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PI3K signaling in the regulation of branching morphogenesis

Wenting Zhu[a], Celeste M. Nelson[a,b,*]

[a] Department of Chemical and Biological Engineering, Princeton University, Princeton, NJ 08544, USA
[b] Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA

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ABSTRACT

Branching morphogenesis drives the formation of epithelial organs including the mammary gland, lung, kidney, salivary gland and prostate. Branching at the cellular level also drives development of the nervous and vascular systems. A variety of signaling pathways are orchestrated together to establish the pattern of these branched organs. The phosphoinositide 3-kinase (PI3K) signaling network is of particular interest because of the diverse outcomes it generates, including proliferation, motility, growth, survival and cell death. Here, we focus on the role of the PI3K pathway in the development of branched tissues. Cultured cells, explants and transgenic mice have revealed that the PI3K pathway is critical for the regulation of cell proliferation, apoptosis and motility during branching of tissues.

1. Introduction

Branching morphogenesis is the process by which new tubes sprout from pre-existing ones. Branching morphogenesis is used to generate functionally efficient, complex, but well-ordered tissue architectures (Hogan, 1999). The formation of ramified trees is a key feature of many tissues, including the epithelium of the mammary gland, lung, kidney, salivary gland and prostate, as well as the nervous and vascular systems. The branching process enables a large increase in the surface area of the epithelium for functional exchange of gases or secreted products (such as in the lung, kidney and mammary gland) or enables distant places in the organism to be reached (such as in the vascular and nervous systems) (Affolter et al., 2009).

It is important to note that although there are many similarities between these branching tissues, at the cellular level the processes are quite distinct. For epithelial tissues, branching morphogenesis involves the collective reorganization of multiple cells into a new branch or bud. Angiogenic sprouting of new blood vessels also involves collective migration, but here the new branch is led by a single tip cell that directs the morphogenesis of the following chain. For neurites, the branches that form are subcellular actin-rich protrusions of a single cell that permit it to form connections with more than one target. The term "branching morphogenesis" is thus often used to describe widely divergent developmental programs that nonetheless build a ramified architecture.

Branching is typically mediated through changes in cell shape, size, division, invasiveness, motility, or some combination thereof (Affolter et al., 2003; Zhang and Vande Woude, 2003). These cell-level processes are tightly controlled by different signals including hormones, growth factors, receptor tyrosine kinases (RTKs), and extracellular matrix (ECM) molecules (Gjorevski and Nelson, 2011; Metzger and Krasnow, 1999). Among them, the phosphoinositide 3-kinase (PI3K)/Akt signaling network (Fig. 1) is of particular interest because it is crucial to many different physiological processes that include cell proliferation, growth, differentiation, apoptosis, metabolism, and motility (Martelli et al., 2006).

Signaling *via* PI3K can be activated by RTKs, such as the hepatocyte growth factor (HGF) receptor, c-Met (Johnson et al., 1995), as well as G protein-coupled receptors (GPCRs) (Schluter et al., 1998). Upon activation, PI3K translocates to the membrane, where

Abbreviations: BAD, Bcl-2-associated death promoter; ECM, extracellular matrix; EGF, epidermal growth factor; FGF, fibroblast growth factor; GDNF, glial cell line-derived neurotrophic factor; GPCR, G protein-coupled receptor; HGF, hepatocyte growth factor; MAPK, mitogen-activated protein kinase; MM, metanephric mesenchyme; MMP, matrix metalloproteinase; MMTV, mouse mammary tumor virus; mTOR, mammalian target of rapamycin; NRG1, neuregulin-1; PAK, p21-activated kinase; PDK1, phosphoinositide-dependent protein kinase-1; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PIKE, phosphoinositide 3-kinase enhancer; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PKB, protein kinase B; PTEN, phosphatase and tensin homology deleted on chromosome ten; RTK, receptor tyrosine kinase; SMG, submandibular gland; SP, surfactant protein; TEB, terminal end bud; TGF, transforming growth factor; UB, ureteric bud; UGS, urogenital sinus; UPS, ubiquitin proteasome system; VEGF, vascular endothelial growth factor; WD, Wolffian duct.

* Corresponding author at: Princeton University, Department of Chemical & Biological Engineering, A321 Engineering Quadrangle, Olden Street, Princeton, NJ 08544, USA. Tel.: +1 609 258 8851; fax: +1 609 258 0211.
E-mail address: celesten@princeton.edu (C.M. Nelson).

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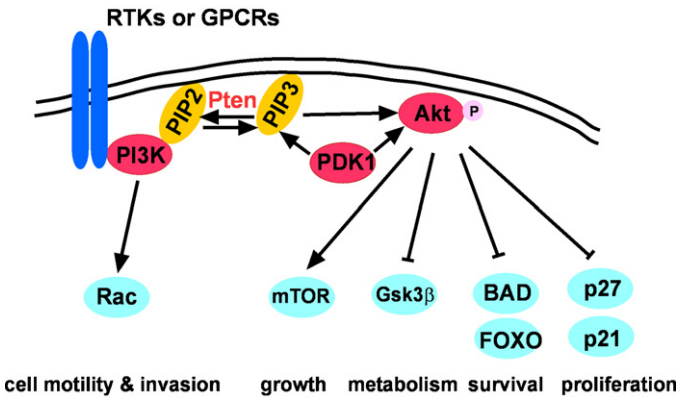

Fig. 1. Overview of the PI3K pathway. Upon activation by RTKs or GPCRs, PI3K translocates to the membrane and converts PIP2 to PIP3. PIP3 then recruits Akt and its activator PDK1 to the membrane where Akt becomes phophorylated and activated. Akt acts as a signaling node and regulates many downstream targets to control multiple cellular processes including cell proliferation, survival, metabolism, growth and motility.

it converts the plasma-membrane lipid phosphatidylinositol-4, 5-bisphosphate (PtdIns(4,5)P2, PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P2, PIP3). PIP3 then recruits both Akt/protein kinase B (PKB) and its activator phosphoinositide-dependent protein kinase-1 (PDK1) to the membrane by binding to their pleckstrin homology (PH) domains, leading to phosphorylation and activation of the serine/threonine kinase, Akt (Matsui et al., 2003). More than 100 nonredundant substrates for Akt have been reported so far, many of which have crucial known cellular functions (Manning and Cantley, 2007). For example, Akt phosphorylates and activates mammalian target of rapamycin (mTOR), which is a central regulator of protein synthesis and cell growth (Wullschleger et al., 2006). Akt also enhances cell survival by blocking the function of proapoptotic proteins including Bcl-2-associated death promoter (BAD) (Datta et al., 1997). Several studies report that Akt inhibits p27, a cyclin-dependent kinase inhibitor, thus promoting cell proliferation (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002). Akt thus acts as a critical signaling node downstream of PI3K.

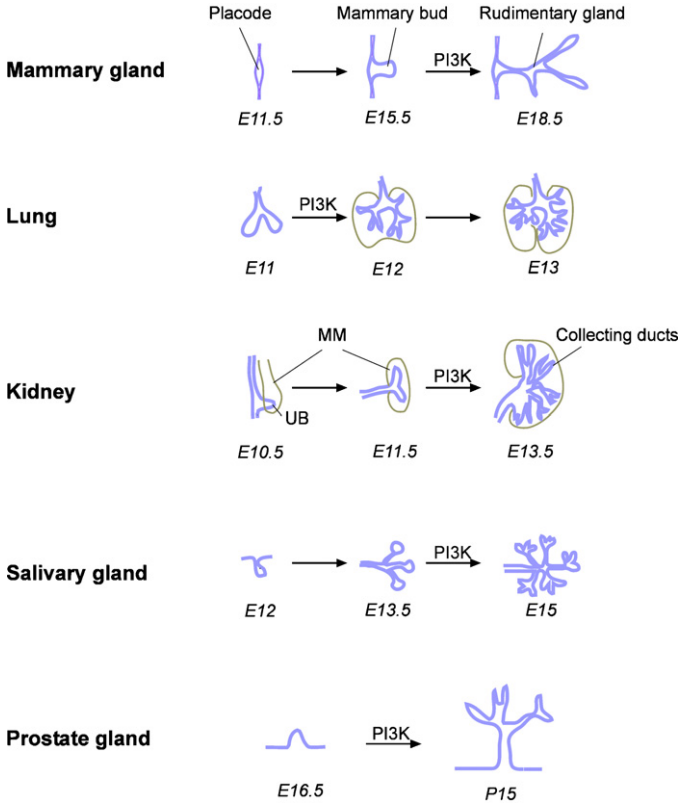

Fig. 2. Branching morphogenesis in epithelial organs.

In addition to Akt, Rac has been indicated as a key downstream effector of PI3K (Kolsch et al., 2008). Activated Rac induces reorganization of the actin cytoskeleton to form lamellipodia and membrane ruffles to facilitate cell motility (Ridley et al., 1992). Rac has also been implicated in the expression of various matrix metalloproteinases (MMPs), secretion of which is important for cell invasion and generating tracks for mesenchymal-type movement (Mack et al., 2011). Due to its effects on cell motility, the regulation of Rac by PI3K is of particular interest for collective

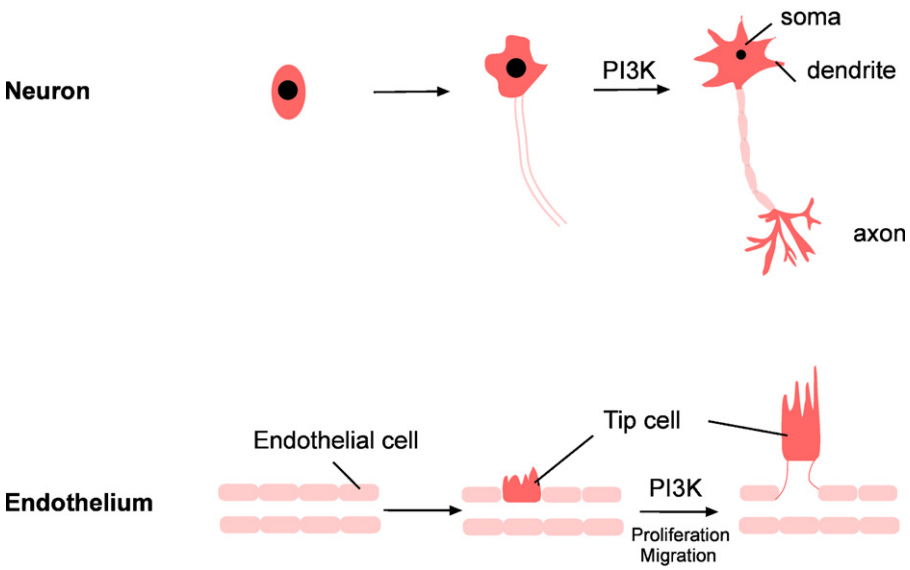

Fig. 3. Branching in the nervous and vascular systems.

migration processes such as those exemplified by branching morphogenesis.

Due to its pro-survival role, the PI3K pathway is tightly regulated by several phosphatases. Among them, phosphatase and tensin homology deleted on chromosome ten (PTEN) is considered the principal regulator of basal cellular PIP3 levels (Leslie and Downes, 2002). PTEN dephosphorylates PIP3 into PIP2, thus decreasing the activation level of the pathway. PTEN was first identified as a tumor suppressor gene located on human chromosome 10q23 (Li et al., 1997; Steck et al., 1997). Mice homozygous for targeted deletions within the PTEN gene (PTEN−/−) die during embryonic development (between embryonic days E6.5 and E9.5), whereas heterozygous mice develop normally but are prone to the acquisition of a wide range of tumor types including cancers of the breast, thyroid, endometrium and prostate as well as T cell lymphomas (Di Cristofano et al., 1998; Podsypanina et al., 1999; Stambolic et al., 1998; Suzuki et al., 1998). As the major negative regulator of PI3K, PTEN is uniquely poised to modulate the effects of this pathway during normal development as well.

In this review, we focus on the role of the PI3K pathway in the branching of epithelial tissues, specifically the mammary gland, lung, kidney, salivary gland, and prostate, as well as angiogenic spouting of the vascular endothelium and axonal and dendritic branching in the nervous system. Tremendous progress has been made in identifying the factors in the pathway that are involved in branching morphogenesis and how these factors affect the cellular activities that control tissue ramification.

2. PI3K Pathway and Epithelial Branching

Epithelial branching morphogenesis is a reiterative process in which a rudimentary epithelial bud extends and bifurcates to form the mature tubular organ (Anderson et al., 2007). Epithelial branching morphogenesis can be subdivided into several steps, including primary bud formation, branch initiation, branch elongation, and reiteration of the branching process (Affolter et al., 2003). Branching is fundamental to the formation of diverse ramiform tissues such as mammary glands, lungs, kidneys, salivary glands and prostate (Fig. 2). Tubular structures can serve many important physiological roles, including control and delivery of gases, nutrients, waste and hormones, and compartmentalization of organ function. Branching of epithelial tissues generates structures that can serve these physiological processes. Tubular epithelia arise from each of the germ layers, ectoderm (*e.g.* mammary gland and salivary gland) (Imagawa et al., 1990; Proctor and Carpenter, 2007), mesoderm (*e.g.* kidney) (Dressler, 2006), and endoderm (*e.g.* lung) (Perl et al., 2002). Although the final forms and functions of these arborized organs differ, many of the major signaling mechanisms that underlie their morphogenesis appear to be conserved.

2.1. Mammary Gland

The ductal network of the mammary gland is formed by mammary epithelial cells. There are three main stages of mammary gland development in both rodents and humans: embryonic, pubertal and adult (Watson and Khaled, 2008). In the mouse, embryonic mammary development begins shortly after midgestation with the formation of five pairs of epithelial placodes that invaginate into the underlying mesenchyme to form the mammary buds, or anlagen (Gjorevski and Nelson, 2011; Robinson, 2007). A limited number of branches then sprout from each anlage to form a rudimentary ductal tree (Sternlicht, 2006). After birth, the rudimentary gland is morphogenetically quiescent and grows just enough to keep up with normal body growth. At puberty, increased levels of ovarian hormones induce the ends of the rudimentary ducts to proliferate and develop into bulbous terminal end buds (TEBs) (Hinck and Silberstein, 2005). The ducts elongate and the TEBs penetrate further into the surrounding mesenchymal fat pad. Clefting of the TEBs results in bifurcation of the ducts to generate new primary ducts. Secondary branches sprout laterally from the primary ducts until the entire fat pad is filled with the epithelial tree (Sternlicht et al., 2006).

Several transgenic and knockout mouse models have been generated to investigate the roles of the PI3K pathway during mammary gland development. It should be noted that since PI3K regulates several critical biological processes, depletion of many of its effectors leads to embryonic lethality, which precludes analysis of the role of PI3K in development and homeostasis of the mammary gland (Vanhaesebroeck et al., 2005). Therefore, tissue-specific overexpression or deletion has been useful in studying the role of this pathway *in vivo* (Table 1). In a mouse model for constitutive PI3K activity, Renner et al. expressed the catalytic subunit of PI3K, p110α, fused to a Src myristoylation sequence, under the control of the mammary-specific mouse mammary tumor virus (MMTV) promoter (Renner et al., 2008). The Src myristoylation sequence forces p110α to be localized to the plasma membrane, which is sufficient to trigger downstream responses characteristic of growth factor signaling, including the activation of Akt, Rac, and S6 kinase (Klippel et al., 1996). These transgenic mice have increased proliferation in the ductal epithelium as compared to their wildtype littermates. These mice also show increased ductal branching, alveolar hyperplasia, and intra-ductal neoplasia, phenotypes which become more obvious as the mice age. Activating PI3K thus appears to promote mammary branching *in vivo*.

As described above, PTEN is a major regulator of PIP3, a direct product of PI3K. Loss of PTEN function either in embryonic stem cells or in human cancer cell lines results in accumulation of PIP3 and activation of its downstream signaling molecules (Leslie and Downes, 2002). Conditional deletion of PTEN in the mammary gland by crossing $PTEN^{loxp/loxp}$ mice with MMTV-$Cre^{+/-}$ transgenic mice results in accelerated ductal extension, leading to earlier filling of the fat pad by the epithelial tree. Excessive lateral branching is also seen in the mutant mice. Deletion of PTEN causes increased cell proliferation in the TEBs and ducts, providing an explanation for the faster ductal extension and enhanced lateral branching in the mutant mice. In addition to excessive ductal branching during puberty, the mice show precocious lobuloalveolar development during pregnancy and delayed involution, coupled with reduced apoptosis. Importantly, PTEN-null mammary epithelial cells are hyperproliferative at all stages, resulting in early onset of mammary tumors (Li et al., 2002). However, in another study in which active PTEN was over-expressed specifically in the mammary epithelium under the control of the MMTV promoter, ductal development during puberty was found to be normal (Dupont et al., 2002). The discrepancy between the two studies may be due to differences in expression, as the level of transgenic PTEN was only twofold higher than in the wildtype littermates in the second study (Dupont et al., 2002). Nonetheless, these mutant mice could not lactate normally and showed a reduced number of alveolar epithelial cells due to a decrease in cell proliferation and an increase in apoptosis, which is consistent with the inhibitory role of PTEN in the regulation of cell proliferation.

In contrast to the effects of PI3K and PTEN, transgenic expression of Akt1 under the control of the MMTV promoter has only mild effects on branching with slight increases observed in one of the two founder lines (Ackler et al., 2002). These transgenic mice have a notable delay in involution upon cessation of lactation, along with increased hyperplasia. There are three isoforms of Akt, all of which are expressed in the mammary gland (Maroulakou et al., 2008). It will be interesting to determine whether disrupting the other

Table 1
Summary of branching defects in mouse models with mutations in the PI3K/Akt pathway.

Genotype	Tissue	Phenotype	References
MMTV- p110α Tg	Mammary gland	Increased ductal branching	Renner et al. (2008)
$PTEN^{loxp/loxp}$;MMTV-$Cre^{+/-}$	Mammary gland	Accelerated ductal extension and excessive side-branching	Li et al. (2002)
MMTV- Akt1 Tg	Mammary gland	Increased tubule branching	Ackler et al. (2002).
$PTEN^{loxp/loxp}$;Nkx2.1-$Cre^{+/-}$	Lung	No branching defects, conducting airway hyperplasia, expansion of stem cell population	Tiozzo et al. (2009)
$PTEN^{loxp/loxp}$;SP-C-$rtTA^{+/-}$; TetO-$Cre^{+/-}$	Lung	Epithelial cell proliferation and hyperplasia as early as 4–6 weeks of age, branching morphogenesis is not disrupted	Dave et al. (2008)
$PTEN^{loxp/loxp}$;SP-C-$rtTA^{+/-}$; TetO-$Cre^{+/-}$	Lung	Alveolar septal and bronchiolar hyperplasia, impairments of branching morphogenesis and distal alveolar epithelial cell differentiation	Yanagi et al. (2007)
$PTEN^{loxp/loxp}$; HoxB7-Cre	Kidney	Aberrant branching patterns, mislocalization of glomeruli and lethality	Kim and Dressler (2007)
$PTEN^{loxp/loxp}$; Nsc-Cre	Neuron	Increased axonal growth, ectopic axonal projections, dentritic hypertrophy	Kwon et al. (2006)
PIKE−/−	Whole body	Reduced dendritic complexity, dendritic branch length and soma size	Chan et al. (2011)
p110α−/−	Whole body	Embryonic lethality, dilated vessels in the head, reduced branching morphogenesis in the endocardium, lack of hierarchical order of large and small branches in the yolk sac, and impaired development of anterior cardinal veins	Lelievre et al. (2005)
$p110\alpha^{loxp/loxp}$; Tie2-Cre	Endothelial	Embryonic lethality by E12.5, severe defects in angiogenic sprouting and vascular remodeling	Graupera et al. (2008)
$PTEN^{loxp/+}$; Tie2-Cre	Endothelial	Increased angiogenesis	Hamada et al. (2005)

isoforms of Akt has any effects on branching morphogenesis of the mammary epithelium.

2.2. Lung

The mammalian lung consists of thousands to millions of airway branches organized in an intricate pattern. Lung development involves two sequential processes: branching morphogenesis of the epithelial tree during the pseudoglandular and canalicular stages (~5–24 weeks in human; E9.5–17.5 in mouse), and development of the air sacs and alveoli during the saccular and alveolar stages (~24 weeks to postnatal in human; E17.5 to postnatal in mouse) (Hilfer, 1996). The pattern of the airway epithelial tree is highly stereotyped; its development in the mouse involves three modes of branching used reiteratively to pattern the entire lung, which have been described as domain branching, planar bifurcation and orthogonal bifurcation (Metzger et al., 2008). During domain branching, daughter branches form in rows or domains at different positions around the circumference of the parent branch, which builds the scaffold of the lung. During planar bifurcation, daughter branches are formed through a series of co-planar bifurcations that are mainly used to form the edges of the pulmonary lobes. During orthogonal bifurcation, branching occurs *via* a series of bifurcations orthogonal to the preceding ones, which fill the spaces inbetween (Affolter et al., 2009). The molecular signaling that distinguishes these three modes of branching remains unclear.

In the embryonic mouse lung, the levels of phosphorylated Akt (pAkt) are the highest at E12 and decrease gradually thereafter, suggesting that pAkt is involved in the earlier stages of lung development (Wang et al., 2005). Mouse embryonic lung culture experiments suggest that treatment with both PI3K inhibitors, LY294002 and wortmannin, decreases the number of terminal buds compared with controls (Wang et al., 2005). Furthermore, the diameter of the developing airways is decreased in LY294002- and wortmannin-treated explants as compared to controls. Inhibiting PI3K decreases epithelial cell proliferation and increases apoptosis in the explants (Wang et al., 2005). The results of this study suggest that the PI3K pathway may regulate the balance between cell proliferation and apoptosis during airway branching morphogenesis.

PTEN-null mouse embryos die during early gestation, indicating a critical role for PTEN in embryonic development (Di Cristofano et al., 1998). A variety of lung-specific PTEN knockout mice have been generated to study the role of the PI3K pathway during lung branching morphogenesis *in vivo*. The conclusions derived from these studies are controversial. The Minoo group deleted PTEN conditionally throughout the airway epithelium by crossing $PTEN^{flox/flox}$ mice with Nkx2.1-cre driver mice (Tiozzo et al., 2009). In these mice, deletion of PTEN leads to airway epithelial hyperplasia but does not affect branching. In the adult, these PTEN knockout mice show expansion of epithelial progenitor cells and are more resistant to airway injury. In a second study, PTEN was deleted by mating $PTEN^{loxp/loxp}$ mice to surfactant protein (SP)-C-$rtTA^{+/-}$ and TetO-$Cre^{+/-}$ mice. Doxycycline was administrated from E0.5 to E14.5. The SP-C promoter specifically deleted PTEN in precursors of bronchiolar and alveolar epithelial cells. Using this system, the authors showed that deletion of PTEN in the developing embryonic lung has no effect on branching morphogenesis, lung maturation, or postnatal development, but instead leads to bronchiolar hyperplasia as early as 4–6 weeks of age (Dave et al., 2008). However, in a third study, which used the same $PTEN^{loxp/loxp}$;SP-C-$rtTA^{+/-}$; TetO-$Cre^{+/-}$ system, the authors found that loss of PTEN results in alveolar septal and bronchiolar hyperplasia, impairments of branching morphogenesis, and defective distal alveolar epithelial cell differentiation (Yanagi et al., 2007). Thus, although PTEN is important for lung development, its specific role in branching remains unclear.

There are several possible explanations for the differences in the phenotypes of the PTEN-null lungs. First, the time points at which PTEN was deleted were different. For example, in the second study, PTEN was deleted from E0.5 to E14.5, whereas in the third study PTEN was deleted from E10 to E16. Second, the genetic background of the mice was different. In the second study, the mice were produced and maintained in a mixed FVBN/129S4/SvJae6 background, whereas in the third study, the mice were in a 129Ola/C57BL6/JF6 background. Genetic background has been shown to affect the onset and severity of tumorigenesis in PTEN-knockout mice (Freeman et al., 2006). This dependence on the genetic background may well apply to the role of PTEN in

organogenesis and could provide another possible explanation for the differences in lung phenotype observed across the three above-described studies.

What is clear is that the PI3K pathway plays an important role in branching morphogenesis downstream of neuregulin-1 (NRG1), Mig-6, and transforming growth factor-beta (TGFβ). Addition of NRG1 to fetal lung explants increases airway branching morphogenesis. Inhibiting PI3K significantly decreases NRG1-induced branching, whereas inhibiting mitogen-activated protein kinase (MAPK) has no effect (Liu et al., 2004). Mig-6 is a ubiquitously expressed adaptor protein that has been shown to negatively regulate signaling downstream of epidermal growth factor (EGF). Mig-6-deficient lungs show increases in airway branching as well as type II alveolar epithelial cell hyperplasia associated with increased levels of pAkt and cell proliferation (Jin et al., 2009). TGFβ inhibits fibroblast growth factor-10 (FGF10)-induced proliferation and morphogenesis of mesenchyme-free lung endoderm in explant culture (Xing et al., 2008). Conditional, endoderm-specific deletion of PTEN overcomes the inhibitory effect of TGFβ on cell proliferation, but does not restore branching morphogenesis (Xing et al., 2008). It is possible that the TGFβ-mediated effects on lung morphogenesis are regulated by other morphoregulatory genes, such as Nkx2.1 (Minoo et al., 1999). Signaling through PI3K downstream of growth factors is thus critical for branching of the epithelium of the mammalian lung.

2.3. Kidney

Embryonic development of the vertebrate urogenital system proceeds through a series of phases, each distinguished by the formation of a more advanced kidney by the intermediate mesoderm: the pronephros, followed by the mesonephros, and finally the metanephros, which persists as the definitive adult kidney (Michos, 2009). Development is initiated when the Wolffian duct (WD) forms from the intermediate mesoderm and grows caudally, first inducing tubules of the pronephric and mesonephric kidneys, and later giving rise at its posterior end to the ureteric bud (UB), a simple epithelial tube and a major component of the permanent kidney. The UB forms at 5 weeks of gestation in the human and E10.5 in the mouse, and then elongates and invades the metanephric mesenchyme (MM) (Bridgewater and Rosenblum, 2009). Mesenchymal signals attract each UB to grow towards the MM while mesenchymal inhibitors discourage branching until each UB reaches the target tissue. Subsequently, the tip of the UB becomes swollen and rounded, forming a structure called the ampulla. The ampulla then divides dichotomously to form two new ampullae, each of which will form a new branch. This is the commencement of extensive UB branching that continues for the duration of kidney development and ultimately forms the collecting ducts of the kidney (Little et al., 2010). Time-lapse analysis of UB branching morphogenesis in kidney organ culture suggests that the UB has a variety of branching patterns, including terminal bifid, terminal trifid, and lateral branching (Watanabe and Costantini, 2004). Understanding the signaling mechanisms that underlie the formation and branching of the UB is very important for gaining insight into the etiology of human congenital renal malformation and kidney diseases.

The signaling pathways that affect branching morphogenesis of the kidney have been widely investigated in both cultured cells and mouse models. HGF is recognized as a potent stimulator of tubulogenesis in renal epithelial cells through the activation of c-Met. The PI3K inhibitor, wortmannin, disrupts HGF-stimulated branching morphogenesis in mouse inner medullary collecting duct cells cultured in collagen (Derman et al., 1995). The PI3K pathway also mediates semaphorin3a-regulated inhibition of branching. Knockdown of semaphorin3a increases UB branching whereas recombinant semaphorin3a decreases branching. Furthermore, when semaphorin3a is overexpressed, the levels of pAkt are reduced, suggesting that semaphorin3a downregulates PI3K/Akt activity to inhibit branching (Tufro et al., 2008).

In addition to HGF, signaling through the Ret receptor by glial cell line-derived neurotrophic factor (GDNF) is required for normal growth of the UB during kidney development (Costantini and Shakya, 2006). When ret9 kidney epithelial cells, which express the full-length human Ret protein, are treated with GDNF, there is an increase in PI3K activity and pAkt levels. This increase in PI3K signaling is essential for the GDNF-mediated response, since the PI3K inhibitor LY294002 blocks cell migration and chemotaxis. Organ cultures of the UB taken from E10.5 embryos demonstrate that inhibition of PI3K, but not MEK1 or p38 MAPK, completely blocks GDNF-dependent outgrowth, which suggests that induction of the UB requires PI3K. PI3K is also essential for branching after the UB has invaded the MM, since LY294002 inhibits branching in E11.5 metanephric kidneys (Tang et al., 2002). Together, these results indicate that Ret regulates UB outgrowth and branching morphogenesis through the PI3K pathway.

This conclusion was corroborated by experiments using cultured kidney epithelial cell lines, which revealed that PTEN inhibits migration towards a source of GDNF (Kim and Dressler, 2007). Similarly, explant cultures of PTEN-null kidneys acquired at different developmental stages showed that at E11.5, PTEN-null kidneys have subtle alterations in branching patterns, particularly at the tips. At the newborn stage, the PTEN-null kidneys have terminal ends that are often dilated and connected to mis-shapen stalks (Bridgewater and Rosenblum, 2009; Kim and Dressler, 2007). PTEN thus plays an essential role in controlling patterning and epithelial migration during kidney branching morphogenesis.

2.4. Submandibular Gland (SMG)

The mouse salivary gland, specifically the SMG, has been widely used as a model of branching morphogenesis due to its clearly defined branching pattern, the relative ease of gland isolation, and the availability of a well-established system for *ex vivo* culture (Hsu and Yamada, 2010). In mouse embryos, the SMG initiates at E12 as a simple spherical epithelial structure attached to a single epithelial stalk and is surrounded by condensed mesenchyme. Clefts subdivide the epithelium into 3–5 buds at E13.5, and branching morphogenesis occurs through repetitive rounds of cleft and bud formation, as well as ductal elongation and lumen formation to create a highly branched tissue by E14 to E15 (Hsu and Yamada, 2010; Patel et al., 2006). The E13 SMG is commonly used for explant experiments because it grows and branches well in serum-free medium and recapitulates the branching pattern that occurs *in vivo* for about 48 h of culture (Hoffman et al., 2002).

So far there are no published studies using transgenic or knockout mice to examine the PI3K pathway in branching morphogenesis of the SMG. Instead, investigators have taken advantage of the well-characterized *ex vivo* culture system to indicate an important role for this pathway. The PI3K inhibitors wortmannin and LY294002 inhibit branching in organ culture by acting directly on the epithelium (Koyama et al., 2003; Larsen et al., 2003). Exogenous addition of PIP3 to the SMG *via* a membrane-transporting carrier in the presence of PI3K inhibitors rescues branching morphogenesis (Larsen et al., 2003). The PI3K pathway seems to be involved in FGF- and EGF-induced branching morphogenesis of the SMG. LY294002 inhibits FGF7 but not FGF10-mediated morphogenesis (Steinberg et al., 2005). Treatment with LY294002 also reduces the stimulatory effect of EGF on branching of the gland (Koyama et al., 2003). Therefore, PI3K activity is required for proper branching morphogenesis of SMG. It will be interesting to examine the downstream

effectors of PI3K in the regulation SMG branching and to validate the findings from the culture studies *in vivo*.

2.5. Prostate Gland

The prostate is a sex-accessory secretory gland present in mammalian males. Although the morphology of the prostate differs considerably between species, epithelial branching morphogenesis is a key feature of its development in rodents and humans (Grishina et al., 2005). In the mouse, the prostate originates from the urogenital sinus (UGS) *via* epithelial budding at E16.5 (Sugimura et al., 1986), and branching morphogenesis begins when the elongating epithelial buds of the UGS contact the mesenchymal pads. Secondary, tertiary, and further branch points are then established with continued proximal-to-distal outgrowth and increase in complexity (Timms et al., 1994). Postnatally, the prostate lobes grow in size, canalize and undergo lobe-specific branching. Morphogenesis is completed between postnatal days 15 and 30, and final maturation occurs at puberty in response to increased levels of androgens (Prins and Putz, 2008; Pritchard et al., 2009; Thomson and Marker, 2006).

Organ explant models have shown that the activity of PI3K increases in the developing prostatic epithelium following androgen stimulation. Akt phosphorylation elevates dramatically upon androgen treatment (Ghosh et al., 2011). Three different PI3K inhibitors, LY294002, wortmannin and PI-103, an ATP-competitive dual PI3K-mTOR kinase inhibitor, all substantially disrupt budding of the prostatic epithelium. In the presence of the inhibitors, there is no decrease in proliferation or increase in apoptotic activity in the epithelial cells, suggesting that processes other than cell division or death are responsible for the effects of these inhibitors on branching. As shown by many studies, PI3K signaling through Rac is important for cell motility and invasion (Ridley et al., 1992). Indeed, prostatic epithelial cell migration is significantly impaired by treatment with LY294002, which suggests that the regulation of cellular migration may contribute to the cellular mechanisms by which PI3K affects branching of the prostate (Ghosh et al., 2011).

3. PI3K Pathway and Neurite Branching

A typical neuron is composed of a cell body/soma, dendrites, and an axon. The soma frequently gives rise to multiple dendrites, but never to more than one axon, although the axon may branch hundreds of times before it terminates (Calderon de Anda et al., 2008). An axon is typically long and thin with a uniform width, and it branches at right angles from the cell body and travels for distances as far as 1 meter in humans and even farther in other species. Dendrites are relatively short, often extending for hundreds of micrometers and undergoing Y-shaped branching multiple times, thus giving rise to a complex dendritic tree (Arimura and Kaibuchi, 2007; Craig and Banker, 1994).

Most axonal branches form at nerve terminals in their target regions. The terminal arbors of these neurons develop after the axons have reached their targets, such as the peripheral skin tissue or the central motoneurons in the spinal cord (Gibson and Ma, 2011). A growing axon generates a new branch either by splitting of the growth cone, the highly motile sensorimotor structure at the tip of an extending neurite, or by outgrowth of a collateral from the rigid axonal shaft, a process also known as interstitial branching (Acebes and Ferrus, 2000; Schmidt and Rathjen, 2010). Dendrites are the main sites of information input into neurons. Though different types of neurons have distinctive and characteristic dendritic branching patterns, there are several general steps that they follow, including neurite initiation, outgrowth and guidance, branching and synapse formation, and stabilization (Portera-Cailliau et al., 2003; Urbanska et al., 2008; Wu et al., 1999). Neurite branch formation is essential for the successful wiring of neurons, and accumulating evidence suggests that PI3K signaling may play an important role in the neurite branching process (Fig. 3).

Cell culture studies have revealed that the PI3K pathway positively regulates the branching of different neuronal cell types (Read and Gorman, 2009), including hippocampal neurons (Jaworski et al., 2005; Kumar et al., 2005; Lim and Walikonis, 2008; Read and Gorman, 2009; Yoshimura et al., 2006; Zheng et al., 2008), dorsal root ganglion neurons (Markus et al., 2002; Mills et al., 2003; Tucker et al., 2006, 2008), superior nerve ganglion neurons (Nakagomi et al., 2003) and hypoglossal neurons (Namikawa et al., 2000). For example, overexpression of Akt increases axon distal branching in the absence of neurotrophin stimulation. The branch-inducing activity is equally pronounced in neurotrophin-treated cultures, with an average of four-fold more branch points developing in cells transfected with constitutively active Akt as compared to controls, strongly suggesting a stimulatory effect for Akt in branching (Markus et al., 2002). The effects of the PI3K pathway have also been investigated for dendritic branching. When several known effectors of branching, including Akt, Rac1 and Arf6, are transfected into hippocampal neurons, only Akt is able to enhance dendritic complexity and the size of the soma. Dendritic complexity is reduced by inhibition of PI3K or RNAi-mediated knockdown of mTOR, indicating that mTOR is the primary mediator of PI3K-regulated dendritic branching (Jaworski et al., 2005). However, studies using PC12 neuronal cells have yielded very different results. Treatment of PC12 cells with LY294002 results in a marked increase in the number of neurite branch points. Consistently, overexpression of a constitutively active Akt decreases branching whereas a dominant negative Akt increases branching. Thus, the effect of the PI3K pathway on neuronal branching may be context- and cell type-specific (Higuchi et al., 2003).

Deletion of PTEN specifically in discrete mature neuronal populations in the cerebral cortex and hippocampus leads to ectopic dendrites and axonal projections with increased synapses. The abnormalities are associated with activation of Akt, mTOR and S6 kinase as well as inactivation of Gsk3β, all of which are downstream effectors of the PI3K pathway (Kwon et al., 2006). These findings suggest that abnormal activation of the PI3K pathway in specific neurons underlies the branching defects in these neurons. Phosphoinositide 3-kinase enhancer (PIKE) binds and enhances the activity of PI3K. PIKE-null neurons show reduced dendritic complexity, dendritic branch length, and soma size. This study also suggests that PIKE depletion ablates neuronal survival but not proliferation. The defects are due to the reduced PI3K activity, because the impaired dendritic arborization can be rescued when the PI3K cascade is augmented in culture or in PIKE−/−PTEN−/− double knockout mice (Chan et al., 2011). PTEN has been shown to be regulated *via* the ubiquitin proteasome system (UPS) by Nedd4, an E3 ligase. Studies using retinal ganglion cells have revealed that Nedd4 downregulates PTEN in the axons *via* UPS when they reach their synaptic targets and thus promotes PI3K-induced cytoskeletal arrangements that bring about branch formation. Overexpressing PTEN in the ganglion cells inhibits axonal branching whereas inhibiting PTEN rescues the branching defect caused by Nedd4 inhibition, indicating that the PI3K pathway is a key mediator of Nedd4-regulated axonal branching (Drinjakovic et al., 2010).

Rac GTPases have also been shown to control axon growth, guidance and branching. In *Drosophila*, loss of Rac GTPase activity leads first to defects in axonal branching, then guidance, and finally growth. Expression of a Rac1 effector domain mutant that does not bind p21-activated kinase (PAK) rescues growth, partially rescues guidance, but does not rescue branching defects of Rac-mutant neurons, which indicates that the interaction between Rac1 and PAK is

critical for proper axonal branching (Ng et al., 2002). Therefore, the PI3K pathway regulates neuronal branching through both Akt and Rac1.

4. PI3K Pathway and Vascular Sprouting

Similar to the nervous system, blood vessels branch into extensive and elaborate networks with distinct local specializations, in this case to supply tissues with nutrients and oxygen. Formation of the primary vascular plexus begins with the differentiation of single cell precursors into endothelial cells and their subsequent assembly into endothelial tubes, a process called vasculogenesis. The primary vascular plexus extends by sprouting outgrowth and remodeling, referred to as angiogenesis (Ruhrberg, 2003; Shima and Mailhos, 2000). In the absence of pro-angiogeneic stimuli, endothelial cells remain in a quiescent state. In the presence of high levels of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), a small proportion of the endothelial cells are selected as tip cells to lead newly sprouting vessels. Invasive tip cells extend numerous dynamic filopodial extensions that are guided by gradients of pro-angiogenic growth factors and various other environmental guidance cues (Gerhardt et al., 2003). During sprout elongation, tip cells are trailed by endothelial stalk cells, which remain connected with the parent vessels and initiate vascular lumen formation (Fig. 3) (Iruela-Arispe and Davis, 2009). Upon contact with other vessels, tip cell behavior is repressed and vessels fuse *via* anastomosis, a process that is aided by associated myeloid cells (Herbert and Stainier, 2011).

Constitutively active PI3K and Akt induce angiogenesis in the chorioallantoic membrane of chicken embryos, a process which is characterized by extensive sprouting of new blood vessels and enlargement of preexisting vessels (Jiang et al., 2000). Both PI3K and Akt lead to increased endothelial cell proliferation. Overexpression of PTEN or dominant-negative PI3K inhibits angiogenesis in the yolk sac of chicken embryos, suggesting that PI3K signaling is required for normal embryonic angiogenesis (Jiang et al., 2000). Mice deficient in the p110α catalytic subunit of PI3K display multiple vascular defects, including dilated vessels in the head, reduced branching of vessels in the endocardium, loss of hierarchical order of large and small branches in the yolk sac, and impaired development of anterior cardinal veins (Lelievre et al., 2005). Endothelial-specific deletion of p110α leads to embryonic lethality by E12.5 due to severe defects in angiogenic sprouting and vascular remodeling. However, inactivation of p110α does not affect endothelial cell proliferation or survival. It is thought that p110α regulates endothelial function by modulating endothelial cell migration through the small GTPase RhoA (Graupera et al., 2008). Moreover, heterozygous deficiency of PTEN in endothelial cells results in enhanced tumorigenesis due to an increase in angiogenesis. In culture, $PTEN^{loxp/+}$; Tie2-Cre endothelial cells show enhanced proliferation and migration (Hamada et al., 2005). Consistent with these findings, PDK1−/− embryoid bodies exhibit developmental and vascular defects that can be attributed to reduced cell migration, whereas overexpression of PDK1 increases endothelial cell migration (Primo et al., 2007).

As mentioned above, Rac can be activated by the PI3K pathway, and signaling through Rac1 has been shown to regulate endothelial branching. Endothelial cells undergo sprouting to form capillary tubes. Inhibiting Rac blocks the cell shape changes that are required for capillary assembly (Connolly et al., 2002). The PI3K pathway is thus critical for angiogenesis and positively regulates this process.

5. Conclusions and Outlook

Branched structures are evident at all levels of organization in living organisms. In principle, a branched structure can be built by the iterative use of a few simple subroutines, including initiation of a bud, extension of the bud and splitting at the end of the bud. Each step is tightly controlled by signaling pathways to ensure the proper formation of the final organ. PI3K has been revealed as a key regulator of branching morphogenesis that controls ramification in similar ways in different organs. For example, inhibition of PI3K, either by pharmacological inhibitors or in genetically engineered mice, leads to a decrease in branching, whereas overexpression of PI3K results in enhanced branching morphogenesis. There are some notable differences in the phenotypes of different organs when signaling downstream of PI3K is modulated. Overexpression of Akt1 only leads to slight increases in branching in the mammary gland, but massive increases in dendritic complexity in hippocampal neurons. The PI3K pathway signals through several downstream effectors, including Akt and Rac, and it is possible that different organs use different effectors to regulate branching.

However, many studies have been performed using cultured cells or organ explants. It would be useful to establish additional transgenic and knockout mouse models to study the role of this pathway in branching *in vivo*. Several major questions remain unanswered. First, branching morphogenesis is a multi-step process. What signals downstream of PI3K control branch initiation and extension? How does PI3K interact with other signaling pathways to regulate branching? Aside from PI3K, several other signaling pathways are also involved in branching morphogenesis, including MAPK (Karihaloo et al., 2001) and Wnt (Dean et al., 2005). Furthermore, mechanical signaling also plays a role in branching (Gjorevski and Nelson, 2010; Nelson et al., 2006), and the PI3K pathway can be activated by mechanical forces including those from shear stress (Rice et al., 2010) and cyclic strain (Hoshino et al., 2007). How do these mechanical signals integrate with PI3K to build the final architecture of the organs? It will be important to answer these questions in a variety of model systems to explore the generality of the underlying pathways. The elucidation of the signaling mechanisms that control branching morphogenesis will provide new insights into the regulation of organogenesis in general and the developmental origins of pathologic processes.

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