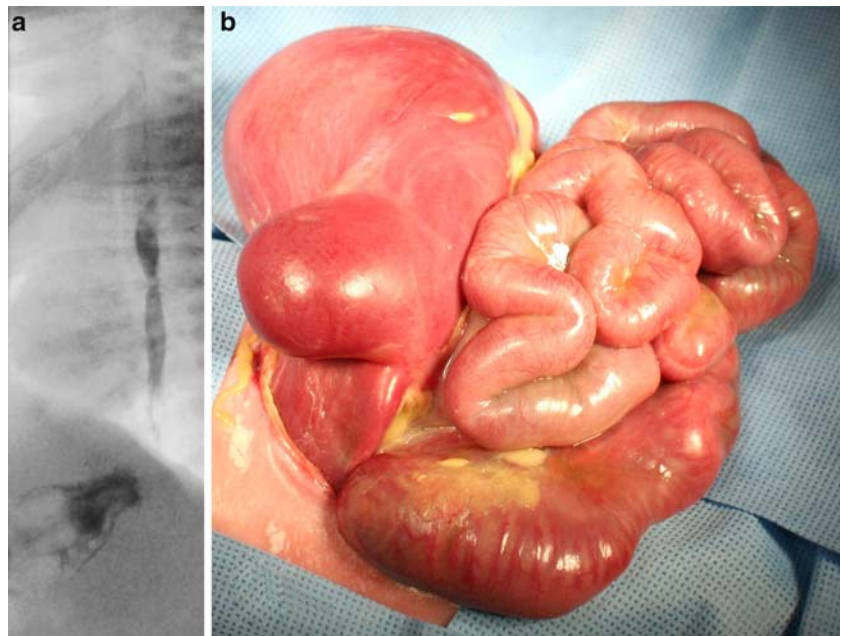

Fig. 2 Surgical models elucidate the biomechanical regulation of lung growth: the gravid ovine uterus has been delivered at laparotomy. Sonography is being used to determine placental and fetal positions prior to hysterotomy. These methods of manipulating the ovine fetus have allowed several models to be created (CDH, obstructive uropathy, gastroschisis and myelomeningocele). Of course, forms of lung hypoplasia generated this way may differ importantly from both teratogenic/transgenic models and indeed humans. Image courtesy of the author

nitric oxide, permissive hypercapnoea and sildenafil for surgical patients [12–17].

An overview of lung development reveals points of clinical importance

Anatomically the mammalian lung develops as a diverticulum from the primitive foregut [18]. Through iterated epithelial branching (branching morphogenesis), the airways take form [18, 19]. Early direct vascular supply from the aorta to the lung normally dwindles: abnormal persistence is observed in sequestrations [20, 21]. Pulmonary vascular development was previously held to follow airway morphogenesis. However recent studies indicate that vascularisation may be an important promoter of airway growth and terminal differentiation [22, 23]. Temporally, the pulmonary diverticulum emerges at around 4 weeks of gestation; failure of the diverticulum to separate normally is thought to explain tracheo-oesophageal fistulae [18, 24]; most airway branching occurs between 6 and 16 weeks [25, 26]; vascular connectivity to establish the respiratory surface for gas exchange emerges around 22 weeks onward (hence the putative limit to survival of preterm birth) [27]; human alveolarisation is in progress by birth and commonly reported to continue through early childhood [25]. Physiologically, the lung undergoes dramatic changes between fetal and neonatal life. Prenatally, the lung is fluid filled and respiratory gas exchange is undertaken instead via the placenta (providing the rationale for the EXIT procedure for, e.g. congenital airway obstructions) [28, 29]. Primitive endodermal cells differentiate into several lineages of which the most studied are the types 1 and 2 pneumocyte and which may also include pulmonary stem cells [30, 31]. Pulmonary epithelium is responsible for lung liquid production (via chloride-secretion): this results in prenatal lung having a small positive intraluminal pressure relative to the thoracic cavity [32]. Abnormal persistence of this lung fluid after birth is held responsible for transient tachypnoea of

the newborn [33]. Surfactant production by type 2 pneumocytes is a further adaptation that assists in lung inflation at birth and resists airspace collapse. Surfactant deficiency is associated with respiratory distress syndrome in premature newborns and may occur in other disorders such as CDH [34, 35]. Estimation of lung maturity has been attempted by assaying surfactant-related metabolites in amniotic fluid [36–38]. Whilst the epithelium has absorbed much scientific interest for conferring tissue specialisation (liquid production, surfactant manufacture), the pulmonary mesenchyme is essential to lung development [39]. Stripping embryonic lung buds of mesenchyme halts their branching; replacing tracheal mesenchyme with that from end-buds restarts epithelial branching at the trachea [40]. Moreover, murine mutants lacking specific mesenchymal gene expression exhibit both lung lesions and diaphragmatic defects with some phenotypic similarities to human CDH [41–43]. These observations illustrate the importance of pulmonary mesenchyme and also support the concept that an *early* mesenchymal lesion in CDH can generate both lung hypoplasia and diaphragmatic defect [44–46] (Fig. 3). As we shall see, the latest evidence suggests that pulmonary mesenchyme and its derivatives have both biochemical and biomechanical roles in the regulation of lung development.

Understanding of lung development derives from a variety of sources

Our understanding of how the lung develops has been aided by comparative biology, transgenic and teratogenic mutants, surgically created models of in vivo physiology and human 'experiments of nature'. Comparative biology has enabled us to appreciate that much of lung development (epithelial branching morphogenesis, vasculogenesis and angiogenesis) is conserved through evolution [47, 48]. This in turn has enabled the similarities with comparable processes in other mammalian organs (pancreas, salivary gland and kidney) to be exploited [49, 50]. Transgenic mutant gene knockout animals have been particularly helpful in establishing the biochemical regulation of airway development by demonstrating the necessary roles of certain growth and transcription factors [51, 52]. In contrast, surgical models of modified lung development have permitted the biomechanical regulation of lung development to be elucidated [10, 11, 53]. Furthermore, human cases have provided vivid illustrations of the biomechanical regulators of lung growth (congenital laryngeal atresia, Potter's syndrome) [54, 55]. Despite the wealth of knowledge generated by these separate approaches, a unifying model that brings together biochemical and biomechanical regulation of

lung growth is still being sought [56–58]. We have been addressing this by studying potential links between biomechanical and biochemical stimuli; in so doing we and others are appreciating new roles for pulmonary mesenchyme in lung organogenesis.

The biochemical regulation of lung development

A key biochemical interaction that underpins lung development is that of fibroblast growth factor-10 (FGF10) and its cognate receptor FGFR2IIIb. Both ligand and receptor have been conserved through evolution and
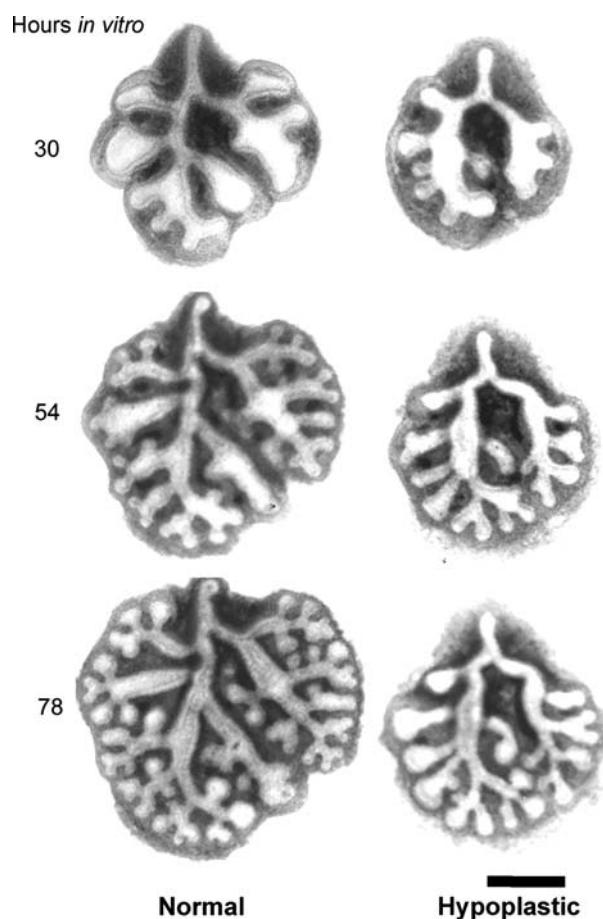

Fig. 3 Non-surgical models of abnormal lung growth are also valuable: teratogenic and transgenic models illustrate that some clinical problems of lung function may have their roots much earlier in development than appreciated from surgical models alone. This shows photomicrographs of whole lung primordia in organ culture. Shown on the *left* of the figure is the longitudinal development of a normal lung rudiment. For comparison the development of a nitrofen-exposed lung rudiment is shown on the *right* at the same timepoints. All specimens are at the same magnification (*scale bar* 400μ m) and the trachea lies superiorly in each case. Nitrofen-exposed lung primordia are growth-impaired prior to the visceral hernia supervening. Image courtesy of the author

are homologous with the *branchless* and *breathless* genes that are essential for airway branching in Drosophila [48]. In a parallel manner, both FGF10 and FGFR2IIIb have been shown to be necessary for lung development in transgenic knockout mice [59–62]. Functional loss of either leads to lung agenesis in the offspring. In vitro lung culture studies have shown that FGF10 induces epithelial branching and can in fact largely substitute in this regard for pulmonary mesenchyme: FGF10 stimulates branching of epithelial only cultures in Matrigel [63]. Abnormalities of FGFs and related heparan sulphate signalling have been demonstrated in experimental lung hypoplasia [64–68]. Several other biochemical regulators of lung morphogenesis are arrayed around the FGF10–FGFR2IIIb interaction (and extensively reviewed elsewhere) [51, 52]. However for the purposes of this review, a focus on this central pathway is sufficient. As we shall see, this archetypal biochemical effector of lung growth may in fact also have a role to play in the biomechanical regulation that we will now survey.

The biomechanical regulation of lung development

Fetal breathing movements, lung liquid production and maintenance of fetal thoracic and amniotic volumes are necessary for normal lung development [55]. Ablation of fetal breathing movements either by phrenic nerve ablation or generation of amyogenic transgenic murine mutants is associated with forms of lung hypoplasia [69, 70]. Excessive lung liquid loss induced by fetal tracheostomy similarly impairs lung growth [71]. Compromise of thoracic volume by visceral herniation in CDH models or due to external fetal compression from oligohydramnios in prenatal uropathies are both associated with forms of lung hypoplasia [11, 72]. Finally, and more difficult to explain, massive abdominal wall defects with extensive liver herniation are also associated with clinically significant lung impairment and often a narrowed thorax [4]. Speculating, it may be that gross reduction in abdominal domain results in commensurate limitation of thoracic growth. Others have suggested that liver herniation draws the diaphragm down leading to major airways becoming kinked across the pulmonary vessels with resulting bronchomalacia [73]. Finally, it is difficult to separate out the deleterious effects of barotrauma resulting from the prolonged ventilation that such babies often require. Having considered the substantial effects that biomechanical stimuli can have on lung development, we can now turn to the focus of our recent work on a biomechanical pulmonary phenomenon that has been long described but largely overlooked.

Airway smooth muscle: 'appendix of the lung' or regulator of growth?

From the earliest stages of lung development and throughout subsequent prenatal life the developing lung is itself vigorously mechanically active [74–77]. Peristaltic waves of airway smooth muscle (ASM) rhythmically propagate throughout the airway tree with resulting propulsion of all important lung liquid (Fig. 4). As a result the growing end buds of the lung (where ASM elaboration is at a minimum) experience phasic distension and relaxation [74, 75]. This curious activity has been observed in airway explants from avian, rodent and human species and they appear to increase in frequency as gestation progresses [74–77]. Why the developing lung should expend so much effort in the synthesis of contractile apparatus and its mechanical activity has remained a mystery. Certainly, rhythmic stretch-relaxation modulates growth and gene expression in a range of cell types including pulmonary epithelium and appears quite distinct from the effects of static stretch [78, 79]. However, why this vibrant peristaltic contractility should suddenly disappear at birth and how it relates to postnatal ASM function and dysfunction are also unknown. Given these uncertainties, we have explored prenatal ASM activity, its regulation and its potential purposes. Most fascinating has been emerging evidence that in ASM peristalsis, we may have an important bridge between biomechanical and biochemical stimuli to lung growth. Finally, the insights gained from our studies hold the promise that strategies to treat prenatal lung hypoplasia and postnatal reactive airway disease can be modified to achieve greater efficacy.

Prenatal ASM peristalsis appears coupled to lung growth

We began with the seemingly simple task of determining where and when in development ASM peristalsis emerges. Our data showed that even embryonic rodent explants equivalent to roughly 5 weeks of human gestation would exhibit powerful peristaltic contractility in vitro [75]. Contrary to expectation, these waves did not arise simply from the trachea and propagate distally. In fact it appears that ASM contraction can arise throughout the proximal airways at this stage but with a clear predilection for the proximal right lung ('pulmonary pacemaker'') [75]. Moreover this area of the developing lung seems to dominate and be autonomous of the remainder of the lung: isolation of other areas of the lung from the influence of the right led to more frequent contractility in the

Fig. 4 Airway peristalsis—a novel regulator of lung growth? *Left hand panels*: fluid flux within the epithelial lumen (*dark*) of cultured lung can be visualised due to the peristaltic propulsion of cellular debris (*leading edge arrowed*) seen here on rapid sequence photography of whole lung. *Right hand panels*: airway contraction (*boxed*) during a wave of airway peristalsis. A video of airway peristalsis from our work is viewable on the web. http://ajrcmb.atsjournals.org/content/vol32/issue2/images/data/118/DC1/Jesudason_Supplement_001.avi
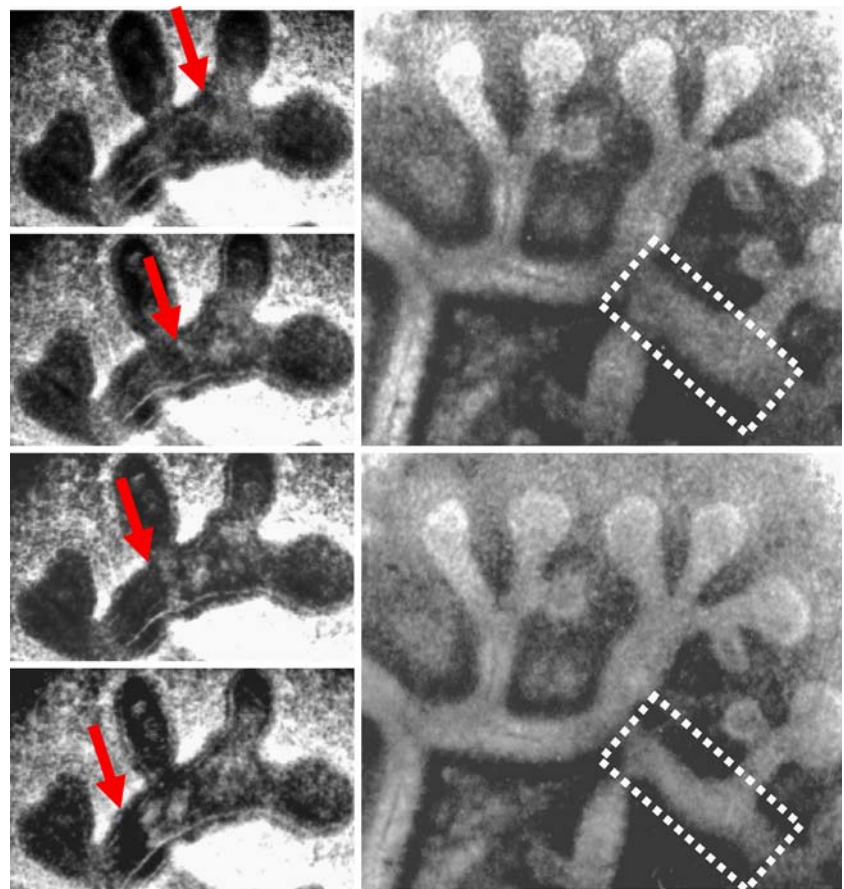

isolated segment; in contrast, isolation of the right lung did not alter peristaltic frequency therein [75]. To ascertain what was driving this activity, we used calcium imaging and c-kit immunoreactivity to show that ASM peristalsis was underpinned by spontaneous propagating calcium waves that arise in the apparent absence of Cajal-type pacemaker cells [75, 80] (Fig. 5a). To explore the purpose of such contractility we alternately stimulated or inhibited either peristalsis or lung growth. In normal embryonic lung we discovered that growth and peristalsis were coupled: modulation of one was reliably accompanied by parallel alteration in the other [75]. In particular, we had an indication of a link between a typical biochemical regulator of lung growth (FGF10) and a biomechanical one (ASM peristalsis): FGF10-stimulated lung growth was associated with significant increase in peristalsis frequency [75]. Hence, these studies showed a degree of coupling between ASM peristalsis and FGF10-driven lung growth and supported the concept that one may regulate the other. Furthermore, given that ASM peristalsis depends on propagating calcium waves, these data indicate that such calcium waves may in turn regulate lung growth [80].

Ca^{2+}: biochemical mechanism for ASM regulation of lung development?

In preliminary studies to explore, how calcium may regulate lung growth we have identified some interesting phenomena. L-type calcium channel blockade with nifedipine reduces intracellular calcium, halts airway peristalsis (AP) (inducing a flaccid paresis) and stunts lung size but without significantly diminishing branching [75, 81]. In contrast, blocking sarcoplasmic calcium reuptake with cyclopiazonic acid raises intracellular calcium leading to a tetanic paresis (and halt of airway contractility) and is associated with a remarkable and complete abolition of branching morphogenesis [82, 83] (Fig. 5b). Moreover this latter effect is associated with loss of smooth muscle actin expression (SMA: an early marker of ASM lineage) [83]. Removal of cyclopiazonic acid leads to resumption of apparently normal branching and SMA expression [83]. Given that ASM progenitors specifically furnish the FGF10 required for airway branching [84], we therefore hypothesise that cyclopiazonic acid may be acting on ASM progenitors to halt both commitment (loss of SMA expression) and also FGF10 production (loss of branching). Supporting

Fig. 5 Ca^{2+} waves underpin airway peristalsis and hence potentially growth: **a** Stacks of pseudo-colour confocal images of Fluo-4–loaded embryonic airways showing propagating Ca^{2+} waves with the initiation site (marked by an *asterisk*) in the trachea (*top row*) and right lung rudiment (*bottom row*); **b** blockade of sarcoplasmic calcium reuptake with cyclopiazonic acid (*CPA*) leads to raised intracellular Ca^{2+} (*y*-axis) and rapid abolition of Ca^{2+} oscillations over time (*x*-axis). As described in the text, this is associated with replacement of peristalsis by sustained airway contraction, halting of branching and loss of SMA expression (not shown); **c** superimposed ASM Ca^{2+} transients measured from normal and nitrofen lung (as labelled) showing altered morphology in lung hypoplasia
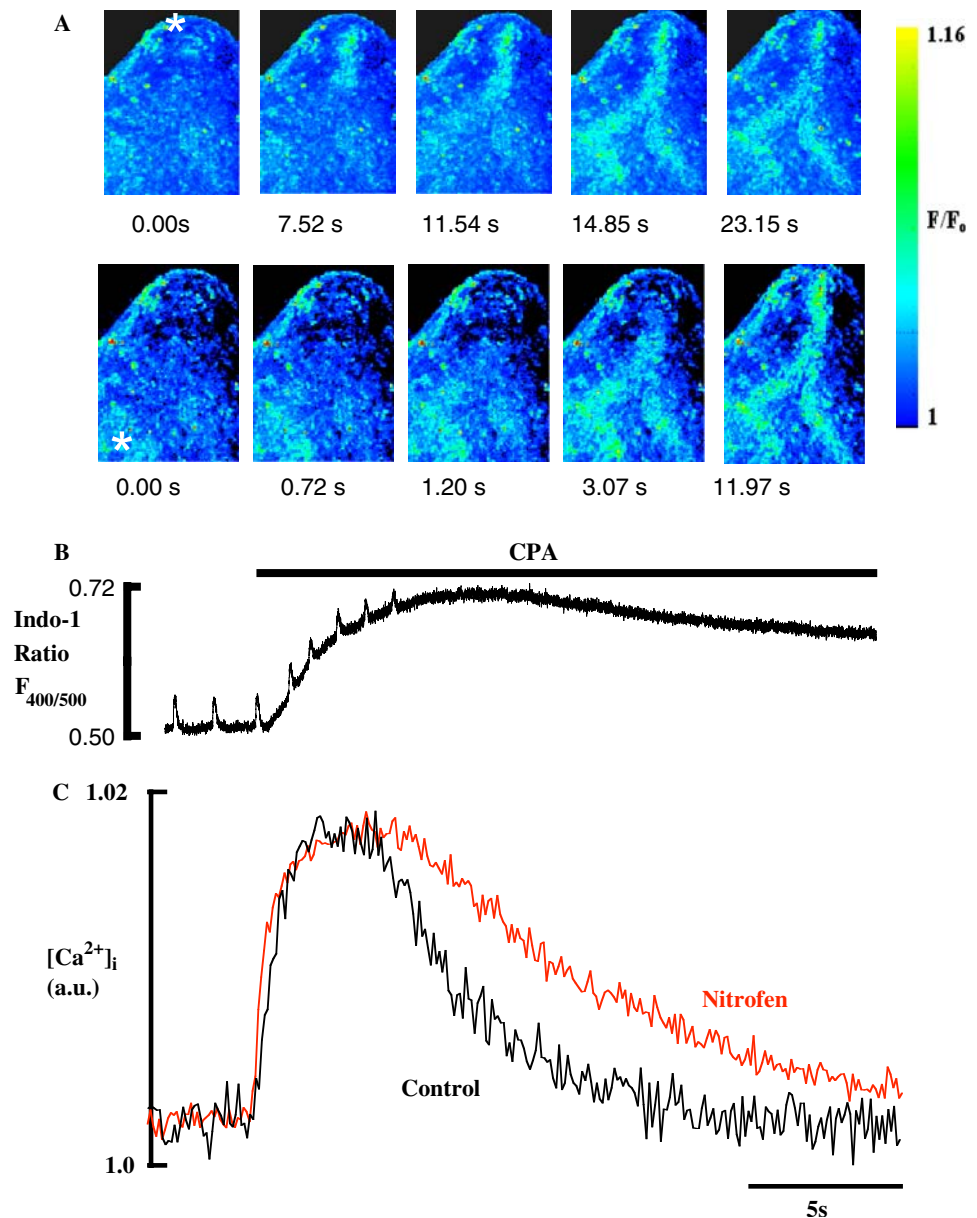
the idea that sarcoplasmic calcium regulates both FGF10-driven branching morphogenesis and ASM elaboration, one of the first described human genopathies relating to loss of sarcoplasmic calcium reuptake is associated with dysregulated proliferation an epithelium that, like the airway, is FGFR2IIIb-bearing and FGF10-responsive [85].

A biomechanical mechanism for ASM regulation of lung development?

We have also argued that coupled elaboration of FGF10 and ASM could operate as a biological feedback mechanism to maintain intraluminal pressures in the growing lung (loss of such pressure is associated with lung hypoplasia) [46]. As the lung lumen increases in volume due to FGF10 mediated growth, maintenance of intraluminal pressure would require increased lung liquid production and/or increased wall tension. The latter (and perhaps even the former—vide infra) could be supplied by increased ASM elaboration and activity: as more ASM progenitors produce FGF10 and growth, more differentiated ASM develops to influence airway wall tension and maintain luminal pressure. Moreover, early work suggests that ASM peristalsis is associated not only with propagating calcium waves but potentially with chloride efflux from ASM. We hypothesise that this chloride could be taken up at the adjacent basolateral epithelial surface to drive epithelial

chloride-secretion led production of lung liquid. Hence ASM peristalsis may regulate growth-related intraluminal pressures not only via its influence on wall tension but also through modulation of all important lung liquid production.

What then are the implications for abnormal lung development? Lung hypoplasia is associated with abnormalities of prenatal ASM-related signalling and postnatal function in human CDH and in experimental CDH lung [8, 86, 87]. We have now shown that the coupling between peristalsis and growth, the underpinning calcium waves and the stereotypical maturation of peristaltic activity are all abnormal in teratogen-induced lung hypoplasia [88, 89] (Fig. 5c). Prenatal ASM may also play key roles in other heterogenous forms of experimental lung hypoplasia: myogenic knockout mice where intercostal musculature +/– diaphragmatic muscularisation are ablated appear to have normal ASM elaboration up to and beyond the point at which fetal breathing supervenes [70]. In only those mutants lacking *both* intercostal and diaphragmatic muscularisation, SMA expression then appears to diminish in late gestation [70]. Although the impact on SMA expression in other thoracic organs (e.g. oesophagus) was not described, it could be that fetal breathing is important for the maintenance of ASM. Assuming the degree of lung hypoplasia manifest in amyogenic mutants is worse than those lacking intercostals alone, the late loss of ASM peristalsis (when it should be most active and widespread) may contribute to this.

Therefore, contrary to the view that ASM is 'the appendix of the lung' with no useful function [90–93], it appears that ASM is critical to aspects of prenatal lung growth: it provides FGF10 (from progenitors), mechanical stretch-relaxation and even perhaps lung liquid production. Encouraged by our discoveries, we began to explore the role of smooth muscle contractility in the development of other hollow organs using gut organ culture and calcium fluorophores. Our idea has been eagerly taken over by colleagues who continue to look at prenatal gut contractility using these techniques.

Future directions and potential clinical applications

Having considered the biological roles of prenatal ASM (and some of the mechanisms by which it may perform them), we can now consider potential clinical applications. In translating prenatal ASM biology from bench to bedside, technological development may have a significant part to play. Improved imaging +/– remote telemetry could allow us to visualise AP and/or measure resulting luminal pressure changes in utero [94, 95]. Given the observed relationship between peristaltic frequency and gestation [76], such measures might be used to provide a readily repeatable, non-biochemical estimate of lung maturity. This would be of great benefit in selecting cases for prenatal intervention or expeditious early delivery. Moreover, lung development following, e.g. tracheal occlusion might be monitored in real time by looking at peristalsis frequency. Following evidence that cyclical tracheal occlusion may be superior to static devices [96], there is also potential for devices that allow balloon pump occlusion of the trachea to optimise resulting lung development ('preventilation''/dynamicPLUG) [46]. Imaging prenatal ASM contractility could even perhaps allow us to identify asthma-predispositions prenatally and discover new drug targets to combat ASM hypertrophy and airway remodelling in asthma (the commonest chronic disease in the developed world) [97].

Finally, the increasing frequency of ASM peristalsis through gestation and the unexplained switch from peristalsis to tonic contractility around birth could be important in the timing of parturition. Teleologically, it would be advantageous for some aspect(s) of fetal lung development to signal readiness for parturition. Could ASM peristalsis play a role in scheduling the changes in uterine contractility that herald labour? If ASM peristalsis is part of a pulmonary parturition clock, could we identify pregnancies at risk of preterm labour from airway contractility patterns long before uterine contractility supervenes [98]? Moreover, could such understanding lead us to appreciate that some lung disease associated with prematurity is cause rather than simply effect?

Closing remarks

We already know that ASM can transform lung morphology postnatally. In asthma, transient ASM constriction contributes to reversible lung hyperinflation; chronic ASM-related airway remodelling then participates in irreversible hyper-expansion supervening [99]. Lung hyperinflation that follows tracheal occlusion [100, 101] could even be seen as mimicking hyperinflation due to ASM-mediated small airway occlusions in asthma. Promisingly, pharmacological suppression of ASM hyperreactivity may prevent, halt or even reverse many of these changes in postnatal lung morphology [93, 99]. Given such dramatic pulmonary sequelae of postnatal ASM activity and the effects of ASM-targeted therapy, it is rational to explore the roles of ASM in prenatal lung morphogenesis (and the potential of ASM-related therapy). Our clinical experience reminds us of the toll that abnormalities of lung growth (prematurity, hypoplasia, CCAM, etc.) take on our patients. Therefore by understanding the role(s) of prenatal ASM, paediatric surgeons can make a further contribution to knowledge and therapy of the lung pathologies that we encounter in daily practice.

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