Heterogeneity, Cell Biology and Tissue Mechanics of Pseudostratified Epithelia: Coordination of Cell Divisions and Growth in Tightly Packed Tissues

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Contents

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Abstract

Pseudostratified epithelia (PSE) are tightly packed proliferative tissues that are important precursors of the development of diverse organs in a plethora of species, invertebrate and vertebrate. PSE consist of elongated epithelial cells that are attached to the apical and basal side of the tissue. The nuclei of these cells undergo interkinetic nuclear migration (IKNM) which leads to all mitotic events taking place at the apical surface of the epithelium. In this review, we discuss the intricacies of proliferation in PSE, considering cell biological, as well as the physical aspects. First, we summarize the principles governing the invariability of apical nuclear migration and apical cell division as well as the importance of apical mitoses for tissue proliferation. Then, we focus on the mechanical and structural features of these tissues. Here, we discuss how the overall architecture of pseudostratified tissues changes with increased cell packing. Lastly, we consider possible mechanical cues resulting from these changes and their potential influence on cell proliferation.

1. INTRODUCTION

Pseudostratified epithelia (PSE) are proliferative tissues that feature elongated epithelial cells. These cells are organized into a single, tightly packed epithelial layer (Fig. 1). PSE give rise to various tissues in a wide range of invertebrate and vertebrate organisms. The term pseudostratified arose from initial observations of PSE, where the various positions that the nuclei of these epithelia occupy along the apico-basal axis were interpreted as cell stratification. However, it later became clear that despite its stratified appearance, all cells within the epithelial sheet are attached to both the apical and basal surfaces of the tissue (Sauer, 1935; Smart, 1972) (Fig. 1). The nuclei in PSE are dynamic and perform apico-basal movements correlated with the cell cycle. These nuclear movements are known as interkinetic nuclear migration (IKNM) (Sauer, 1935). IKNM consists of different modes of nuclear movement: Shortly before mitosis, nuclei move to the apical surface in a rapid and directed manner (Kosodo et al., 2011; Norden et al., 2009; Strzyz et al., 2015; Tsai et al., 2010). This apical migration is highly conserved among organisms and appears in all cells within PSE before cell division. Consequently, all mitotic events in PSE are localized to the apical surface of the tissue. Following division, daughter nuclei are displaced from the apical surface and localize to more basal positions. In most systems studied so far this basal translocation is slower than apical migration (Kosodo et al., 2011; Norden et al., 2009).

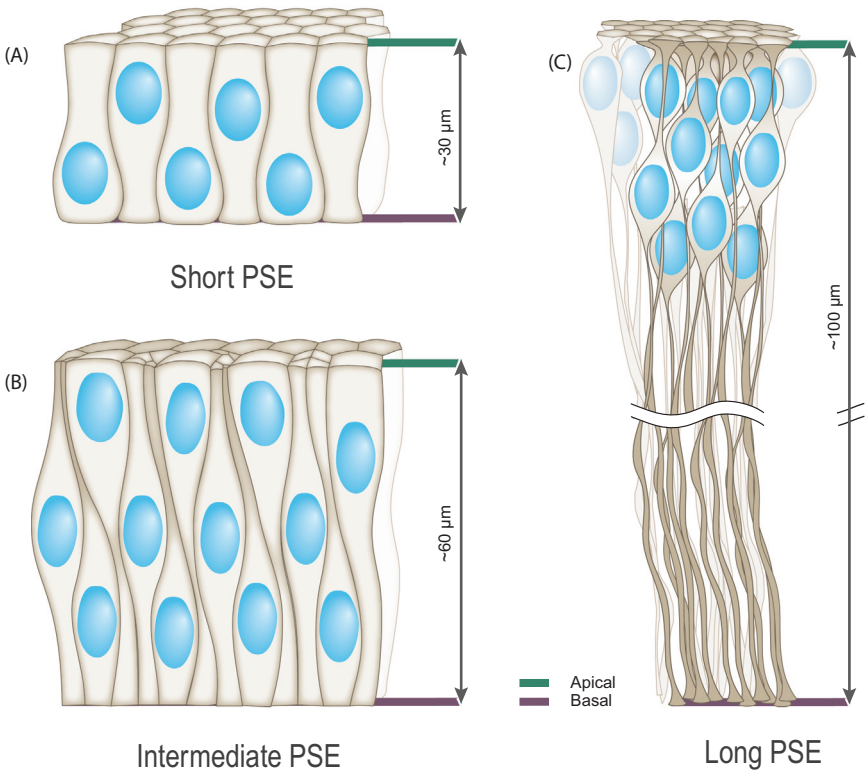

Figure 1 Pseudostratified epithelia (PSE) can be categorized into three major types. (A) The short PSE in which cells are 20–30 µm long and their nuclei organize into two to three layers inside the tissue. Examples include the vertebrate endodermal organ buds (Bort et al., 2006) and the Drosophila optic lobe (Rujano et al., 2013). (B) The intermediate PSE [eg, fly imaginal discs (Meyer et al., 2011) and zebrafish retinal neuroepithelium (Norden et al., 2009)] in which cells are up to 60 µm long and the tissue typically has four to five nuclear layers. (C) The long PSE found in, for example, in the neural tube of higher vertebrates at later stages of development. This tissue comprises eight or more nuclear layers and its cells span up to 100 µm (Smart, 1972). An example of extremely thin and elongated PSE is found in the developing neocortex, where the length of the radial glial cells exceeds 200 µm (Taverna and Huttner, 2010).

As cells within pseudostratified tissues are arranged in a tightly packed epithelial sheet, it has to be considered that nuclear movements during IKNM do not occur in isolation. On the contrary, nuclei move and divide within a tissue, which progressively expands and increases its cell density with every round of division. As cells proliferate, their morphology within the PSE also changes. They progressively elongate and decrease their apical

surface (Smart, 1972). As a consequence, proliferation in pseudostratified tissues influences the overall tissue organization and most likely has implications for tissue maturation, and consequently, continued proliferation might affect the tissue-scale mechanical properties and responses to mechanical stimuli in the PSE (Humphrey, 2003). Even though research has just touched upon it in the PSE, mechanical stimuli are well known to modulate cellular behavior, including proliferation [see (Benham-Pyle et al., 2015; Pathak et al., 2014)]. Changes in cell numbers occurring upon proliferation might therefore directly influence the mechanical properties of PSE, thereby creating a regulatory loop that feeds back to proliferation and/or differentiation.

1.1 Widespread Presence of Pseudostratified Epithelia in Diverse Developmental Contexts

PSE are commonly seen during the development of many different species. In vertebrates, they give rise to a plethora of tissues, including the liver, lung and pancreas buds, gut, nasal placode epithelia, otic placode/vesicle, lens placode/vesicle and the central nervous system. Recently, this list has been further expanded by showing that PSE also play important roles during very early mammalian development, for example by forming the epiblast of the gastrulating mouse embryo (Ichikawa et al., 2013). Furthermore, pseudostratified tissues are not restricted to vertebrates. They are also found building the embryonic ectoderm of the sea anemone Nematostella (Meyer et al., 2011), imaginal discs of fly larvae [precursors of legs, wings and antennae (Meyer et al., 2011)], as well as the fly optic lobe neuroepithelium [precursor of the visual processing centers of the fly brain (Rujano et al., 2013)]. This means that pseudostratified tissues precede the formation of many organs in both vertebrates and invertebrates. Therefore, studying their biology should generate insights on how these organs originate and develop. Additionally, most likely the tissues described as PSE to date do not exhaust the full spectrum of pseudostratification occurring in nature. Thus, further studies of various organisms and tissues at different developmental stages are needed to fully understand the role these tissues play during ontogenesis.

1.2 Heterogeneity of Pseudostratified Epithelia

As mentioned previously, PSE can be found throughout the animal kingdom. Although all PSE share the dispersed, "layered" nuclear arrangement, they can largely vary with respect to their cell length. Different PSE can span apico-basal distances from less than 30 micrometers to hundreds of

micrometers and even millimeters (Fig. 1). Furthermore, the degree of pseudostratification of a PSE, which describes how many nuclear layers are stacked within the epithelial sheet, can also vary. In the remainder of the review we will refer to this layering feature as tissue packing. One of the most prominent and most studied pseudostratified tissues is the developing neocortex in rodents and other mammals. During the development of the cortex, cells progressively elongate and their length is highly dependent on their developmental stage (Huttner and Brand, 1997; Florio and Huttner, 2014; Schoenwolf and Alvarez, 1989; Sauer, 1935). Therefore, we will use the central nervous system as an example to illustrate the large variability of cell lengths in pseudostratified tissues and to subdivide PSE into categories with varying degrees of cell elongation and nuclear stacking.

The vertebrate central nervous system originates from the neural plate (Schoenwolf and Alvarez, 1989; Sauer, 1935; Smith and Schoenwolf, 1989; Smith et al., 1994). Cells forming the neural plate are relatively short, measuring 20–30 μm and their nuclei arrange into only two to three nuclear layers. A similar tissue organization is found in the neuroepithelium of the optic lobes in Drosophila (Rujano et al., 2013), vertebrate otic vesicle (Clendenon et al., 2009; Hoijman et al., 2015), and endodermal organ buds (Bort et al., 2006). In all these tissues in which nuclei are stacked into only two or three layers, cells show a rather columnar morphology. For the purpose of this review, we will refer to these tissues as short PSE (Fig. 1A).

As the development of the brain progresses, the neural plate forms the neural tube and the brain vesicles. These structures are built from proliferative neuroepithelial cells, initially spanning apico-basal distances of 30–60 μm. In these more elongated epithelia compared to the short PSE, progenitor nuclei arrange on average into four to five layers (Jeong and McMahon, 2005; Nagele and Lee, 1979). A similar tissue organization is observed in retinal and hindbrain neuroepithelia of the zebrafish (Leung et al., 2011; Norden et al., 2009), the epithelium of the developing mouse intestine (Grosse et al., 2011), the fly imaginal discs (Meyer et al., 2011) and the ectoderm of the sea anemone Nematostella (Meyer et al., 2011). Cells in these tissues are discernibly less columnar than those in simple PSE; however, they still retain some cytoplasm in their apical and basal processes. In this review, they will be referred to as intermediate length PSE (Fig. 1B).

At later stages of development, intermediate length neural progenitors elongate further. For example, in the neural tube and the retina of higher vertebrates, these cells reach a final length of up to 100 μm, arranging their nuclei into eight or even more layers (Iulianella et al., 2008;

Nasonkin et al., 2011; Smart, 1972). We will define these as long PSE (Fig. 1C, upper half). In the developing neocortex, PSE elongation is even more extensive and accompanied by specification of neuroepithelial cells and their transformation into the radial glial cells (Bystron et al., 2008). These cells can be extremely elongated, measuring mms in primates (Miyata, 2008; Rakic, 1972). The ratio of their length to width is very high and their processes contain very little cytoplasm. The only bulky region of the cell is found around the nucleus. This gives radial glial cells a "bead on a string" appearance (Taverna and Huttner, 2010). Markedly, the nuclei in radial glia are not dispersed along the entire apico-basal axis of these highly elongated cells, but reside in a restricted zone within 150 μm of the apical surface, where they are densely packed, exceeding ten layers (Miyata, 2008; Okamoto et al., 2014; Smart, 1972) (Fig. 1C). Radial glial cells achieve extreme degrees of cell elongation that are not comparable to any other pseudostratified tissue described here, and we will therefore consider them separately.

In sum, PSE are a diverse and heterogeneous group of tissues, exhibiting a wide spectrum of cell lengths and nuclear layering. The differences in cell length can be correlated to differences in the overall cell architecture and nuclear stacking within the tissue. More specifically, cell elongation seems to correlate with a thinning of apical and basal processes. Additionally, in more elongated cells, nuclei are stacked into more layers, leading to increasing pseudostratification of the tissue. During development, both the degree of tissue pseudostratification and cellular length increase, with the most extreme example of this change being found in the neural tissue of higher vertebrates.

Despite the heterogeneity of PSE, one common characteristics of all PSE is that their nuclei move toward the apical side before mitosis. This apical nuclear migration occurs irrespectively of cell length or initial nuclear position. As a result, all divisions in PSE take place at the apical surface. How this nuclear movement is accomplished in diverse PSE will be discussed in the following sections. Additionally, we will consider potential explanations of its conservation in all PSE.

2. APICAL NUCLEAR MIGRATION IN PSEUDOSTRATIFIED EPITHELIA

In the 1930s, it was first noted by Sauer that nuclei in PSE move to the apical surface before mitosis (Sauer, 1935). Since then many studies focused on characterizing the phenomenon of nuclear movements in PSE. It is now

clear that apical nuclear migration is an active process dependent on cell intrinsic forces generated by cytoskeletal components (Norden et al., 2009; Spear and Erickson, 2012a; Strzyz et al., 2015; Tsai et al., 2010). Furthermore, apical nuclear movements have been shown to occur in a fast, persistent and directional manner in many systems (Kosodo et al., 2011; Norden et al., 2009; Okamoto et al., 2014, 2013). Additionally, apical nuclear movements are linked to cell cycle progression and occur specifically in the G2 phase of the cell cycle (Hu et al., 2013; Kosodo et al., 2011; Leung et al., 2011; Spear and Erickson, 2012a). It was suggested that this apical nuclear migration can cause passive displacement of other surrounding nuclei following division (Kosodo et al., 2011; Leung et al., 2011). Together, this means that the apical surface represents the "proliferative zone" of the tissue, in which mainly mitotic nuclei reside. Consequently, apical nuclear migration appears to be a key event, responsible for the spatial organization of proliferation in the PSE. For this reason, we propose the introduction of a novel term to refer to this important phenomenon. This will highlight the link between apical nuclear migration and mitosis and distinguish it from other nuclear motion occurring during IKNM. As nuclei migrate apically, only shortly before mitosis and this movement is characterized by high directionality and fast kinetics, we suggest the term *P*re-mitotic *R*apid *A*pical *M*igration (*PRAM*). Further, we will depict in more detail how PRAM occurs and discuss its importance for proliferation in PSE.

2.1 Machineries Responsible for PRAM

Although PRAM occurs in all PSE observed so far, the molecular mechanisms driving nuclear movements vary depending on the tissue investigated. They can either depend on microtubules (MTs) or the actomyosin contractile system or a combination of both. MTs drive PRAM in radial glia of the rodent neocortex (Hu et al., 2013; Tsai et al., 2010; Xie et al., 2007) as well as in the elongated neuroepithelial cells of the chick neural tube (Spear and Erickson, 2012a). In brief, PRAM in these systems occurs via dynein recruitment to the nuclear envelope. This is followed by the active transport of the nucleus along MTs toward their minus ends which are anchored at the apically localized centrosome (Baffet et al., 2015; Hu et al., 2013; Kosodo et al., 2011; Tsai et al., 2010). In radial glia cells this dynein recruitment is a two-step process that involves two nuclear envelope components in combination with their respective adaptor proteins. Notably, both these pathways are activated consecutively and are both necessary to ensure that nuclei reach

the apical surface for mitosis (Hu et al., 2013). In the chick neural tube, PRAM also occurs in two steps. However here, the second part of the nuclear movement is driven by actomyosin and not by dynein recruitment (Spear and Erickson, 2012a).

Interestingly, in short and intermediate length PSE actomyosin contractility has been shown to be the major force generator of PRAM (Meyer et al., 2011; Norden et al., 2009; Rujano et al., 2013). However, the exact molecular mechanisms underlying actomyosin-driven apical migration are so far not well understood. What is known is that in preparation for mitosis, cells undergo extensive remodeling of the actomyosin cytoskeleton, resulting in the acquisition of a rounded cell shape [reviewed in (Heng and Koh, 2010)]. As apical nuclear migration and mitotic rounding occur at about the same cell cycle stages and both depend on actomyosin contractility, it has been suggested that apical nuclear translocation represents mainly an initial step of cell rounding before mitosis (Meyer et al., 2011; Nagele and Lee, 1979; Spear and Erickson, 2012b). Although this might be the case in shorter PSE cells, it was recently demonstrated that PSE cells of intermediate length in the zebrafish retina can enter mitosis and undergo cell rounding at nonapical positions (Strzyz et al., 2015). Additionally, in zebrafish retinal neuroepithelia (Strzyz et al., 2015) as well as the epithelium of Drosophila wing disc (Liang et al., 2014), apical nuclear migration was still observed after mitotic entry at basal positions. This indicates that at least in intermediate length PSE apical migration of nuclei and mitotic rounding in preparation for mitosis are separate processes.

In addition to the question how exactly actomyosin generates the forces resulting in PRAM, it is furthermore not understood how and why different machineries underlying PRAM in different kinds of PSE arose. To date, MT-based PRAM has been mainly observed in long PSE, whereas actomyosin-based PRAM is more prominent in short and intermediate PSE. This might indicate that the differences in the PRAM machinery are related to different PSE tissue thickness. What could be the reason for this switch in PRAM machineries upon increasing cell elongation? It is tempting to speculate that the two different cytoskeletal machineries are able to generate different amounts of forces to bring nuclei to the apical side. It is, for example, possible that the forces generated by actomyosin are sufficient to drive nuclear migration over relatively short distances. Following this line of argument, it can be imagined that in the short PSE of the Drosophila optic lobe for example, actomyosin-based cell rounding is sufficient to move nuclei apically. However, with progressive cell elongation and the need of nuclei to cover

longer distances before reaching the apical side, PRAM might have emerged as a separate nuclear translocation mechanism independent of mitotic rounding. In the intermediate length epithelia, actomyosin seems to be sufficient to generate such movements. Once PSE further elongated, reaching the length scales of radial glia cells and having processes almost devoid of cytoplasm, it is possible that actomyosin is not able to generate enough force any longer to cover the increasing length scales of PRAM. In these cases, dynein-dependent migration along MTs might have evolved as the more effective force generator to drive nuclei toward the apical side. This would imply that tissues which display progressive changes in the degree of pseudostratification during development switch from moving nuclei in concert with cell rounding to actomyosin-driven PRAM and finally to MT-based PRAM. It would be very exciting to test this hypothesis in forthcoming studies in tissues ideally from the same organism but with increasing ratios of elongation.

2.2 Nuclear Migration in Tightly Packed Tissue

As mentioned earlier, a striking hallmark of PSE is that the nuclei are tightly packed into multiple layers and cells are elongated, with high length to width ratios. Consequently, nuclei in PSE cells do not move in isolation. On the contrary, migration to the apical side in G2 from within the depths of the PSE tissue necessitates the movement of the translocating nucleus through the crowded environment in which neighboring cells also feature bulky nuclei moving in different directions. Despite this crowded tissue packing, nuclei undergoing PRAM keep their directed, apical trajectory. To achieve this, the cytoplasm and organelles of the cell undergoing PRAM are likely to be displaced and neighboring nuclei rearranged. Furthermore, the plasma membrane of the PRAM-cell, as well as of the adjacent cells, will be deformed by the moving nuclei. This means that proliferation in PSE leads to significant local nuclear and tissue rearrangements (Fig. 3).

So far, it is not known whether and how nuclear dynamics and rearrangements in the PSE correlate to tissue packing. It is possible that the dynamicity of nuclei is needed to generate pseudostratification in the first place, for example, by allowing nuclear displacements within columnar cells. However, it is also possible that nuclear movements are a consequence and not a cause of pseudostratification. To date, multiple functions of overall apico-basal nuclear dynamics in PSE have been proposed, including a role in cell fate specification (Baye and Link, 2007; Del Bene et al., 2008), a role in

shaping the developing organs (Hoijman et al., 2015; Langman et al., 1966) and optimizing proliferation by preventing local cell over-crowding (Okamoto et al., 2013). Here, we will focus on the function of PRAM during the proliferative phase in which an expansion of the PSE takes place. As mitosis in these tissues is directly preceded by PRAM, in the next paragraphs we will first discuss links of PRAM to cell cycle events and revisit the current hypotheses of how PRAM might influence proliferation in different PSE.

2.3 PRAM and Its Links to Cell Cycle

So far, we described the machineries responsible for PRAM in different types of PSE. Another important question is how these machineries are regulated to reproducibly move nuclei apically exclusively before mitosis. Recently, different groups provided evidence that PRAM is tightly linked to cell cycle progression via the activity of the cell cycle kinase CDK1 (Baffet et al., 2015; Leung et al., 2011; Strzyz et al., 2015). CDK1 is a master regulator of cell cycle continuation and plays a role in the transition from the G2 phase into mitosis (reviewed in Vermeulen et al., 2003). It was shown that CDK1 activity is both necessary and sufficient for PRAM (Strzyz et al., 2015). Interestingly, CDK1 has been demonstrated to play a role in PRAM of both, intermediate length neuroepithelia of the zebrafish retina as well as in the long epithelium in neocortex (Baffet et al., 2015; Strzyz et al., 2015). Hence, it seems that CDK1 can link cell cycle dynamics to the cytoskeleton independently of which cytoskeletal element is used for nuclear translocation. How exactly does CDK1 regulate PRAM in these different tissues? CDK1 phosphorylates multiple cytoskeleton related proteins [(Sivars et al., 2003) reviewed in (Enserink and Kolodner, 2010)] and thus influences their localization and function. In the radial glia cells CDK1 directly phosphorylates a specific nuclear envelope protein, and only this phosphorylated protein can efficiently bind to a dynein adaptor protein and recruit dynein. CDK1 activity has been further shown to influence subcellular localization of another adaptor protein of the dynein complex, regulating its cytoplasmic distribution, which defines whether it binds to the nuclear envelope or is sequestered within the nucleus (Baffet et al., 2015). Most likely, similar mechanisms of modifying protein interactions and their localization by CDK1 phosphorylation also operate during actomyosin-driven PRAM and allow localized actomyosin contractility. However, the details of these interactions still need to be explored.

Therefore, PRAM is a phenomenon that depends on cell cycle progression and invariably occurs in the G2 phase of the cell cycle. Notably, CDK1 activation is a key step necessary for the initiation of PRAM, despite differences in cytoskeletal components responsible for nuclear movement, making it a common regulator of PRAM in different systems.

Together, we so far illuminated the cytoskeletal machineries that drive PRAM and how they are linked to cell cycle progression. However, an important question remains: Why do all PSE move their bulkiest organelle, the nucleus, toward the apical side to undergo mitosis? Why is this apical nuclear migration, which might at first sight appear as a waste of energy, found in all PSE studied to date? In the next chapters, we will outline some possible explanations for this seemingly counterintuitive phenomenon.

2.4 PRAM and Establishment of Proliferative Zone at Apical Surface

As cells acquire a rounded morphology in preparation for cell division, they occupy more lateral space during mitosis than in the interphase (Smart, 1972). For this reason, it has been suggested that nuclei move to the apical surface because it might provide more space for mitotic events (Fig. 2B). Consequently, by alleviating the constraints of tight nuclear packing, this apical restriction of mitotic nuclei achieved by the reproducibility of PRAM could help these cells to proliferate (Fish et al., 2008; Schenk et al., 2009). In some PSE, like the neuroepithelium of the retina, due to its tissue curvature, the apical surface indeed provides more space for the mitotic nuclei than the basal surface. In PSE with a flat apical surface, however, which is seen for most other types of central nervous system neuroepithelia, the basal side and the apical side harbor the same amount of space, and the conservation of the apical surface as the mitotic zone of the tissue cannot be explained by providing more space. This becomes even clearer when we look at the apical surfaces of the brain that have a negative curvature. Here, the apical surface is much smaller than the basal surface, but the nuclei undergo PRAM despite the apparent low apical-to-basal surface ratio. Additionally, cell rounding should be considered, which is a robust process that increases surface tension and decreases adhesion. It is to be expected that in such a tissue, in which the actomyosin cortex of the surrounding cells is not as rigid as in the mitotic cells, the mitotic cell can easily "make space" by the process of rounding and thereby deform neighboring cells (Fig. 2B and C). Hence, robust division in the tightly packed tissue would not require the formation of an apical

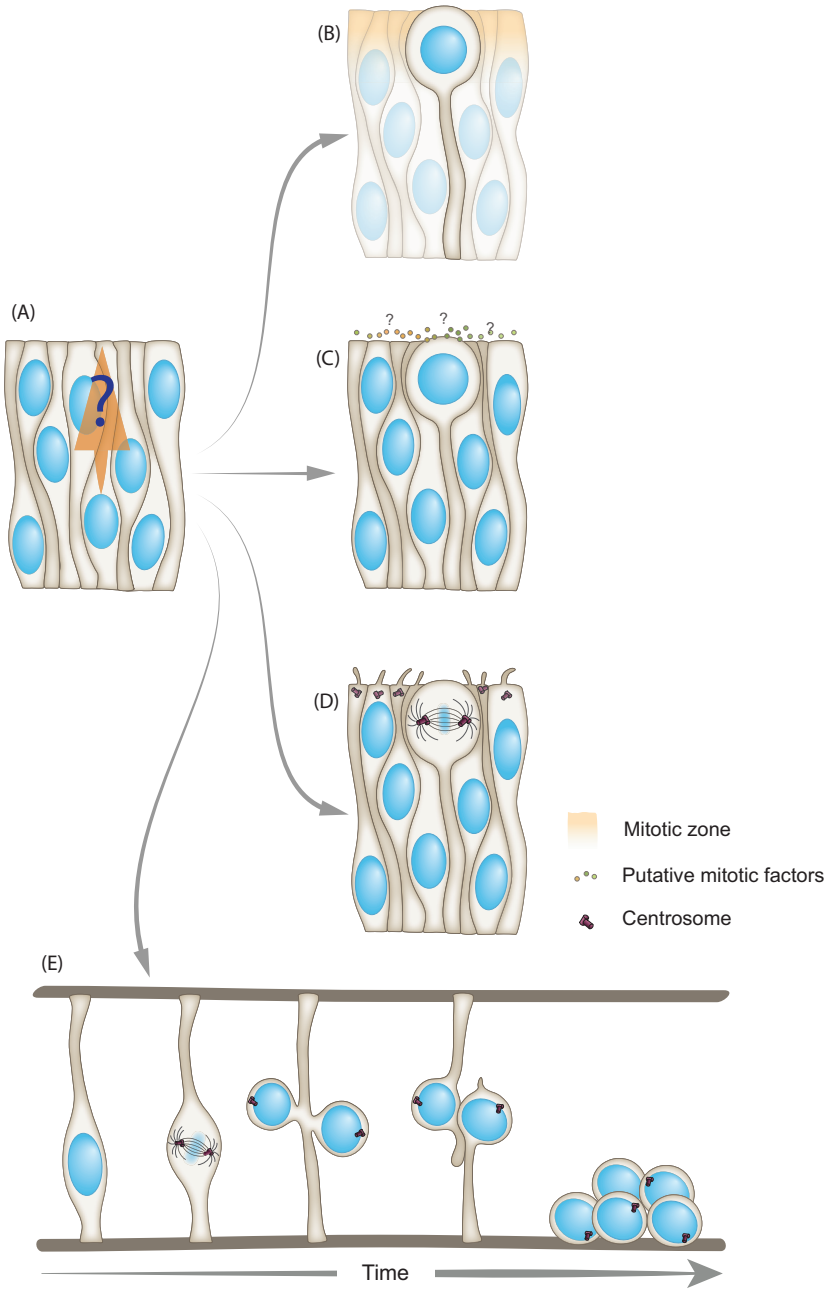

Figure 2 Theories on the purposes of PRAM and apical mitosis. (A) The nucleus of a PSE cell will rapidly migrate through the tightly packed tissue prior to mitosis (indicated by the *gray arrow*), in order to divide at the apical surface. (B) More space for cell rounding

"mitotic only zone" (Fig. 2B). This argument is supported by data which has demonstrated that in some contexts cells can divide at nonapical locations, despite tight nuclear packing (Strzyz et al., 2015; Weber et al., 2014).

Thus, compartmentalization of mitotic nuclei to a particular apical zone might not be absolutely necessary for successful cell proliferation in PSE. Still, such a restriction of mitotic nuclei to a defined tissue region of the apical side, from which interphase nuclei are usually displaced, might allow cells to divide more freely, without the interfering presence of nonmitotic nuclei in the vicinity. Therefore, moving nuclei apically might contribute to the optimization of cell proliferation in a densely packed tissue.

2.5 PRAM and Mitotic Entry

In rat radial glial cells it was observed that when PRAM was blocked during the final stages of nuclear movement, just before the nucleus had reached the subapical region, the cells were not able to enter mitosis and remained in a premitotic state (Hu et al., 2013). On the basis of these observations, it was speculated that the apical side might provide some signals essential for mitotic entry (Fig. 2C). This would mean that successful PRAM is critical for the proliferation of cells in the PSE, as it would represent a prerequisite for mitotic entry. However, in other systems including the mouse neocortex, chick neural tube, as well as the intermediate lengths PSE of the Drosophila wing disc and the zebrafish retina, it has been shown that cells within the intact PSE can enter mitosis also at nonapical locations (Liang et al., 2014; Spear and Erickson, 2012a; Strzyz et al., 2015; Tsuda et al., 2010; Yang et al., 2012). Therefore, it seems that PRAM is not absolutely necessary for mitotic

◀ at the apical side than within the tissue. Due to the apical endfeet attachments and/or displacement of interphase nuclei from this region upon rounding, a mitotic zone devoid of neighbor nuclei might thereby be created apically and aid in PSE proliferation (Fish et al., 2008; Schenk et al., 2009). (C) By entering mitosis at the apical surface, a cell might gain contact to putative factors that ensure proper completion of mitotic events (Hu et al., 2013). The identity of such factors, as well as whether they are intra- or extracellular is so far unknown. (D) PRAM might be important to allow contact between the nucleus and the apical centrosome (Fish et al., 2008; Miyata, 2008). In vertebrates, the centrosome is associated with the primary cilium at the apical side of the cell during interphase. Upon mitotic entry, the cilium is resorbed and the centrosome participates in spindle formation. (E) Apically dividing proliferative cells have a horizontally aligned spindle, and both daughters reintegrate into the tissue after mitosis. Nonapical divisions, however, impede cellular reintegration and cause perturbations in PSE architecture, suggesting that PRAM helps to maintain tissue integrity (Strzyz et al., 2015).

entry in many PSE. Nevertheless, certain signals stimulating mitotic entry might be provided by the apical surface and some tissues might rely more on these signals than others.

2.6 PRAM and Centrosome Position

In PSE the centrosome remains apically of the nucleus during the whole cell cycle (Fig. 2D). In vertebrates, this apical centrosome is involved in nucleating the primary cilium, which serves as a signaling hub during interphase (Chen et al., 1998; Miyata, 2008; Müsch, 2004; Rodriguez-Boulan and Macara, 2014). Although in cuboidal and columnar epithelia the distance between nucleus and centrosome is often in the range of one to five micrometers, in PSE these distances can reach up to tens of micrometers depending on nuclear position before PRAM (Fig. 2A). In mitosis however, the centrosome and the nucleus need to meet, as centrosomes are important factors for the organization of the mitotic spindle (Nigg and Raff, 2009). Consequently, it has been speculated that one reason for the occurrence of PRAM is to bring the nucleus into the vicinity of the apically localized centrosome for mitosis (Fig. 2D). This, in turn, would ensure that the centrosome can serve as a basal body for the primary cilium in interphase and as a spindle organizer in mitosis (Fish et al., 2008; Miyata, 2008, 2015; Schenk et al., 2009). Therefore, it was suggested that the apical position of the centrosome is the major cause for PRAM. This indeed seems to be the case in elongated PSE as well as neocortical radial glia, since in these tissues the apical centrosome is involved in arranging the MT tracks, along which the nucleus is transported apically. However, in intermediate length PSE, PRAM has been shown to occur independently of centrosome position. It was recently demonstrated that in the zebrafish retinal neuroepithelium PRAM still occurs following centrosome mispositioning or ablation. It even ensues after nonapical centrosome-nucleus association (Strzyz et al., 2015). Similarly, in the Drosophila wing disc apical mitoses take place even in the absence of centrosomes (Poulton et al., 2014), indicating that also in this tissue PRAM occurs independently of centrosome position or existence. This means that apical centrosome localization is not an absolute prerequisite for PRAM in intermediate length PSE. Nevertheless, by moving nuclei apically to meet the centrosome in the control scenario, mitotic entry is most likely facilitated. As the constant apical position of the centrosome is important for robust signaling from the primary cilium, this might enable the cell to orchestrate signaling and mitotic events (Fig. 2D).

It is further possible that the apical position of the centrosome also has additional functions in interphase independent of primary cilia function, as cells in Drosophila PSE lack primary cilia, but still show apical positions of centrosomes throughout the cell cycle (Meyer et al., 2011). In this context, it is imaginable that apical centrosomes are important for maintaining the characteristic apico-basal organization of the MT cytoskeleton observed in PSE (Norden et al., 2009; Z. Xie et al., 2007; Yang et al., 2012) and thereby for intracellular trafficking and organelle positioning in these tissues.

2.7 PRAM and Efficient Cell Reintegration Into Tissue Following Division

Despite the fact that in many PSE nuclei can enter mitosis and even divide nonapically (Liang et al., 2014; Spear and Erickson, 2012a; Strzyz et al., 2015; Tsuda et al., 2010; Yang et al., 2012), PRAM and subsequent apical divisions are highly conserved in all PSE. A hallmark of cell divisions during progenitor expansion in PSE is the perpendicular cleavage plane orientation with respect to the apical surface (Cui et al., 2007; Das et al., 2003; Kosodo et al., 2004; Nakajima et al., 2013; Sauer, 1935; Y. Xie et al., 2013) (Fig. 2D). This control of the cleavage plane positioning is important for the bisection of the apical membrane and thereby can influence the distribution of apical components into both daughter cells. In some examples of PSE, namely mouse radial glia cells, the chick neural tube and the Drosophila wing disc, perturbation of cleavage plane orientation can cause one daughter cell to lose its apical attachment leading to cell delamination (Konno et al., 2007; Morin et al., 2007; Nakajima et al., 2013). Importantly, components such as aPKC, LGN, NuMa, SCRIB/DLG, which regulate cleavage plane orientation in various PSE localize apically in these tissues (Cui et al., 2007; Horne-Badovinac et al., 2001; Konno et al., 2007; Morin et al., 2007). Consequently, PRAM appears to be necessary to ensure perpendicular divisions and thus the generation of two daughter cells that both inherit parts of the apical cellular compartment. Additionally, it has recently been suggested that the importance of PRAM in maintaining overall PSE integrity reaches beyond the need of cleavage plane orientation. It was shown in the intermediate length PSE of the zebrafish retina that here the interference with the mitotic cleavage plane does not induce cell delamination while the inhibition of PRAM does. In this case, perturbation of PRAM led to nonapical mitotic entry followed by nonapical cell division. This in turn resulted in the generation of cells that were not able to efficiently reintegrate into the

tissue following division. Consequently, cell delamination and ectopic proliferation were observed (Fig. 2C). Such ectopic divisions majorly interfered with retinal layer formation and subsequent organ morphogenesis (Strzyz et al., 2015). Together, these insights reveal that by ensuring apical mitosis, PRAM allows the cells in diverse PSE to maintain or reestablish their apical contact following division. Thus, PRAM serves as a mechanism that safeguards PSE tissue integrity and architecture (Fig. 2E).

In conclusion, while it is clear that PRAM allows cells to reproducibly divide at the apical location, the impact of interference with this process varies depending on tissue type. Generally, PRAM appears to optimize the proliferative output of the tissue, support mitotic entry and allow the centrosomes to meet the nucleus apically for mitosis. Furthermore, PRAM seems to play a major role in maintaining PSE tissue integrity as well as preventing cell delamination (Fig. 2). However, further cross-organismal studies are necessary to better understand functions of PRAM.

3. TISSUE-WIDE PROPERTIES OF PSE

To date, most studies of PSE focused on single cells and their dynamics, as discussed in the previous chapters. Tissue scale mechanical properties, however, are so far only scarcely explored, but tissue-wide understanding of proliferation, mechanical properties, and responses to stimuli is necessary to fully comprehend the development and function of these important tissues. In the final part of this review we will therefore offer some ideas regarding the crosstalk between tissue architecture, mechanics and cell proliferation, aiming to open new grounds for future studies in the field of PSE biology.

3.1 PSE Tissue Packing

As noted previously, PSE are tightly packed, increasing cell density with increasing PSE elongation. When cells round up at the apical side in order to undergo mitosis, they take up several times more apical surface area than their apical endfeet do during interphase (Smart, 1972) (Fig. 3B). Because of this, the available apical area could represent a constraint to PSE proliferation and division rates. This means that the number of layers of interphase nuclei that will eventually divide apically must be coordinated with the availability of this apical surface and packing of the PSE might serve as readout of proliferative

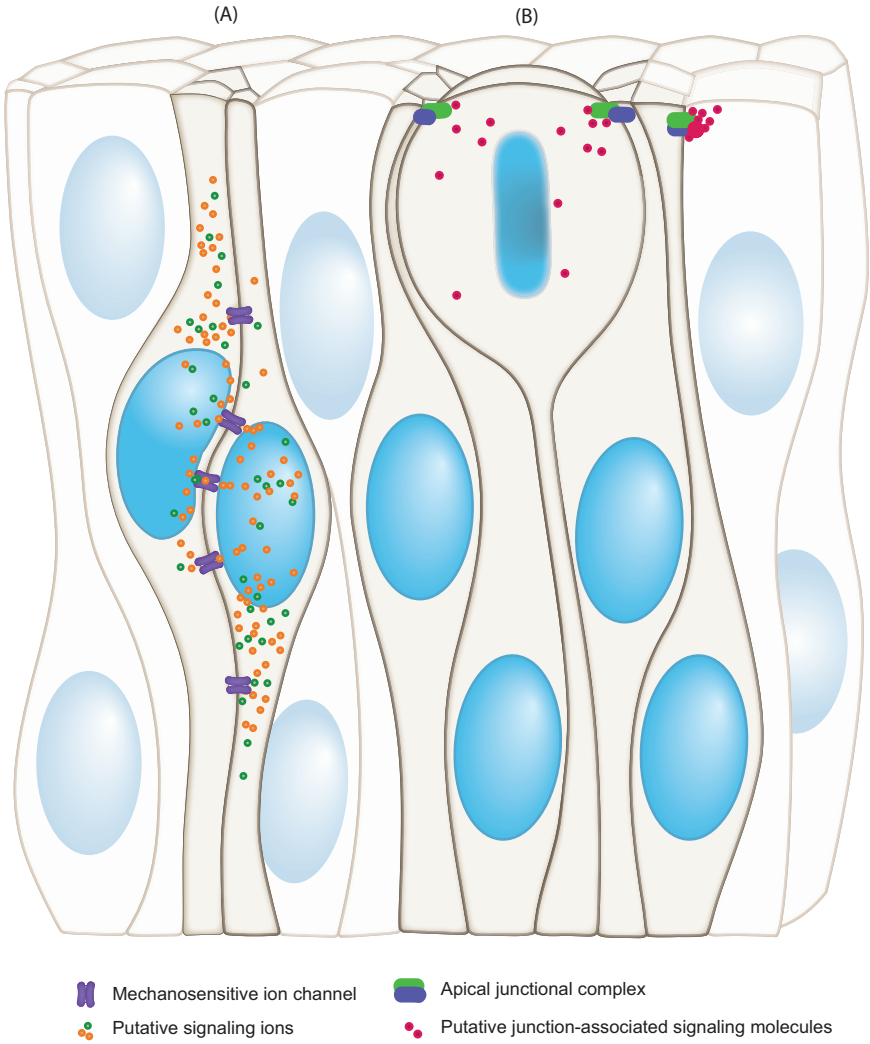

Figure 3 Mechanical stimulation and responses to it can originate in cell deformation during PRAM and mitosis. (A) By mechanosensitive ion channels such as Piezo localized throughout the lateral cell membranes, lateral forces arising during PRAM might regulate proliferation and proliferation-driven growth of the PSE. (B) Upon mitosis, the rounded cell has increased surface tension (Stewart et al., 2010) and takes up several times more space at the apical side than the endfoot of an interphase cell (Smart, 1972, 1973). In this way, rounding might displace apical junctions between the rounded cell and neighboring, interphase cells. Depending on the amplitude of this displacement, rounding could result in the release of a junction-bound signaling molecule, such as a components of the Hippo pathway (Benham-Pyle et al., 2015), signaling to the cell cycle machinery and thus controlling proliferation rates throughout the tissue.

capacity. To understand the limitations to PSE proliferation and the maximal number of nuclear layers that can make up a specific PSE, it is important to know how much of the apical space a mitotic cell inhabits and for how long it occupies this apical surface unit. Additionally, it is important to identify how long interphase nuclei need to reach the apical surface. Building upon this idea Fish et al. (2008) defined the optimal packing as the maximal number of proliferative layers that can be packed under the apical surface of a specific PSE, without altering its cell cycle parameters. This number of nuclear layers is expected to be proportional to the total cell cycle length and inversely proportional to the length of mitosis. It is important to note that Fish et al. treat proliferation as the only contributor to PSE packing. With respect to this, one might argue that both proliferation and minimization of cell extrusion/death lead to increased packing. However, cell death is not widespread in most PSE investigated to date (Dzafic et al., 2015; Milán et al., 1997; Naruse and Keino, 1995) and might often be negligible when it comes to its effect on total cell number and growth. Proliferation, on the other hand, is the major contributor to PSE cell numbers and we will thus continue our discussion with the assumption that cell extrusion/death can be neglected.

Due to the fact that M phase and total cell cycle length, as well as the size of the rounded, mitotic cell can differ between tissues, the optimal packing introduced previously will also vary between different PSE. As a simple illustration of this difference, one can consider two theoretical PSE tissues, A and B, that both have a packing level of 10 (ie, they harbor 10 layers of nuclei stacked in an apico-basal tissue column). We assume that A and B both have equal cell cycle and M phase lengths of respectively 12 h and 30 min. What they differ in, though, is the relative size of mitotic cells. In tissue A, the mitotic cell is relatively small compared to tissue B—it takes 2 times more apical surface than an interphase nucleus does, whilst in tissue B the M-phase cell takes up three times as much apical surface as an interphase nucleus. Taking these facts together this would mean that PSE A has the highest proliferative efficiency at its packing optimum of 12 layers, and PSE B at the optimal 8 layers. Since they both harbor *10* nuclear layers, PSE A would result as *under-* and PSE B as *overpacked*. The underpacked tissue might occur earlier in development and could further increase its proliferative efficiency by packing more nuclear layers. Here, a positive feedback loop mechanism might act, sensing the tissue packing state and allowing cells in these tissues to further increase their proliferative capacity. Conversely, tissue B, because it is overpacked, might be a more developed PSE at the onset of differentiation. In this scenario the apical surface might become overcrowded with mitotic

cells and consequently hinder its own proliferation through a negative feedback loop. Still, tissue B could potentially reach its optimal packing level, provided that M-phase is shortened and/or overall cell cycle length prolonged. In conclusion, packing of the PSE might serve as readout of proliferative capacity as mentioned previously, but only if the tissue's optimal packing level is already known from measuring all cell cycle parameters. Following this rationale Fish et al. (2008) calculated the packing level that allows maximal proliferation of mammalian radial glia cells. Using values of cell cycle parameters and M-cell size obtained from fixed samples, they concluded that, in the PSE of the mammalian cortex, radial glia proliferation seems most efficient when eight layers of nuclei are stacked in a nuclear column beneath the apical surface. This layering thus represents the optimal packing level of this particular PSE tissue (Fish et al., 2008).

However, so far these thoughts are merely theoretical. It would therefore be now important to experimentally verify whether packing of different PSE tissues follows these predictions. This can be achieved by examining proliferation in presumably over-, under-, and optimally packed PSE, as well as by investigating the links between packing and tissue development. As PSE tissue packing has so far not been systematically analyzed, many fundamental questions regarding this feature remain: Whether, and, if so, how does the proliferation rate in different PSE scale with this increase in tissue packing? Can PSE sense and increase their packing levels? Assuming that changes in packing also affect PSE mechanical properties, can this feed back to proliferation control mechanisms? If yes, how could this mechanofeedback be regulated? As suggested here and in the example above, intricate feedback loops might be at the center of coordinating growth and cell cycle parameters to keep proliferation of a PSE at its intrinsic optimum. It would be fascinating if such mechanisms would indeed be identified, and the upstream cues and downstream molecular cascades involved in their regulation dissected. Further, we provide a discussion on how PSE packing under spatial constraints could potentially be controlled raising outlooks for future studies.

3.2 Packing Increase in PSE

In the *underpacked* state, the apical surface unit might accept a higher mitotic frequency, meaning that the tissue could robustly proliferate and further increase its packing, as illustrated in the previous section. More nuclear layers could, in turn, result in growth by increasing tissue thickness. This might

further raise the tissue's proliferative capacity and total cell number. Therefore, it could be beneficial for the developing PSE to maximize its packing by increased proliferation instead of remaining in the underpacked state.

However, as this way of packing more nuclear layers would eventually lead to overall tissue expansion, it might be most efficient only in case when growth is spatially unrestrained. Additionally, with increased number of cells and packing, proliferation would rise as well, resulting in the need for more (apical) space to accommodate mitotic cells. Tissue growth is, however, typically limited in the developing organism, for example, it might be internally restricted by the tissue's apical surface tension (Okamoto et al., 2013). Consequently, proliferation and packing within the PSE might be affected by significant external spatial constraints (Streichan et al., 2014) imposed by the limited space in which the tissue develops. Hence, in order to increase packing, these limiting effects would need to lessen (LeGoff and Lecuit, 2015). Cell elongation and/or cell volume decrease could be potential strategies that might be employed to increase packing upon constrained growth. It is known that PSE tissues indeed elongate and pack more nuclear layers as they mature. In Smart's 1972 study of the mouse diencephalon, a 10-day-post-conception (E10) PSE had a thickness of 90 μm with six layers of nuclei. At day 11 the tissue was 110 μm thick, with eight nuclear layers, to grow up to 150 μm in thickness and a twelve-layer pseudostratification at day 12 (Smart, 1972). In order to undergo such changes in degrees of pseudostratification, cells would have to undergo multiple structural rearrangements. Specifically, they would need to elongate and thin their cellular processes, reorder their nuclei as well as decrease the attached endfeet areas (Miyata, 2015) in a tissue-wide, coordinated manner. With respect to this, mechanical tests have shown that apical endfeet are contractile in the PSE of the mouse cerebral wall (Okamoto et al., 2013). Active deformations such as endfoot shrinkage would thus most likely be mediated by actomyosin cortex contractions, which are known to play a central role in controlling cell shape (Salbreux et al., 2012). Such changes might also be largely enhanced by the abundant dynamicity of neighboring cells. Cell elongation, on the other hand, might be a more complex change, as it would imply both thinning of cellular processes, and elongation leading to the thickening of the entire tissue. Thus, cell elongation might require greater forces and might be governed by both cortical contractions/rearrangements, and microtubule polymerization throughout the cell body. However, cell elongation as a packing strategy might be an option only if spatial constraints acting perpendicular to the tissue plane are mild, as it would also result in tissue growth

and an increase of tissue thickness. With even further increase in packing prior to reaching the maximum, these constraints might become impossible to overcome. At this point, a decrease in cell and nuclear volume might be the only strategy allowing further increase in layering. With respect to this, it seems that cells do possess autonomous mechanisms to measure and adjust their size in accordance to the environment and cell cycle (Ginzberg et al., 2015). With this in mind, introducing cell size-related research to the PSE field and relating it to tissue-wide packing studies would largely contribute to our understanding of mechanisms governing PSE proliferation.

Because of the phenomenon of packing, growth in the PSE might not be directly coupled to proliferation, making research both experimentally and theoretically challenging. Nevertheless, it would be fascinating to understand how proliferation, elongation and volume decrease relate to each other and temporally overlap. So far nothing is known about the mechanisms behind any of these three possible packing strategies or about their developmental regulation in the PSE. Hence, multiscale, tissue-wide developmental studies, focusing on architectural changes in different PSE, are needed to link proliferation, elongation, and volume decrease to tissue expansion.

3.3 Linking Mechanical Stimulation to PSE Responses

The research field of tissue mechanics emerged in the 19th century, founded by D'Arcy Thompson, who was interested in the mechanical forces shaping biological systems. During the last decade it has reemerged as a highly multidisciplinary field of developmental biology (Mammoto and Ingber, 2010), owing to advances in live imaging and biophysical methods. It is now well known that diverse mechanical stimuli play major roles in proliferation, tissue development and function. Examples of animal development being affected by sensing mechanical stimuli include the developing zebrafish heart and nephrons, the developing mouse lung, and chick neural tube closure (reviewed in (LeGoff and Lecuit, 2015; Mammoto and Ingber, 2010)). The PSE, a highly dynamic epithelium with tightly packed elongated cells could serve as a model system providing novel insights into the role of mechanical cues in tissue development and function.

Recently, more pathways underlying the mechanisms of translating mechanical stimuli into cellular responses have been unraveled (Provenzano and Keely, 2011). In the recent years, studies have also touched upon mechanosensation in different pseudostratified tissues

(Mao et al., 2013; Porazinski et al., 2015; Schluck et al., 2013). Upon the application of force the studied PSE altered their proliferation levels or exhibited developmental changes. For example, a study of a fish mutant in the Hippo signaling pathway demonstrated organism-wide responses to gravitational force (mechanostimulation) (Porazinski et al., 2015). The mutant developed malformed (flattened) and misaligned organs, including the neural tube and optic cup, both of which derive from a PSE. In another study, direct links between mechanical strain and PSE proliferation were investigated (Schluck et al., 2013). By mechanically stretching Drosophila wing disc epithelia, it was confirmed that a proliferation increase by mechanical tension can exist in this PSE. With these two studies in mind, we can speculate that PSE are indeed mechanosensitive and can respond to mechanical forces such as strain by changing proliferation rates or other developmental parameters. Furthermore, several studies have dealt with mechanosensation by the cell nucleus (Dahl et al., 2008). This central organelle might be a possible mechanotransducing element in the PSE due to the tight packing of nuclear arrangement. Further, we discuss two exemplary mechanosensing pathways that might also be important regulators of PSE proliferation.

3.4 Hippo Pathway as Example of Mechanosensitive Signaling Pathway

The Hippo signaling pathway is well known to play a central role in regulating cell proliferation through its response to mechanical stimuli. Because its constituents have also been found to affect the development of PSE tissues (Porazinski et al., 2015), we believe that more detailed research focusing on the PSE could provide interesting insights into how Hippo regulates proliferation of complex tissues.

The Hippo pathway [reviewed in (Yu and Guan, 2014)] is conserved across the animal phylogeny, with orthologous genes studied in models ranging from Drosophila to mouse. It comprises a signaling cascade that affects tissue growth and homeostasis by controlling cell number by proliferation, growth, differentiation and death. As a result of these essential functions, the constituents of the Hippo pathway, their cascades and activities are extensively studied. Many Hippo signaling components localize to the cellular junctions (eg, PAR6, β-catenin) and several have been confirmed to interact with the cytoskeleton (eg, α-catenin, Zyxin) in both mouse and Drosophila (Yu and Guan, 2014). Upstream factors known to affect this

pathway are cell polarity, G-protein-coupled receptor (GPCR) signaling and mechanotransduction. In the latter, the master mediators of signal transduction seem to be cellular tension and the actin cytoskeleton (Yu and Guan, 2014). A mechanotransduction pathway following the cascade formed by tension sensors, Hippo and proliferation (Fig. 3B) is of special interest to this review because of the tightly packed state of the pseudostratified tissue. Therefore, a crowded, dynamic and polarized environment such as the PSE, with its PRAM and apical mitoses, likely provides a plethora of mechanical stimuli and the need for their efficient transduction throughout the tissue (Fig. 3).

It has recently been demonstrated in an epithelial cell culture study that mechanical strain controls proliferation via junction-associated YAP and ß-catenin (Benham-Pyle et al., 2015). Stretching quiescent epithelial sheets resulted in the relocation of ß-cat and YAP from the tight junctions to the nucleus. This, in turn, resulted in the activation of transcription leading to cell cycle reentry and increased proliferation. In an epithelial tissue such as the PSE, with the important feature of highly efficient proliferation, a similar Hippo-associated pathway might play a role and mediate cell cycle responses to proliferation, packing or nuclear dynamics (Fig. 3). In this regard, it would be helpful to have tension-sensing and strain-inducing in vivo methods in the PSE, to serve as read-out of the tissue's physical properties and test its Hippo pathway response to mechanical stimulation.

3.5 Piezo Channel as Example of Mechanosensitive Cellular Response Element

As another possible PSE mechanosensitive mechanism, we here describe the Piezo mechanosensitive ion channel (Coste et al., 2012). Although not yet studied in the PSE, Piezo might be distributed along lateral cell membranes and act as the mechanoresponsive element able to respond to forces arising from PRAM,

Mechanosensitive channels (Guharay and Sachs, 1984) are membrane-bound force-transducing molecules, whose working principles rely on their ability to respond to a wide range of external and internal local mechanical stimuli {eg, flows, (osmotic) pressure changes, stretching or position information) [reviewed in (Kung, 2005)]}. Following the stimulus, diffusion of ions (cations in eukaryotes) through the channel triggers an intracellular signaling cascade. Two current principal models of their gating mechanism are (1) the lipid bilayer tension or stretch model, in which

membrane tension induces a conformational change and opens the pore and (2) the spring-like tether model, in which tethers connect the channel to the ECM or the cytoskeleton (Lumpkin and Caterina, 2007), and the channel opens upon their displacement by local extra- or intracellular forces.

The Piezo is a pore forming protein of a mechanosensitive cation channel, conserved in mouse, zebrafish, and Drosophila (Coste et al., 2012; Eisenhoffer et al., 2012). With its highly specific structure, this channel opens upon changes in membrane tension, allowing the influx of Ca^{2+} ions. By promoting subsequent nuclear localization of YAP (Benham-Pyle et al., 2015; Pathak et al., 2014), Piezo represents another link of mechanical force transduction to cellular responses via the Hippo pathway. In the highly proliferative PSE, a mechanosensitive ion channel such as Piezo might form a signaling cascade involving Hippo, as well as downstream cell cycle regulators (Fig. 3). By localizing mechanosensitive ion channels throughout the lateral cell membranes, the tissue could respond to mechanical stimuli that are not directly related to apical junction displacements (Fig. 3A). In this way, lateral forces might also regulate proliferation and proliferation-driven growth of the PSE.

3.6 Possible Origins and Effects of Mechanical Forces in PSE

As mentioned previously, mechanosensing plays an important role in proliferation control of the Drosophila wing disc and development of the zebrafish neural tube and optic cup. Employing both junctional localization of Hippo pathway components and lateral membrane localization of mechanosensitive channels such as Piezo, the dynamic PSE would be "fully equipped" to respond to mechanostimuli originating anywhere along the apico-basal cell axis (Fig. 3). These tension-altering stimuli in the PSE might arise from local events such as PRAM (Fig. 3A), apical mitotic cell rounding (Fig. 3B), or from more global events such as crowding of the apical surface or increased tissue packing. For example, during apical mitosis, the rounded mitotic PSE cells could change their physical properties by increasing surface tension and weakening the junctions, similar to cells in culture (Stewart et al., 2010). Rounded cells could influence their neighbors as well, by compressing their apical endfeet. Furthermore, lateral membranes of the elongated PSE cells are subject to various deformations resulting from nuclear dynamics. Together, apical mitosis and PRAM might cause displacements and tension changes in the membrane-bound

apical polarity components (Fig. 3B), as well as in the neighboring cell lateral membranes under tension (Fig. 3A). Such shape changes could act as powerful signaling sources by, for example, changing the tension of plasma membranes (Tsujita et al., 2015). Additionally, overall changes in tissue packing result in significant alterations of PSE architecture as discussed previously, and could feed into mechanosensitive pathways. An important first step in future studies should be to investigate whether Piezo and/or other mechanosensitive channels act in the PSE. Furthermore, it will be important to demonstrate if and how PSE-specific events mentioned previously influence junctional components and the localization of Hippo pathway constituents such as YAP.

Altogether, mechanical stimulation in the PSE could lead to specific, tissue-wide coordinated cellular responses that affect proliferation. Recent work done on mechanosensing via the Hippo pathway and the Piezo channel opened new exciting research possibilities for PSE mechanobiology. Furthermore, research focusing on the localization, dynamics and function of Hippo components and mechanosensitive channels in the PSE will most likely shed light on the mechanical control of proliferation and growth. To accomplish this, developmental cell biological in vivo studies should be complemented with PSE in vitro research. Both in vivo and in vitro studies in the PSE, however, have their own advantages and limitations. Tissue-scale studies represent a challenge for live imaging due to the very tight packing and tissue thickness, but would allow unprecedented insights into tissue-wide dynamics of, for example., YAP or ion currents upon mechanical stimulation. On the other hand, maintaining a PSE as epithelial sheets or organoids in vitro is possible, but by no means trivial (Eiraku et al., 2011). These cells exhibit most of the PSE features (IKNM, differentiation) and might ease both imaging and manipulations. In the future, reproducible biophysical methods including tension sensors and packing alterations, together with powerful live imaging methods, would help to understand local and global physical properties of this tissue in both in vivo and in vitro environments. Further, investigation of possible feedback loops between mechanical forces and proliferation and growth at both the tissue as well as the cellular levels will be important. We believe that the PSE with its tight packing, nuclear dynamicity and developmental relevance represents an excellent model to expand our knowledge of tissue mechanics to more complex epithelial tissues and will provide new insights into general regulation of cell proliferation and tissue growth.

4. CONCLUSIONS

Until now studies of PSE mainly focused on single-cell behavior. As a result, we gathered a significant understanding of the PSE cell biology, including valuable insights into nuclear dynamics occurring in these cells as well as their importance for cell and tissue proliferation. What we need to explore in much more detail, however, is how the dynamic events occurring in single-cells, such as PRAM and apical mitoses translate to tissue-wide changes. Future studies of mechanisms linking mechanics to PSE proliferative dynamics are essential to fully comprehend the development and function of these tissues. In addition, large-scale comparative quantitative studies are necessary to understand the versatility of PSE types and their packing levels. Finally, we believe that a transition toward a more mechanobiological approach, together with a shift toward a more comparative, tissue-scale perspective in the field of PSE biology should be underway. These additional routes in PSE research will result in unprecedented insights into the developmental and evolutionary causes and consequences of pseudostratification itself.

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