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Review

mTOR signalling, embryogenesis and the control of lung development

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ABSTRACT

The existence of a nutrient sensitive "autocatakinetic" regulator of embryonic tissue growth has been hypothesised since the early 20th century, beginning with pioneering work on the determinants of foetal size by the Australian physiologist, Thorburn Brailsford-Robertson. We now know that the mammalian target of rapamycin complexes (mTORC1 and 2) perform this essential function in all eukaryotic tissues by balancing nutrient and energy supply during the first stages of embryonic cleavage, the formation of embryonic stem cell layers and niches, the highly specified programmes of tissue growth during organogenesis and, at birth, paving the way for the first few breaths of life. This review provides a synopsis of the role of the mTOR complexes in each of these events, culminating in an analysis of lung branching morphogenesis as a way of demonstrating the central role mTOR in defining organ structural complexity. We conclude that the mTOR complexes satisfy the key requirements of a nutrient sensitive growth controller and can therefore be considered as Brailsford-Robertson's autocatakinetic centre that drives tissue growth programmes during foetal development.

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1. Early understanding of growth and nutrient sensing processes in developmental biology

Joseph Needham's *Chemical Embryology* published in 1931 [1] gives a fascinating and exhaustive perspective of the history and cultural understanding of foetal development beginning with Ancient Egyptian methods of artificial bird egg incubation through to the rather lurid descriptions of foetal fluids and membranes,

summarised in 1814 by J.F. John in his "*Chemische Tabellen des Tierreichs*". At the time Needham's book was published, much attention was given to defining a universal quantitative formula for the relationship between tissue size and foetal weight and Needham comprehensively covered this debate arriving at a rather conflicting set of conclusions:

i) Mitotic index falls with increasing age of the foetus, yet, within this constraint,
ii) Different cells of the body mature and slow at different rates. For all that, the gross pattern of foetal weight gain over time shows,
iii) S-shaped kinetics that is characteristic of an endogenous growth controller, a so-called "autocatakinetic" response.

This last view is probably the most interesting since it is the first acknowledgement that a critical control point governs the size of growing biological systems. The main proponent of this "autocatakinetic hypothesis" was Thorburn Brailsford-Robertson, a Scots-born Australian, who in 1908 argued that "*a master reaction…would act as the limiting factor of growth, and would impress its own particular character on the general appearance of the whole process from the outside*" [2]. With considerable foresight, he argues that this master reaction would likely be an endogenous cell regulator whose key characteristic being that it was nutrient sensitive and capable of directing the related processes of what we now know to be gene expression and protein synthesis, a concept dismissed by Needham as being "*definitely out of court*". It was therefore clear from the early 20th century that individual cells possessed some kind of nutrient-sensitive regulator of cell growth and differentiation; 83 years later, Heitman et al. [3] made the link between rapamycin, a macrolide inhibitor of cell growth produced by *Streptomyces hygroscopius*, and yeast kinases, target of rapamycin (TOR) 1 and 2 [4,5]. 4 years later a single mammalian homologue was identified which shared the nutrient and rapamycin sensitive properties of its yeast ancestors and was ultimately named mTOR [6–8]. Thus, although modern day understanding of eukaryotic TOR signalling originated from yeast biology, the philosophical origins of a nutrient sensitive "autocatakinetic" regulator of cell growth is firmly rooted in developmental biology and has a documented history that dates back 5000 years to the beginnings of Egyptian agrarian civilisation and mass food production. With this in mind, and as tribute to the foresight of Brailsford-Robertson and his predecessors, the purpose of this review is to identify the key roles played by mTOR during embryogenesis and development. By taking the lung branching morphogenesis programme as an example, we show how this kinase regulates the formation of complex organ structures by impressing "*its own character on the general appearance of the whole process from the outside.*" [1].

2. The initiation of protein synthesis and growth in the fertilised oocyte

The first moment of embryonic development begins with the controlled reversal of metaphase II growth arrest in oocytes in response to the acrosome reaction at the head of the sperm that enables gamete fusion and exocytosis of the sperm nucleus into the egg. In mammals, this event is coordinated by a series of calcium signalling events which begin with the interaction between the sperm head and an oocyte surface matrix glycoprotein, zona pellucida 3 (ZP3) [9]. ZP3 stimulates production of phosphatidylinositol-(3,4,5)-triphosphate in sperm membranes inducing downstream Akt signalling (a regulator of mTOR but unconfirmed in sperm) and release of phospholipase Cζ, leading to

Ca^{2+} spikes in the oocyte which persist for hours after fertilisation. These Ca^{2+} oscillations are necessary and sufficient for orchestrating all fertilisation events including exit from metaphase II (MII) arrest and the initiation of cell cycle activity and so can be thought of as the cue that mediates the very first induction of transcription and translation. MII arrest is sustained in quiescent oocytes by activation of the cyclinB/cell division cycle protein 2 kinase (cdc2) heterodimer, collectively known as maturation promoting factor (MPF). This prolonged activity is unique to MII and is maintained by a cytostatic factor (CSF) known as early mitotic inhibitor 2 (Emi2) which prevents the targeted degradation of cyclinB by sequestering cdc20, an activator of the anaphase promoting complex/cyclosome (APCC) ubiquitin ligase (Fig. 1). On fertilisation, Ca^{2+} oscillations induce cyclic calmodulin kinase II (CamKII) activity which phosphorylates Emi2 at a RSST motif spanning amino acids 192-5, inducing a strong interaction with polo like kinase-1 (Plk1) leading to a secondary Emi2 phosphorylation within its N-terminal phosphodegron domain ($DSGX_nS$)[10,11]. In this configuration, Emi2 is ubiquitylated by SCF(βTrCP) and its clearance permits cdc20 to activate APCC and degrade cyclinB enabling the first mitotic cell division [10–15] (Fig. 1).

De novo protein synthesis is required to activate and then sustain zygote gene expression and so represents a critical first step for initiating embryonic development [16]. Interestingly, the form and character of protein expression is influenced by the frequency and duration of Ca^{2+} spikes [17] raising the possibility that CamKII transduces these events towards a programmed induction of translation. Since de novo gene transcription does not occur during oocyte MII arrest, the first phase of protein synthesis occurs by translation of mRNA derived from the maternal genome, retained in the oocyte as stable dormant transcripts through sexual maturity and ovulation. Cytoplasmic polyadenylation binding element protein-1 (CPEB-1) plays a central role in this process by mediating the silencing, storage or activation of maternal transcripts through its interaction with 3′-untranslated mRNA regions. Studies in zebra fish demonstrate that CPEB isoforms mediate a cascade of events from initial activation of the transcriptome in the fertilised oocyte through to the establishment of tissue patterns during embryogenesis [18]. A more detailed analysis of this cascade in fertilised mammalian embryos revealed CPEB1 initiates a positive feedback loop involving up-regulation of RNA binding proteins including DAZL (deleted in azoospermia-like) whose expression is essential for regulating other proteins involved in mitotic spindle assembly and function [19]. CPEB-1 is also a calcium-activated CamKII substrate whose phosphorylation in non-embryonic tissues has been shown to promote its interaction with 3′ untranslated region (UTR) of mRNA, induce its polyadenylation and so increase the efficiency of gene expression [20,21]. Though yet to be demonstrated in 1-cell embryos, this protein seems to show the type of acutely responsive calcium-dependent regulation that could co-ordinate calcium signalling intensity with the expression of distinct maternal mRNAs as the first step in protein synthesis.

Protein synthesis cannot occur, however, without ribosome assembly and the initiation of a translational cap complex and so CEBP-1 function must be partnered with regulators of this process. mTOR is well established as the kind of nutrient-sensitive regulator of cell growth which fits beautifully with Brailsford-Roberson's concept of an "endogenous, nutrient-sensitive autocatakinetic growth regulator". mTOR forms the catalytic core of two distinct multi-protein complexes, mTORC1 (composed of mTOR, mLST8/GβL and regulatory-associated protein of mTOR (raptor)) and mTORC2 (mTOR, mLST8/GβL and rapamycin-insensitive component of mTOR (rictor)). mTORC1 displays acute sensitivity to the mTOR inhibitor, rapamycin and phosphorylates the key proteins in the initiation of ribosome assembly, (S6 kinase 1 (S6K1) and translational initiation, eIF4E binding protein 1 (4EBP1)). mTORC2,
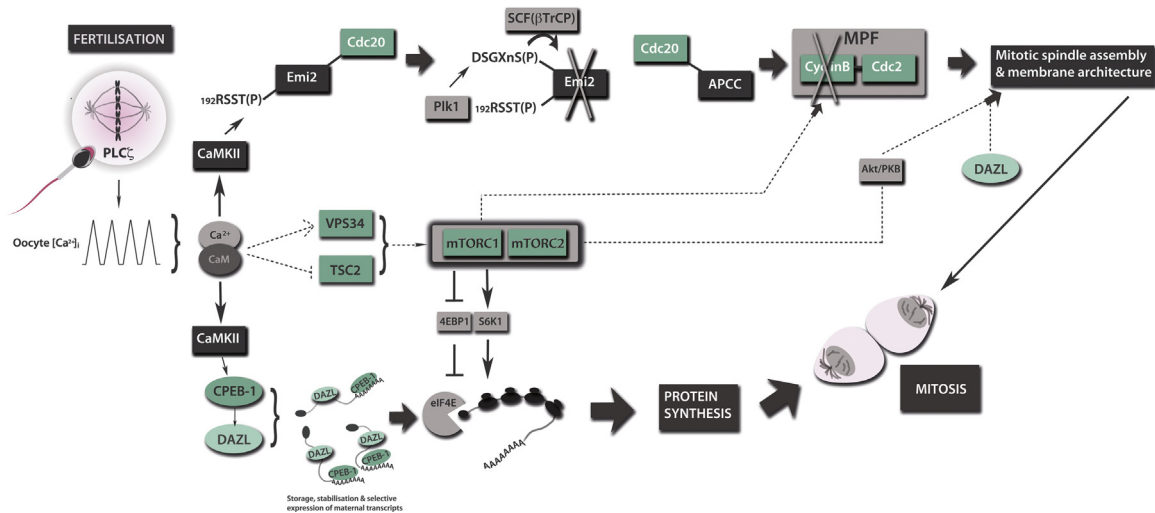
Fig. 1. Role of the mTOR complexes in the first embryonic cell division. On fertilisation, the acrosome reaction induces PLCζ activity which initiates Ca^{2+} oscillations in the oocyte, the intensity and periodicity of which control the reversal of MII arrest, genome activation, translation of oocyte transcripts and subsequence cell division. The schematic shows the Ca^{2+}/CaM/CaMKII pathway as an important mediator of these events which, 1) relieves MII arrest by promoting a cascade of phosphorylation events which culminates in APCC-dependent cyclin B destruction (see text for details) and, 2) activates the expression of maternal transcripts through phosphorylation of CPEB-1 and its downstream regulation of DAZL (lower branch of figure: see text for details). Calmodulin/CamKII are known to induce mTOR complex signalling through VPS34 and TSC2 pathways in other systems and, once active, may drive cap-dependent translation (mediated by 4EBP1 and S6K1), influence cell cycle activity (mTORC-1 regulation of cyclin-B) and promote mitotic spindle assembly and cytoskeletal re-organisation during cell division (regulated by mTORC2). The central position of mTOR in controlling translational activation and the close association between Ca^{2+} oscillation periodicity and genome activation implies a fundamental link between these two signalling pathways that drives the first cell division of life. The dotted lines highlight candidate signalling interactions that may be at the heart of this link.

on the other hand phosphorylates Akt/PKB at Ser473 and works in tandem with phosphoinositide-dependent kinase 1 (PDK1)-catalysed phosphorylation at Thr308 to induce full activation of the enzyme [22]. Thus, mTORC2 regulates the involvement of Akt/PKB in cell proliferation, growth, survival, and metabolism. As with CEBP1, however, it is maternal mTOR transcripts and protein rather than de novo mTOR expression which drives protein synthesis through the first cell division. This is demonstrated by germ line knockouts of mTOR, mLST8, rictor or raptor which each fail to prevent maturation of the fertilised oocyte from zygote to blastocyst stages and only begin to disrupt embryonic growth from early implantation and beyond (E5.5 in mice) [23–25]. Interestingly, experimental knockdown of either rictor or raptor in fertilised mouse 1-cell embryos suggests that maternal mTORC1 and mTORC2 may play distinctly separate roles in the initiation of the first mitotic cleavage. mTORC1-dependent induction of S6K1-T389 phosphorylation appears necessary for MPF degradation and, more generally, may influence cdc2 activity during mitosis [26]. mTORC2-mediated Akt/PKB Ser473 phosphorylation, on the other hand, is indispensable for the first mitotic cleavage, acting through Rho GTPases to organise the mitotic cytoskeleton [27,28]. So how might mTOR complex activity be linked to the Ca^{2+} spikes that initiate the first cell division of life? Evidence for direct CamKII regulation of the type needed to co-ordinate mTOR signalling with Ca^{2+} spike amplitude and frequency during and following fertilisation is weak. Calmodulin (CaM), an essential component of CamKII activation, is known to interact with the carboxyl-terminus of Tuberous Sclerosis Complex 2 (TSC2) which functions as an upstream repressor of mTOR [29]. Mutation or deletion of this site repressed steroid/nuclear receptor transcription, however, the viability of animal models carrying mutations that disrupt the TSC2 gene (the EKER rat, for example [30]) argues against a significant role for this mode of Ca^{2+}-regulated mTOR function in oocyte fertilisation. It may be that other Ca^{2+}-regulated inputs to the mTOR pathway mediate the primary response of protein synthesis to Ca^{2+} spike amplitude and frequency. One strong candidate for this role is the class III phosphatidylinositol-3 kinase (PI3K) regulator, vacuolar protein sorting protein 34 (Vps34), that

phosphorylates phosphatidylinositol at the 3-position to produce phosphatidylinositol-(3)-phosphate (PI(3)P), a membrane marker for both intracellular trafficking and autophagy [31]. In non-foetal systems, increased nutrient availability (amino acids, for example) has been shown to induce Ca^{2+} signalling and CaM binding to Vps34 that is required for lipid kinase activity and increased mTORC1 signalling [32,33]. Though unexplored in the context of oocyte fertilisation, Vps34 may be the missing link between Ca^{2+} spike amplitude, mTORC1 and translation that controls the first cell divisions of early embryonic life. Fig. 1 highlights the key roles of mTORC1 and mTORC2 during the reversal of MII arrest, initiation of maternal RNA translation and organisation of the mitotic cytoskeleton and hypothesises VSP34 and TSC2 as possible links with calcium oscillations which occur during fertilisation.

3. mTOR, stem cells and embryonic nutrient supply

A key implication of Brailsford-Robertson's concept of auto-catakinetic foetal growth regulation is that it must link nutrient supply to the behaviour of stem cells in the ectodermal, endodermal and mesodermal germ layers. This was one of the more controversial hypotheses proposed at the time but we now know that both mTOR complexes regulate cell growth and autophagy in response to various nutrient cues and so play key roles in determining embryonic germ layer formation and the establishment of embryonic nutrient supply. Knockout studies show that endogenous (i.e. foetal-derived) mTOR complex function supersedes maternal mTORC1 and 2 activity during blastocyst development around the time that inner cell mass (ICM) and trophectoderm cells proliferate to form foetal germ layers and the placental interface respectively. Guertin et al.'s [25] study of the effects of raptor, rictor and mLST8/GβL knockout demonstrated that all mTORC1 components are essential for embryonic progression beyond the blastocyst stage and die shortly after implantation (E5.5) whereas individual knockout of rictor or mLST8/GβL tend to delay but not prevent further embryogenesis (E10.5). This has led to a particular interest in the role of mTORC1 as a critical link between the factors that control

embryonic stem cell proliferation, fate and nutrient supply to the developing foetus.

Embryonic stem cells (ESC) grow from the inner cell mass (ICM) to form the epiblast layer of the late blastocyst and participate in a highly organised developmental process that establishes all somatic foetal tissues. This begins before embryonic implantation into the uterine wall and is influenced by the hypoxic (PO_2 <13 mmHg), glucose-restricted but amino acid rich qualities of uterine fluid [34–36]. mTOR, presumably in its mTORC-1 configuration, plays a critical role in maintaining ESC pluripotency and proliferation by stabilising the transcriptional activities of Sox2, Oct-4 and NANOG, repressing cell cycle inhibition by cyclin G2 and PDCD4 and expression of differentiation-promoting transcription factors such as Mix1 homeobox-like 1 (MIXL1), T gene, and the homeobox protein PITX2 [37]. Its inhibition promotes ESC lineage differentiation towards mesoderm and endodermal cell fates together with an increase in cell motility and lowered rates of proliferation; conversely, recovery of mTOR activity stabilises stem cell pluripotency. This places mTOR as a signalling hub that controls the relationship between nutrient availability, ATP supply and the metabolic demands of ESC growth (see [38] for a recent review). The subtlety of this relationship was recently demonstrated in a non-embryonic setting by Yilmaz et al. [39] who showed that mTORC1 is essential for transducing variation in calorie intake with intestinal stem cell proliferation. In this system, Paneth cells at the base of the intestinal crypts act as paracrine regulators of neighbouring intestinal stem cells (ISC) through mTORC-1 regulated silencing of bone stromal protein-1 (Bst-1) and cyclic ADP ribose (cADPR) release. Calorie restriction suppresses mTORC-1 activity and so relieves this silencing effect enabling paracrine cADPR induction of mTORC1 in neighbouring ISCs which induces their non-differentiating proliferation. The capacity of this system to link ISC proliferation with calorie intake is profoundly illustrated in periodic feeders (Burmese pythons, for example) which experience a near 5-fold increase in villi length and doubling of gut mass within 24 h of ingesting a meal [40]. Whether a similar kind of process operates in ESC cells of the blastocyst during early development in vivo is unknown, however, foetal stem cell progenitors show a clear link between nutrient availability and proliferation versus determination of cell fate [41]. The evidence generally supports mTOR as an "autocatakinetic" centre which links nutrient availability to stem cell proliferation and pluripotency in a number of systems including ESC.

4. mTOR and the establishment of placental nutrient transport

Given its key role in nutrient sensing, calorie-monitoring and the determination of embryonic cell fate, it is unsurprising that mTOR signalling is central to the development of the placenta as the nutrient and gas exchange interface for the foetus. The placenta develops by penetration of trophoblast cells lining the perimeter of the blastocyst into the uterine endometrium and involves staged transitions of trophoblast behaviour beginning with adhesion to the uterine epithelium, phagocytosis of the epithelial layer followed by invasion of the underlying decidual stroma. These events coincide with increased expression of the system $B^{0,+}$ amino acid transporter [42] which initiates trophoblast mTOR signalling by uptake of leucine or arginine [43]. The mTOR complex constituent knockout studies of Guertin et al. [25] confirm the earlier observations of Martin and Sutherland [43] that mTOR activation is essential for trophoblast outgrowth and point towards a mTORC1-like protein assembly involving mTOR, raptor but not mLST8 as the main regulator of this process. Knockout of rictor and mLST8 only show lethality at E10.5 well after the onset of placental nutrient exchange and so

appear to be dispensable for trophoblast adhesion, migration and proliferation.

mTOR plays varied roles in each staged transition of trophoblast behaviour. Focal adhesion of trophoblast cells to the uterine epithelium involves the expression of extracellular matrix bridging proteins in both cell populations [44,45]. Of the numerous such proteins induced during the implantation period, the secreted integrin receptor binding protein known as secreted phosphoprotein 1 (SPP1 or osteopontin) plays a particularly key role in directing mTOR signalling among a plethora of integrin-specific responses ranging from cell and ECM adhesion, cytokinesis and cell survival. SPP1 interaction with the trophoblast integrin heterodimer aVb6 and uterine epithelial integrin aVb3 induces focal adhesion molecule assembly that may be particularly essential for stabilising embryo implantation and trophoblast invasion of the uterine stroma [46]. From here, focal adhesion kinase (FAK) orchestrates PI3K, Akt, mTOR, ERK1/2 and p38 MAPK signalling to regulate temporal cell adhesion and cytoskeletal remodelling during cytokinesis and trophoblast expansion [47,48].

mTOR is implicated during this time as a link between paracrine and nutrient cues that influence trophoblast cell growth. Upregulation of glucose and amino acid transporters in trophoblast cells and uterine epithelial cells by progesterone and secreted interferons induce cell proliferation by increases in mTOR signalling and regulated genes in each of these compartments [49,50]. This close link between nutrient transport, placental growth and mTOR function explains why maternal calorie intake or variation in placental metabolite exchange can lead to foetal growth insufficiency or foetal hypersoma (gigantism) and further underscores the role of mTOR kinase as a primary centre for co-ordinating nutrient and growth signals in foetal and maternal compartments during pregnancy.

5. Gastrulation and early organogenesis

Gastrulation is the first major morphogenetic event in embryonic development which establishes the distribution of the mesoderm and endoderm layers that produce patterned body axes. This process requires a balance between cell movement across specific regions of the embryo followed by regulated growth to establish the basic tissue architecture necessary for organ development. The fundamental requirement for mTORC1 in early embryonic growth masks the more subtle roles that this kinase may play in tissue patterning during gastrulation, however, several signalling pathways that converge on mTOR are implicated in the process. Fibroblast growth factor (FGF) signalling to tyrosine kinase receptors is a fundamental aspect of foetal morphogenesis and, in gastrulation, regulates mesodermal cell fate and cell cytokinesis through ERK1/2-dependent regulation of the transcription factor Brachyury and its genomic target, Wnt11 [51–53]. Polarised cell movement induced by FGF also activates protein kinase C (PKC) and the generation of Ca^{2+} waves to regulate localised Rho GTPase activity and the outgrowth of cell protrusions [54–56]. Variations in ERK1/2 and PI3K signalling downstream of the tyrosine kinase receptor, ErbB, act sequentially to regulate different aspects of cell behaviour, with PI3K activity necessary for cell adhesion and spreading whereas ERK1/2 drives cytokinesis [57]. Since PI3K and MAPK pathways feed-back to one-another and input to mTOR signalling, this hub acts as a co-ordination centre for the regulation of cell movement and proliferation during gastrulation and the beginnings of organogenesis.

The relationship between PI3K and mTOR signalling in determining post-gastrulation cell fate is particularly well described during neural tube development. Early studies demonstrated that loss-of-function mutations in mTOR led to failure of forebrain

expansion in mice [58] whereas constitutive activation of mTOR by loss of TSC2 caused excessive forebrain development [59]. This association between mTOR function, neuronal cell differentiation and population expansion explains the appearance of tuberous brain projections that are composed of mixed cell phenotype in Tuberous Sclerosis Complex (TSC) patients carrying mutations in the mTOR repressor genes, TSC1 or 2 [60]. This suggests that temporal and spatial mTOR activity in brain determines tissue patterning and cell fate during neuronal development. Neural tube development is the earliest stage of neurogenesis in late gastrulation and PI3K-mediated signalling to mTOR influences the number and distribution of differentiated neurones during this process. Loss of PI3K signalling to Akt/PKB and mTOR by, for example, inactivation of PDK1, results in fewer neurones and axon tracts whereas more direct inhibition of mTOR using rapamycin maintains neuronal progenitor cell cycle activity but suppresses neuronal differentiation and disrupts axon migration of ready-differentiated cells [61]. Moreover, in an echo of the role played by mTOR in controlling intestinal stem cell turnover (Section 3), Love et al. [41] demonstrate that mTOR acts as a nutrient dependent restriction point that determines the proliferation-differentiation programme of neuron replacement in the foetal retina. Here, slow stem cell growth gives rise to a limited number of progenitor cells that undergo rapid expansion and neuronal differentiation. mTOR inhibition caused by nutrient deprivation exclusively blocks the proliferation and differentiation of progenitor cells without affecting the stem cell population. This suggests a nutrient-sensitive restriction point balances nutrient availability with neuronal cell turnover but does not jeopardise overall regenerative capacity by preventing stem cell growth. By implication, Brailsford-Thompson's "autocatakinetic" centre resides exclusively at the level of mTOR activity in progenitor cells that are primed for proliferation and differentiation and is not located in the basal stem cell population. This characteristic is vital for the sustained growth of complex tissue structures for it retains a reservoir of founding stem cells whilst simultaneously committing a sub-population of progenitors, primed by mTOR signalling, to specific differentiation programmes.

6. mTOR and the development of tissue complexity: lessons from the branching morphogenesis programme of the lung

From gastrulation to birth, foetal organs undergo highly specified programmes of growth and morphogenesis involving the generation of new tissue layers, folding, physical twists and changes in size proportions. This is perhaps best exemplified by the interdependent growth of the lung airway and vascular system which develops as a semi-fractal, branched network of endoderm- and lateral plate mesoderm-derived tubules that ultimately converge at the alveolar blood-gas barrier. Weibel and Gomez [62] were among the first to measure the fractal branching pattern of the airways noting that the decrease in airway diameter for each new branch declined by a constant factor (0.85 as re-analysed by Mauroy et al. [63]). This fractal scaling factor, referred to here by the Greek symbol, φ, is slightly shallower than the predicted "optimal" rate (0.79) suggesting that there is a built-in excess of dead space within the airways which accommodates physiologic change in airway diameter due to exercise, inflammation or disease [63].

The pulmonary vasculature closely follows the course of the conducting airways, sharing the same rate of branching and tubular diameter decline up to the 15th branch generation. Beyond this, vascular branching diverges at a greater rate proceeding through 28 generations to create the microvasculature surrounding each alveolus [64]. The outcome of this branching morphogenesis programme is a compact, yet high gain-of-structure, low resistance gas exchange system of truly colossal proportions: in the airway,

23 branch generations form a conducting network consisting of 17 million branches generating a combined length and gas exchange surface area of 7 km and 130 m^2 respectively, yet the pathway for gas movement from atmosphere to alveolus is not more than 45 cm; these impressive dimensions are eclipsed, however, by the pulmonary vasculature whose fractal pattern and accelerated rate of tubular branching in the gas exchange region of the lung generates an estimated total length of 2–6 thousand km [65].

6.1. The airway branching process

Is there a role for mTOR in the pacing and coordination of this branching morphogenesis programme? Airway outgrowth is induced by fibroblast growth factor-10 (FGF-10) which is secreted from discrete regions of the mesenchyme ahead of each prospective airway branch. On contact with the airway epithelium the tyrosine kinase fibroblast growth factor receptor-2b (FGFR2b) stimulates airway tube elongation towards the FGF-10 signal and so, as the branch grows, a gradient of FGF-10 extends from the growing tip towards the proximal point of each branch [66]. mTORC1 is active among the epithelial progenitor cells at the tip of the airway that are closest to the FGF-10 induction signal. Treatment of nascent lungs with rapamycin consequently blocks cell cycle activity in this region and, in an echo of the signal dampening that produces a controlled reduction in size for each subsequent branch generation, raises non-proliferative airway branching so that the dimensions of each new branch become disproportionately small [67]. Thus mTORC1 activity influences both forward elongation of the airway as well as the branching process.

The duration of forward growth is determined by feedback mechanisms involving sonic hedgehog (Shh) whose secretion from the advancing epithelial tube inhibits FGF-10 expression in the mesenchyme immediately in front of the airway bud causing this induction signal to move laterally, and Sprouty2 (Spry2), an endogenous regulator of FGFR2b downstream signalling. Little is known about the links between mTOR and Shh signalling in lung although associations between these pathways have been shown in other contexts. In brain, for example, Shh directs neurogenic patterning of the developing cerebellum by locally increasing expression of one mTOR substrate (eukaryotic initiation factor 4E (eIF4E)) and suppressing the activity of another (S6K1). Here, Shh gradients set the stage for neural precursor proliferation in some areas of brain and neural differentiation in others [68]. In cancer, Shh and mTOR may also act co-operatively to increase proliferative growth under conditions where TSC2-mediated inhibition of mTOR and the cell cycle checkpoint protein p27^{kip1} is defective [69]. In lung development, shh has an additional role to play in regulating canonical growth factor signalling through bone morphogenetic protein 4 (BMP4), followed by noggin, that establishes the pattern of parabronchial smooth muscle deposition around the airway tubes [70]. It is tempting to speculate that the kind of shh-mTOR signalling seen in brain development can contribute to this process in lung and, indeed, may drive the pathological deposition of pulmonary smooth muscle seen diseases linked to sustained mTOR signalling such as lymphangioleiomyomatosis (LAM) [71].

Spry2 plays a much more direct role in delineating airway epithelial tubular growth patterns in response to FGF-10/FGFR2b signalling. Spry2 is well known to antagonise tyrosine receptor kinase signalling to ERK1/2 and its knockout in lung results in airway epithelial branching defects [72–74]. Its activation centres on a conserved N-terminal tyrosine residue at position 55 (Y55) whose phosphorylation by FRS2α-activated Src kinase [75] is necessary for its interaction with both protein phosphatase 2A (PP2A) and the ubiquitin ligase, cCbl (reviewed [76]). Although
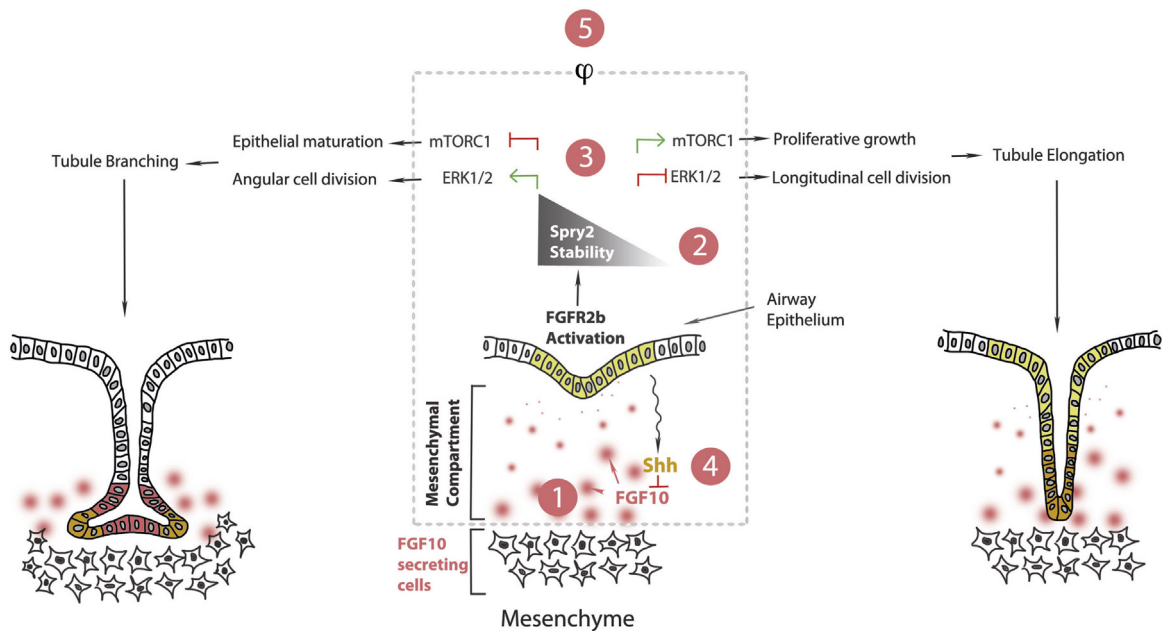
Fig. 2. The molecular basis of φ in the airway branching programme of the lung. Temporal and spatial interactions between FGF-10, epithelial FGFR2b, Spry2 and sonic hedgehog (Shh) establish the fractal patterning of airway branching. Each signalling cycle proceeds as follows: 1) The concentration of FGF-10 secreted from mesenchyme cells ahead of prospective airway branches determines the duration of FGFR2b activity in the epithelial cells of the airway tube (yellow indicates FGFR2b-active airway epithelial cells). 2) FGFR2b evokes Spry2 activation, observed as loss of Spry2 stability as upstream inducers of ERK1/2 (e.g. Grb2, [76]) or repressors of mTORC1 (e.g. TSC2, [67]) form complexes with the Spry2-cCbl ubiquitin ligase and are targeted for degradation. The result is antipodal regulation of ERK1/2 and mTORC1 (note that this mechanism is unlikely to be reproduced by experimental Spry2 knockdown (e.g. [73,74]). 3) Tubular outgrowth occurs when Spry2 is destabilised (active) promoting epithelial mTORC1 activity and suppressed ERK1/2 activity. Conversely, branching epithelial growth is promoted when Spry2 is stable (inactive) and ERK1/2 activity is at its height, promoting re-orientation of the spindle angle from the longitudinal axis of growth (see [74]). 4) Secretion of Shh from airway epithelium induced as a consequence of sustained FGFR2b activation suppresses the production of FGF-10 ahead of the growing airway tube causing the FGF-10 signal to move laterally and promote a new cycle of growth. 5) A scaling factor, φ, whose complete molecular identity is currently unknown, dampens the rate of signalling through this cyclical pathway with successive cycles and establishes the fractal pattern of the conducting airways of the lung.

the precise mechanism of Spry2 activation remains uncertain, it seems that PP2A de-phosphorylates two serine residues in a serine rich region of the protein (Ser115 and Ser118) to induce a conformational change that reveals a proline rich tail capable of binding SH3 domain containing proteins and their partners [77]. By acting as an adaptor protein for the cCbl ubiquitin ligase, Spry2 therefore targets specific cell signalling components for ubiquitylation and degradation. In airway epithelium, FGF-10 promotes Spry2 association with the FRS2α/Grb2/Raf signalling complex and disrupts signalling to ERK1/2 by targeting Grb2-associated proteins for proteasomal destruction [76–79]. However, under the same conditions, Spry2 also forms a complex with TSC2 and cCbl which induces Rheb-GAP activity and so promotes mTORC1 activity that is coordinated with the airway branching process [67]. Thus, far from simply antagonising tyrosine kinase receptor signalling, Spry2 acts as a FGF-10-regulated switch that partitions signalling between mTORC-1 and ERK1/2 and so plays a crucial role in linking the airway outgrowth cues with downstream growth responses, a central feature of any molecular regulator of fractal growth. Recent work has developed the airway branching model further by showing that ERK1/2 activation increases the mitotic spindle angle of epithelial cells away from the longitudinal axis of tubular growth. Spry2 therefore functions to orchestrate proliferative forward growth associated with mTORC1 activation and lateral branching growth associated with ERK1/2 signalling along the FGF-10 gradient [67,74]. By extension, the interaction between FGF-10, FGFR2b, Spry2 and Shh and downstream effects on TORC1 and ERK1/2 activity can be described as part of the branching periodicity "clock" mechanism that drives branching morphogenesis of the airways and which acts as a molecular engine that defines φ in the lung (Fig. 2).

6.2. The vascular branching process

Vascular growth follows the airway branching programme and is initiated by the secretion of vascular endothelial growth factor-A (VEGF-A) from the epithelial cells of the advancing airway tube. VEGF-A gene expression is regulated at multiple levels (transcription factor binding at a 1.2 kb (mouse, rat) to 2.362 kb (human) 5′ promoter region; mRNA stability through protein interaction with its 3′ untranslated region; mRNA translation via internal ribosome entry site (IRES) sequences in the 5′ UTR) and is intrinsically sensitive to external factors such as oxygen and growth factors (reviewed [80]). Low oxygen tensions are a persistent feature of the foetal environment, ranging from 0 to 13 mmHg during embryonic implantation to ~30 mmHg throughout gestation (reviewed, [81]). Raising oxygen partial pressures beyond this range inhibits vascular growth [82] and is a contributing factor to neonatal conditions such as bronchopulmonary dysplasia. Oxygen-sensitive VEGF-A expression is regulated by hypoxia inducible factor (HIF) binding to a HIF consensus site (TACGTGGG) located in the 5′-proximal VEGF-A promoter. HIFs are heterodimeric transcription factors comprising an oxygen regulated α subunit and a nuclear translocating β subunit. In hypoxia, the α subunit is stabilised, dimerises with the β subunit and is translocated to the nucleus by association with the members of the nuclear importer family, importin α1, 3, 5 or 7 [83]. Three HIFα isoforms are expressed in the developing lung. HIF-1α occurs in the branching airway epithelium and its homozygous knockout in mice causes developmental failure of the vasculature and death by E10.5 [84]. HIF-2α occurs in the epithelium and interstitium and its knockout produces vascular defects during alveolar septation [85]. HIF-3α is a truncated isoform that lacks the C-terminal transactivation domain necessary for interaction with
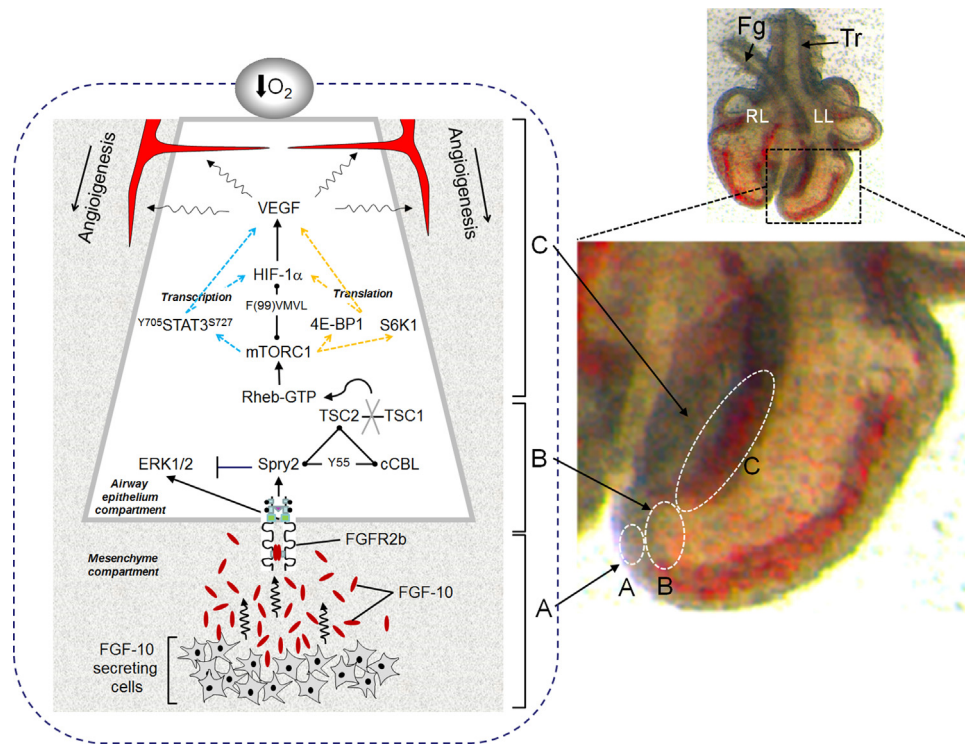
Fig. 3. mTORC1 and the co-ordination of airway and vascular growth in the foetal lung. Modified from Scott et al. [64] (with permission) to reflect the varied mTORC1 inputs into HIF-1α signalling and VEGF-A production [94,102]. Dotted arrows indicate pathways inferred from non-developmental systems. Right hand panel shows an embryonic stage rat lung where perfused vasculature can be seen surrounding the airway branches and proximal to the airway tip (red hue in expanded image). Signalling in three zones of airway and vascular growth (A, B and C) are indicated: Zone A: FGF-10 is expressed in the mesenchyme ahead of nascent airway buds and diffuses towards the airway epithelium. Zone B: FGF-10 induces FGFR2b autophosphorylation and signalling to Spry2 whose activation inhibits signalling to the ERK1/2 pathway and also induces TSC2 clearance enabling Rheb to bind GTP and activate mTORC1. Zone C: HIF-1α, maintained stable by the low oxygen partial pressure of the foetal environment, is regulated through the FGF-10-Spry2-mTORC1 signalling axis by a direct interaction involving the putative FVMVL TOS motif between amino acids 99–103. In addition, it is likely that HIF1α transcription and translation is augmented by: i) phosphorylation of STAT3 at Y705 and the mTORC1 substrate site S727 and/or ii) co-operative activation of 4E-BP1 and S6K1. These mechanisms are also implicated in VEGF-A transcription and translation. Fg, foregut; Tr, trachea; RL, right lung lobules; LL, left lung lobules.

p300/CBP and so is thought to act as a competitive repressor of HIF-1α and 2α activity [86]. Thus, HIF-1α plays the primary role in controlling vascular signalling during lung development, whereas HIF-2α function is predominantly associated with the maturation of vascular structures and the generation of the blood-gas barrier [87]. Although growth factors are well known to regulate VEGF-A expression through other transcription factors (e.g. STAT3, Sp1, AP2; [80]), it seems that HIF-1α and 2α-dependent regulation of vascular growth and blood vessel maturation is critical for generating the type of vascular growth, reported by Lazarus et al. [88], that is necessary for three dimensional lung branching. Proline hydroxylase domain (PHD) proteins critically regulate HIF-1–3α stability during lung development, as their inhibition augments HIF target gene expression and promotes blood vessel growth and formation of the blood gas barrier [89–91]. Of the three PHD isoforms, however, PHD2 appears to exert primary regulation of HIF-1α as its knockdown exclusively induces HIF-signalling activity and gene expression in normoxia, whereas knockdown of PHD1 and 3 does not [92]. Although airway and vascular development is supported better at foetal rather than postnatal PO_2, and hyperoxia causes vascular defects due to silencing of the HIF signalling system, oxygen gradients around the airway tip are too shallow to direct localised HIF regulation of VEGF-A (~15 mmHg: difference between the PO_2 of the umbilical vein (30 mmHg) and the amniotic fluid (15 mmHg; [93]). The low PO_2 of the foetal environment therefore primes the HIF system by stabilising the α-subunit with local regulation mediated by the growth factors and repressors of the airway branching periodicity "clock" mechanism that drives branching morphogenesis (Fig. 3).

6.3. mTOR and the co-ordination of airway and vascular branching

Scott et al. [67] investigated the mechanism that links the FGF-10 airway growth cue with HIF-1α driven VEGF-A secretion in foetal airway epithelial cells. When foetal airway epithelial cells were cultured at the low PO_2 that naturally reflect the foetal lung environment, FGF-10 augmented HIF-1α activity above the level produced by hypoxia alone, was kinetically matched by Spry2 destabilisation and mTORC1 activation and was entirely abolished by rapamycin. mTORC-1-dependent amplification of HIF-1α activity is widely observed in response to growth factor cues and has been demonstrated in primary and cell line culture systems with widely ranging phenotypes. A functional interaction between the Raptor scaffold protein of mTORC1 and the N-terminus of HIF-1α has been reported to amplify basal hypoxic induction of HIF-1α by increasing its interaction with the transcriptional co-activator CBP/p300 [94]. Raptor binds mTORC1 to its substrates by interacting with conserved penta-peptide sequences known as mTOR signalling (TOS) motifs (FDL/IDL and FEMDI within S6K1 [95] and 4E-BP1 [96,97] respectively). Mutation of the highly conserved phenylalanine at position 1 of the TOS motif prevents mTOR binding and subsequent phosphorylation of its target residues. In an echo of this, a TOS-like dependency for a series of residues between 99 and 103 of HIF-1α (FVMVL) is necessary for growth factor-sensitive regulation of HIF-1α transcriptional activity [67,94,97]. In this case, alanine mutation of the leading phenylalanine residue retains the hypoxic induction of HIF activity but abolishes growth factor regulation of this transcription factor and its efficient interaction with
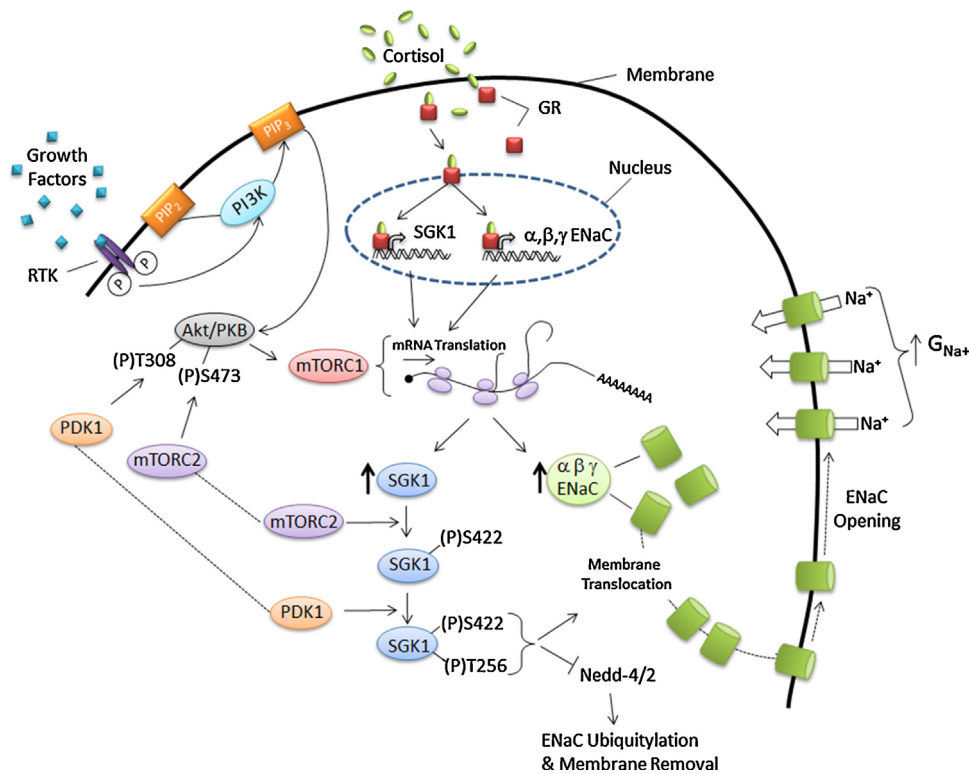
Fig. 4. mTORC1 and mTORC2 play discrete roles in glucocorticoid- and growth factor-induced epithelial Na⁺ transport. Glucocorticoids (cortisol) activate Na⁺-driven fluid clearance from the lung at birth. This schematic illustrates the central role of the mTOR complexes in linking growth factor and glucocorticoid pathways to the expression and membrane translocation of the ENaC subunits. Here, cortisol induces transcription of SGK1 and α, β, γ ENaC subunits whose translation is subject to regulation by mTORC1 signalling to 4EBP1 and S6K (not shown). Simultaneous activation of PI3K signalling induces ENaC subunit trafficking to the membrane through a process that involves mTORC2 and PDK1 at two points: 1) Akt/PKB phosphorylation at S473 (by mTORC2) and T308 (by PDK1), and 2) SGK1 phosphorylation at S422 (by mTORC2) and T256 (by PDK1). Dual phosphorylation of SGK1 induces an inhibitory phosphorylation of the Nedd4/2 ubiquitin ligase and so promotes ENaC trafficking to the cell membrane [111–114]. At birth, ENaC opening increases Na⁺ absorption from the lung lumen, promoting fluid clearance from the lung and the first breaths of life.

CBP/p300 [67,94]. Thus, spry2 mediated mTORC1 may link airway outgrowth with vascular signalling by directly controlling the efficiency with which the HIF-CBP/p300 transcriptional complex catalyses histone deacetylation and DNA de-condensation along the VEGF-A gene.

A number of alternative modes of HIF-1α regulation by mTORC1 have also been proposed including: 1) stabilisation of HIF-1α [98], 2) regulation of HIF-1α mRNA translation by 4E-binding protein 1 (4E-BP1) [99] or ribosomal protein S6 kinase 1 (S6K1) [100] and 3) feedback regulation of mTORC1 regulation of HIF-1α by REDD1 (regulated in development and DNA damage responses 1), a hypoxia-inducible factor-1 target gene [101]. Dodd et al. [102] have consolidated these diverse inputs into HIF-1α signalling by exploring the association between mTORC1 and its downstream regulation of 4E-BP1, S6K1 and the signal transducer and activator of transcription-3 (STAT3) transcription factor. They demonstrate that, whilst HIF-1α translation rate and protein abundance are set by mTORC-1 regulated co-operative signalling to 4E-BP1 and S6K1, growth factor (insulin)-regulated HIF-1α transcription is driven by STAT3, ready-primed by phosphorylation on Tyr705 (by, for example, ciliary neurotrophic factor [103,104]) to enable mTORC1-dependent ser727 phosphorylation and HIF-1α transcription. Thus mTORC1 signalling inputs into STAT3, 4E-BP1 and S6K1 orchestrate HIF-1α and VEGF-A gene expression by controlling the overall efficiency of HIF-1α transcription and protein translation. Perhaps the most significant implication of this study is the emerging evidence for several mTORC1-mediated inputs into VEGF-A gene expression, for STAT3 interacts co-operatively with HIF-1α at the VEGF-A promoter to drive VEGF-A expression and angiogenesis [80,105,106].

The FGF-10-Spry2-mTORC1-STAT3/HIF-1α-VEGF-A signalling axis represents an essential link between the processes of airway and vascular branching morphogenesis and the current evidence supports a role for mTORC1 in, i) regulating patterns of cell proliferation at the airway tip, ii) influencing branching rate and tubular size and, iii) potentiating the HIF-1α system to drive VEGF-A expression according to the magnitude and distribution of the FGF-10 airway outgrowth cue. These concepts are encapsulated in the model proposed in Fig. 3 which places the interaction between Spry2 and mTORC1 at the centre of the signalling pathway that defines co-ordinated airway and vascular growth in the lung.

7. mTOR, birth and the onset of pulmonary gas exchange

In addition to its stem cell niche and organ structural roles, mTOR controls physiological ion transport events in the pulmonary epithelium which are essential for the foetus to complete the transition from placental to pulmonary gas exchange at birth. Fluid secretion into the airways during development provides a distending pressure which supports the formation of the three dimensional structure of the lung. This is rapidly cleared at birth by a cortisol and catecholamine-driven Na⁺ absorption process [107,108] that regulates the expression, assembly and membrane insertion of epithelial Na⁺ channel (ENaC) α, β, and γ subunits (e.g. [109]). Consequently, factors that prevent the proper membrane trafficking and assembly of these subunits as a functional Na⁺-selective ion channel cause perinatal respiratory distress and risk of death due to fluid retention in the lung (reviewed [110]). Serum glucocorticoid kinase-1 (SGK1) is a PI3K-regulated component of this

pathway whose activation by growth factors works synergistically with the glucocorticoid stimulus to sustain Na$^+$ absorption by preventing ENaC subunit ubiquitylation and clearance through the Nedd4/2 pathway [111]. Watt et al. [112] used a new generation of selective kinase inhibitors to dissect this relationship and found that sequential inhibition of PI3K (PI103), SGK1 (GSK650394) and mTORC2 (TORIN1) abolished the growth factor-(insulin) and glucocorticoid-induced Na$^+$ current whereas short-term mTORC1 inhibition with rapamycin had no effect. Further analysis revealed that TORIN1 effectively abolished Akt/PKB and SGK1 phosphorylation at S473/T308 and S422 respectively, unveiling an exclusive requirement for mTORC2 in the signalling cascade that links PI3K to SGK1 and ENaC membrane recruitment. Similar mTORC2 control over SGK1 and Na$^+$ conductance has been reported in kidney although, here, mTORC1 also regulates ENaC subunit abundance by controlling the translation of ENaC transcripts [113,114]. Fig. 4 summarises the distinct roles of the mTOR complexes in controlling ENaC expression and membrane recruitment. By transducing the endocrine signals which determine fluid absorption capacity in the lung and other epithelial tissues, the mTOR complexes, and in particular, mTORC2, play key permissive roles in controlling events essential for postnatal life.

8. Perspective and conclusions

mTOR complexes 1 and 2 beautifully fit Brailsford-Robertson's concept of a nutrient-sensitive "autocatakinetic" regulator of foetal growth. Maternal mTORC1 drives translation during the first cleavage steps of early embryonic life with newly expressed foetal mTORC1 taking over at the blastocyst stage to define the behaviour of cells in the stem cell niche. Foetal mTORC1 also establishes the placenta as the first developmental organ of nutrient and gas exchange and lays the structural foundations for organogenesis during gastrulation. In the formation of complex structures such as the lung, mTORC1 regulates patterns of tissue growth within the branching morphogenesis programme and is a contributing factor in the molecular timing mechanism that establishes the fractal pattern of the airways. Through its varied interactions with the HIF signalling pathway, mTORC1 links the growth of the pulmonary vascular network to the airway branching morphogenesis programme and so maintains a continuum of nutrient supply to the growing regions of the lung. As the foetus approaches full term, mTORC2 establishes the conditions for fluid withdrawal from the lung so that the first breaths of life can take place in the moments following birth. Far from being "*definitely out of court*" as Needham put it, to extend his tennis analogy, mTOR signalling lays out the pitch, selects the players and umpires play over the net as conditions vary around the court. mTOR is a master regulator of tissue growth and organ complexity at all stages of foetal life.

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