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Review

3D *in vitro* cell culture models of tube formation

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ABSTRACT

Building the complex architecture of tubular organs is a highly dynamic process that involves cell migration, polarization, shape changes, adhesion to neighboring cells and the extracellular matrix, physicochemical characteristics of the extracellular matrix and reciprocal signaling with the mesenchyme. Understanding these processes *in vivo* has been challenging as they take place over extended time periods deep within the developing organism. Here, I will discuss 3D *in vitro* models that have been crucial to understand many of the molecular and cellular mechanisms and key concepts underlying branching morphogenesis *in vivo*.

Contents

1. Introduction

The functional architecture of organs such as the lung, kidney, pancreas, mammary gland and salivary gland consists of three-dimensional (3D) branching networks of epithelial tubes which transport fluids and serve as barriers between physiologically distinct compartments. All tubes are characterized by a central lumen enclosed by polarized epithelial or, in case of the vasculature, specialized epithelial cells called endothelial cells. Often, they end in spherical caps called acini (mammary gland) or alveoli (lung), or, in special cases such as the vasculature, form a continuous endothelial network. The main tubular organs form by branching morphogenesis, a highly dynamic and complex developmental process which involves coordinated cell migration, cell shape changes, apical-basal polarization, proliferation, differentiation, apoptosis and reciprocal interactions between the epithelial and the surrounding mesenchyme. Branching morphogenesis is regulated by many transcription factors, hormones, growth factors, and chemical and physical cues from the extracellular matrix (ECM), which often act in a highly localized fashion [1–6]. This complex regulation of branching morphogenesis has complicated the elucidation of cellular and molecular mechanisms underlying this process using *in vivo* models. *In vitro* 3D cell culture models of cells grown in ECM recapitulate many aspects of branching morphogenesis and have provided crucial insights in common mechanisms and key concepts involved in tube formation. As the various models currently in use each have their strengths and limitations, the appropriateness of the model depends on which aspects and cellular behaviors

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in branching morphogenesis are being analyzed. Here, I will discuss general design principles and signaling mechanisms involved in tube formation to provide context on factors that should be taken into account when choosing a model. Second, a number of commonly used models based on established cells lines will be discussed, along with strengths and weaknesses of commonly used types of ECM.

2. Overview of tube formation *in vivo*

2.1. Mechanism of tube formation

As described in detail elsewhere [2,7,8], tubes form by different mechanisms, which can be subdivided into two types, depending on state of cell polarization during the process: (1) tubes that emerge from pre-existing polarized epithelial sheets and maintain epithelial polarity and lumen topology during the process, and (2) tubes that rely on *de novo* polarization during their formation. In processes called wrapping, budding or clefting, tubes form from pre-polarized epithelia. Wrapping involves cell shape changes mediated by apical constriction that cause parts of an epithelial sheet to move inward to form a groove, which eventually seals to form a tube parallel to the originating epithelium. An example of wrapping is neural tube formation. Budding also involves initial invagination of cells *via* apical constriction, and causes formation of a new tube outward, which subsequently extends by cell migration and/or cell division [2,7]. Examples of budding include branching morphogenesis of organs like the lung [9] and the kidney ureteric bud [4]. Clefting is a variant of budding that subdivides an epithelial bud into two new buds, and is involved in the formation of the mouse salivary gland [10]. Tube formation involving *de novo* apical-basal polarization and lumen formation includes processes called cavitation, cord hollowing and cell hollowing. In cavitation, tubes form from a cluster of cells, in which the outer cells will polarize, and luminal space is created by eliminating the inner cells. An example of cavitation includes the clearance of the lumen by apoptosis in the terminal end bud (TEB) of the developing mammary gland [11,12]. In contrast, cord hollowing does not depend on cell death, but involves formation of apical surfaces and small lumens between opposing cells in cords with a diameter of a few cells. Merging of these small initial lumens subsequently separates cell layers and leads to a continuous lumen along the center of the cord. An example of cord hollowing is the formation of the zebrafish gut [13]. Finally, cell hollowing follows the same principle, but involves the merging of a large intracellular vacuolar compartment at adjoining areas of the plasma membrane, which leads to formation of a continuous central lumen extending through the middle of a chain of single cells. The best example of lumen formation by cell hollowing is formation of capillaries of the vasculature [14].

2.2. Tube architecture

The common final architecture of all mature tubes is a circular lumen enclosed by a layer of epithelial cells that are polarized with apical membrane surfaces facing the lumen, basal surfaces contacting the ECM or underlying tissue, and lateral surfaces that interact with adjacent epithelial cells through specialized cell–cell junctions. These junctions include adherens junctions, which physically link cells to each other, and tight junctions, which separate the apical and basolateral membrane domains and provide a tight seal between neighboring cells. Cell polarization is not only critical for the physiological functions of mature tubular organs, including barrier function and vectorial transport across luminal epithelia; it is also required for their initial development. Thus, when tubes form *de novo*, delivery of apical membrane precedes the formation of lumens [7,15]. Furthermore, tubes arising from pre-polarized epithelia depend on polarization for apical constriction [16–18], maintenance of cell–cell adhesion and tube extension [19]. In addition, basal targeting of ECM-interacting proteins control adhesion, active remodeling and mechanochemical sensing of ECM, thereby reinforcing apical-basal polarization [20], and together with soluble signals locally control branching morphogenesis [21–23].

2.3. Regulation of branching morphogenesis by growth factors

As a main concept, branching morphogenesis involves locally induced initiation of branching at distal tip areas, coupled to inhibition of branching activity elsewhere [24]. Upon initiation, tubes elongate, which depending on the organ, relies on cell migration, combined with cell elongation, cell proliferation and recruitment of additional cells. Further patterning involves side-branching, or bifurcation, which splits a growing tube into two. Many growth factors, almost exclusively ligands for receptor tyrosine kinases (RTK), promote tube initiation and elongation [1]. For some tubular networks, like the Drosophila tracheal system or the vertebrate vasculature, branching relies on one or two dedicated tip cells, which exhibit protruding filopodia and actively migrate to guide the elongating tube [6]. Tip cells form in response to locally secreted ligands, including fibroblast growth factor (FGF)/Branchless in the trachea and vascular endothelial growth factor (VEGF) in vascular endothelial cells. Cells with the highest receptor activity will become tip cells, which are genetically and morphologically very distinct from stalk cells that are located behind, and linked to tip cells *via* cell–cell adhesions. Tip cells inhibit induction of additional tips and lateral branching at the stalk *via* Delta-Notch signaling [6].

Branching of organs and glands that form by budding, such as the lung, kidney ureteric bud and the mammary gland is less well understood. Here, morphologically discernible tip cells are absent, but high branching activity and proliferation rates, and distinct gene expression patterns in distal bud areas compared to regions behind the bud indicate that these branches have functionally distinct tip and stalk or trunk regions as well [25–27]. Branch initiation and elongation of budding tubes depends on inductive and reciprocal interactions with cells and ECM of the surrounding stroma or mesenchyme. The mesenchymal RTK ligands that induce branching [3,5,22] often involve members of the FGF family (lung, kidney, mammary- and salivary glands) [1], but also include glial cell neurotrophic factor (GDNF) (kidney), hepatocyte growth factor (HGF) (lung, mammary gland) [28,29] and members of the epithelial growth factor (EGF) family (mammary gland) [30]. Inhibitory signaling is still poorly understood, but involves complex reciprocal signaling with the mesenchyme. For example, mesenchymally secreted FGF10 initiates outgrowth of lung epithelial buds by FGF receptor 2 (FGFR2)-mediated proliferation and migration of distal tip cells. Negative feedback signaling involves paracrine inhibitory signaling in which FGFR2-induced secretion of Sonic hedgehog (SHH) by distal lung buds inhibits production of FGF10 by the mesenchyme [31]. In addition, FGFR2-induced expression of the RTK antagonist sprouty2 provides an autoinhibitory feedback loop in the lung epithelial buds [9,32]. Reciprocal signaling may also promote positive paracrine feedback signaling, for example during branching of the kidney ureteric bud. Here, branching is mainly induced *via* the activated Ret receptor in response to secretion of its ligand GDNF by the metanephric mesenchyme [3,33]. Signaling is amplified by Ret-mediated induction of Wnt11 secretion, which increases GDNF expression in the mesenchyme [34]. Intracellular negative regulation includes GDNF-Ret-induced epithelial expression of sprouty1, which inhibits Ret activity [35]. Tubules of the mammary gland mostly form postnatally and involve a bilayered trunk region of a layer of luminal cells covered by a layer of myoepithelial cells, and a distal multilayered TEB, which controls

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