Inductive signals in branching morphogenesis – lessons from mammary and salivary glands

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Abstract

Branching morphogenesis is a fundamental developmental program that generates large epithelial surfaces in a limited three-dimensional space. It is regulated by inductive tissue interactions whose effects are mediated by soluble signaling molecules, and cell-cell and cell-extracellular matrix interactions. Here, we will review recent studies on inductive signaling interactions governing branching morphogenesis in light of phenotypes of mouse mutants and *ex vivo* organ culture studies with emphasis on developing mammary and salivary glands. We will highlight advances in understanding how cell fate decisions are intimately linked with branching morphogenesis. We will also discuss novel insights into the molecular control of cellular mechanisms driving the formation of these arborized ductal structures and reflect upon how distinct spatial patterns are generated.

Introduction

The mammalian kidneys, lungs and many of the exocrine glands such as salivary and mammary glands develop through a process of branching morphogenesis where an epithelial rudiment elongates and branches iteratively to give rise to an arborized tree-like structure. Each type of organ ends up with a unique shape, which reflects organ-specific differences in its construction. Firstly, the structure of the tree hierarchy is determined through usage of two different modes of branching: splitting of the branch tip (tip furcation or clefting) and budding from an existing duct (lateral/side branching). Secondly, branching can take place according to a stereotyped routine as seen in the lung, where each lung adheres to a near identical order of branching events, or following stochastic principles as suggested for the mammary gland, where each mammary gland exhibits obvious differences in overall branching appearance [1,2]. Finally, the frequency of branching yields trees of different densities exemplified by the tightly packed salivary gland and sparse pre-pregnancy mammary architecture [3,4] (Figure 1).

The development of the salivary and the mammary gland begins by formation of an invaginating bud, which elongates and starts to branch as a solid epithelial structure that is progressively hollowed into a bilayer of luminal and basal cells. Branching is not stereotypical, and the existence of local paracrine signals acting as guidance cues has not been confirmed. Submandibular salivary gland branching morphogenesis is characterized by sequential bud enlargement and clefting, which is completed during embryogenesis [4]. Embryonic mammary gland development is hormone independent and yields a rudimentary ductal tree, whereas development in puberty and pregnancy is driven by reproductive hormones whose effects are, however, mediated by locally produced growth factors [3]. The pubertal stage is characterized by invasive ductal tips termed terminal end buds (TEB) that consist of a single basal layer (cap cells) and a multilayered luminal cell population (body cells).

Two experimental approaches, genetically modified mouse models and *ex vivo* organ culture systems, have been highly informative in identifying the critical signals controlling branching morphogenesis. However, the explicit functions of signaling molecules are often difficult to define, because the arborization of the epithelial tree is an integrated process of cell proliferation, branch point generation, and branch elongation. In this review, we reflect upon the recent mechanistic insights of inductive signals guiding branching morphogenesis, with an emphasis on the mammalian mammary and salivary gland.

Cell fate and branching

Inductive signals regulate cell fate decisions as well as morphogenetic cell behaviors and recent studies have linked branching potential with differentiation state. The importance of Fibroblast growth factor 10 (Fgf10) in salivary gland development has been recognized for a long time [4].

Chatzeli *et al.* revealed that salivary gland branching morphogenesis is initiated when mesenchymal Fgf10 induces specification of distally located progenitors in the salivary bud [5] (Figure 1A). The branching potential may be limited to these cells, since when mechanically separated, only the distal part, not the proximal stalk, can initiate branching. In addition to signal input coming from the nerves [4], the fate of the proximal progenitors depends on the activity of Yap, a Hippo pathway transcriptional regulator, which mediates its effects, at least in part, by inducing the Epidermal growth factor (Egf) family member Epiregulin [6**]. The Yap deficient salivary glands had fewer end buds, a branching defect shown to result from domain specification defects rather than compromised proliferation. Therefore, although distal cells may be competent to initiate branches [5], coordinated behaviors of both distal and proximal cells are required for branching morphogenesis.

The segregation of early mammary progenitors into distinct proximal-distal populations during embryonic mammary branching morphogenesis remains largely unexplored. Lineage tracing and single-cell RNA-sequencing data suggest that the first lineage segregation takes place along the basal-luminal axis around birth, i.e. after a couple of rounds of branching has already taken place [7**,8]. Lilja *et al.*, however, provided evidence that the basal and luminal lineages become primed already when branching begins and suggested that the two processes may be mechanistically linked [9**]. Ectopic activation of the Notch1 receptor drives cells towards luminal fate [9**], and transcription factor ΔNp63 towards basal fate [7**], but the identity (chemical or mechanical) and origin of signals that specify the lineages remain to be identified (Figure 1B).

Later stages of mammary branching morphogenesis are principally fueled by unipotent luminal and basal stem/progenitor cells, although existence of multipotent stem cells has not been ruled out [10]. A distally located stem cell pool has been proposed to lie within TEBs, whose progeny is left behind in the trailing duct as the TEB advances [11**] (Figure 1C). Based on this model, branching activity at puberty is driven by the bifurcating tips only. Additionally, the proximal ductal area houses stem cells that remain quiescent during puberty, but contribute to pregnancy-induced epithelial expansion [12]. Whether mammary epithelial cells in different parts of the gland possess intrinsically different morphogenetic potential or whether the ability to branch is bestowed by transient signals, remains an open question.

Epithelial growth and maintenance

Cell proliferation is necessary to produce building blocks for the growing organ and expansion of a nascent branch. One of the best characterized pathways regulating proliferation is the Fgf/Erk pathway: stromally produced Fgfs, in particular Fgf10, are essential for both salivary bud outgrowth and mammary TEB formation [3, 4, 13]. Additionally, loss of epithelially produced Fgf20 compromises mammary ductal growth [14]. Intriguingly, the expression of Fgf20 wanes after embryogenesis, yet the phenotype only arises at puberty. The underlying mechanism remains to be untangled but one possibility is that absence of Fgf20 leads to qualitative changes in the stem/progenitor cells that only manifest when robust stem cell activation is needed. Fgf20 is not expressed in developing salivary glands (our unpublished data).

During recent years, the Tumor necrosis factor (Tnf) family member Ectodysplasin (Eda) has emerged as an important mesenchymal cue: its loss compromises growth and branching, while its overexpression has the opposite effect in both salivary and mammary glands [14-17]. Eda signaling activity is confined to the epithelium where it regulates the expression Fgf20, as well as ligands of many other pathways (Egf, Wnt, Sonic hedgehog) [18] suggesting that Eda's effects are likely mediated by multiple signaling pathways. Loss of Fgf20 attenuates the Eda overexpression hypergrowth phenotype thereby confirming the importance of Fgf20 downstream of Eda signaling [14].

Although many of the molecular mechanisms regulating mammary and salivary gland branching are shared, the Wnt pathway is an exception. Salivary gland end buds are devoid of Wnt activity, but mesenchymal Wnt signaling is thought to regulate salivary gland growth indirectly by inducing the expression of paracrine factors such as Eda [15]. On the other hand, epithelial Wnt signaling has an established function as a regulator of TEB number and size and is suggested to maintain the stem/progenitor status in the TEB [13]. The study by Sreekumar *et al.* [19**] revealed new mechanistic insights into Wnt action by showing that Wnts promote survival of the TEB cap cells by preventing the nuclear accumulation of the pro-apoptotic FoxO transcription factors. Macrophages, which enrich around TEBs and are largely fetal-derived [20], were shown to be the source of Wnts [21**]. A positive feedback loop between cap cells and macrophages was identified: the former produce Notch ligand Delta Like Canonical Notch Ligand 1 (Dll1) which activates Notch2/3 receptor-mediated signaling in macrophages, leading to increased production of Wnts [21**].

A completely new molecular mechanism regulating epithelial proliferation was identified by Hayashi *et al.* [22**], who showed that mesenchymally produced micro-RNA containing exosomes regulate proliferation in developing salivary glands. miRNA-133b-3p was identified as the critical cargo and proposed to downregulate DIP2B, a protein involved in DNA methylation, to epigenetically control the expansion of distal progenitors. Weather exosome-mediated tissue interactions also regulate mammary gland morphogenesis is currently unknown.

Branch elongation

From the construction point of view, branches are elongated by deposition of cells from the tip to the duct or by cell division and/or rearrangement within the duct itself. Mammary ductal cells are the progeny of stem/progenitor cells proliferating in the TEB, suggesting that elongation is driven from the tip [11**]. The progenitors in the salivary tip also give rise to the distal ducts [23], but their proliferation contributes relatively more to tip enlargement than ductal elongation, which has been attributed to the range of the growth-inducing signal [24]. The proximal ducts of the salivary gland are sustained by their own supply of cells, suggesting that they elongate on a different basis than the terminal branches [23].

Ducts may change their aspect ratio through convergent extension, which has been reported for elongation of the kidney tubules where Wnt9b regulates planar cell polarity (PCP) activity [25,26]. Vangl1/2 membrane proteins are essential mediators of convergent extension and oriented cell divisions through PCP. About half of embryonic mammary glands with compromised surface expression of Vangl proteins (*Vangl2* Looptail mutant) fail to sprout and branch and instead form a large bud-like structure suggesting involvement of cellular rearrangements in elongation [27]. Yet, morphometric analysis of postnatal glands suggests that the length of ducts becomes fixed, implying that mammary ducts do not intrinsically elongate [11**].

Although cell proliferation at the tip feeds cells into the duct, it is not clear whether proliferation *per se* drives tip displacement. Mammary ductal elongation has been modeled purely based on cell proliferation/apoptosis in the TEB, given that the geometry of the TEB remains constant [28]. In this model, cell crowding in the TEB creates a flux of cells to the duct, pushing the TEB forward, but this has not been experimentally confirmed. Experimental studies on mesenchyme-free organoids have revealed that displacement of the tip may also be regulated by collective cell migration and proliferation only defines the number of migratory cells [29] (Figure 2A). The migratory behavior is regulated by a gradient of Erk signaling pathway activity, where the fastest cells are found at the tip front. On the other hand, the migratory behavior of salivary epithelial cells follows a different pattern and is characterized by faster outer-bud cells and slower inner-bud cells, as revealed by organ culture studies [30] (Figure 2B). The lateral mode of

movement of the outer-bud cells may be related to remodeling of the basement membrane (BM) at the epithelial-stromal interface, which is required for radial expansion of the bud [31].

Although mammary organoid studies have implicated that the mechanism of ductal elongation can be intrinsic to the epithelium, the stroma clearly plays a role *in vivo*, not only as a source of growth factors but also through its mechanical properties. Stromal collagen fibers have been proposed to guide ductal elongation and branching based on their pre-patterned orientation in the ECM [32]. Peuhu *et al.* [33] proposed that proper stromal stiffness and collagen fibrillogenesis by stromal fibroblasts is instrumental for ductal elongation at puberty. The basis for this is enigmatic as there is no evidence that mammary epithelial cells would utilize the stromal ECM directly as a substrate for e.g. cell migration *in vivo*. Fibrillar collagens associated with mammary ducts are also substrates for enzymatic digestion by matrix-metalloproteinases (MMPs) [13]. MT1-MMP/Mmp-14-mediated collagen remodeling by periductal fibroblasts rather than by epithelial cells, was shown to be essential: its absence blocked ductal elongation and branching at puberty [34**]. How fibroblasts are induced to perform these activities locally remains to be investigated, although ECM remodeling can be regulated at least via EgfR signaling [35].

Branch point generation

A number of cellular mechanisms have been shown or proposed to govern branching in different organs: oriented cell divisions, collective cell migration, differential growth, epithelial folding, and ECM-driven tissue shape changes [36]. The mechanism of tip splitting has been most extensively studied in salivary glands where it is associated with appearance of narrow clefts. The cue inducing cleft initiation is unknown but the Egf pathway has emerged as a candidate [37,38]. Cleft progression is driven by deposition of fibronectin and involves actomyosin contractility [4]. Organ culture studies revealed that fibronectin deposition leads to focal induction of BTB/POZ domain-containing protein 7 (Btbd7) (Figure 2B) [4], and generation of the *Btbd7* null mouse model confirmed its requirement for branching morphogenesis *in vivo* [39*]. Btbd7 affected cell motility specifically in outer (basal) cells by inducing E-cadherin ubiquitination and degradation providing a mechanistic explanation for the ability of Btbd7 to control cell adhesion and migration. Loss of Btbd7 disrupts branching morphogenesis also in the lung and kidney [39*], but its role in mammary gland development is unknown.

Lateral branching in postnatal mammary glands occurs in the context of an epithelial bilayer and therefore likely depends on a different morphogenetic program than tip bifurcation (Figure 2A). Side branching is well documented to occur during estrous cycle and pregnancy downstream of progesterone whose effects are mediated, at least in part, by Wnt4, and the Tnf family member Rankl [3]. Transcription factor Id2 is another mediator of progesterone receptor-mediated signaling, which appears to control side branching through differentiation of bi-potent luminal progenitors into cells expressing CD61 that was identified as a marker for side branches [40*]. In contrast to canonical Wnt pathway, non-canonical Wnt5A/Ror2 signaling suppresses side branching, a function attributed to its ability to modulate the actin cytoskeleton [41,42].

Morphological analysis of wildtype and several genetically modified mice suggest involvement of side branching also during pubertal mammary gland development [43], although this view has recently been challenged [11**]. The distance between branch points in the nipple proximal area does not decrease between 5 and 8 weeks of age as would be expected if side branching was to occur [11**]. Further, by whole-mount EdU analysis, Scheele *et al.* [11**] detected proliferation only in TEBs. Previous studies have, however, reported significant cell proliferation also in ducts during puberty by analyzing Ki67 or BrdU incorporation in sections [44,45]. If side branch formation is not primarily driven by locally enhanced cell proliferation, but rather e.g. by cell shape changes as shown for the avian lung [46], it might be difficult to

conclusively detect an incipient lateral branch in fixed samples, and once formed, could not be distinguished from TEB formed by tip splitting.

Patterning

How spatial patterning and the geometry of branched networks are generated and to what extent are the construction principles shared between branched organs remain outstanding questions in the field. One appealing approach to tackle this question is computational modeling [47,48]. Recently, branch pattern formation in the mammary gland, kidney and prostate was proposed to be governed by simple generic rules involving the collective dynamics of progenitors present at ductal tips that drive ductal elongation and stochastic tip bifurcation [2,49]. Tips, however, compete for space and get terminated in regions of high ductal density, and hence branching could be regarded as a stochastic, self-organized process rather than a hard-wired genetic program. In the kidney, cessation of tip growth was proposed to occur when nephrons differentiate at individual tips [2]. However, more recent studies found no evidence for stochastic cessation of tip growth by nephrogenesis, or any other mechanism, and 3D renderings do not reveal ureteric bud tips "inside" the growing kidney [50,51]. Indeed, in the lung and kidney, the initial rounds of branching are highly stereotyped [1,50], arguing for a deterministic rather than stochastic process. Ligand-receptor-based Turing type of mechanism involving a positive signaling feedback loop in combination with the tissue-restricted expression of the ligand and receptor has been proposed to pattern both the lung [52] and kidney [53*], Fgf10-FgfR2b forming the key ligand-receptor pair in the former and GDNF-Ret in the latter. Whether a similar principle also applies to salivary and embryonic mammary gland pattering – both critically dependent on stromal Fgf10 and epithelial FgfR2b – remains to be explored.

Concluding remarks

The relative ease of culturing embryonic salivary glands in combination with advanced live microscopy has enabled major leaps in understanding the cellular behaviors induced by inductive cues. However, in mammary gland research, current knowledge is largely based on static images of fixed 2D samples, although stroma-free organoid studies have been highly informative in dissecting many cellular level details in 3D [29]. Classic tissue recombination experiments, however, underscore the importance of stroma by showing that mammary gland epithelium recombined with salivary mesenchyme results in salivary-like epithelial morphogenesis [3]. The development of an ex vivo culture method for the embryonic mammary gland [16], clearing protocols for 3D analysis of postnatal mammary glands, intravital imaging of postnatal glands at single cell resolution [11**] together with the single-cell omics revolution is expected to lead to major discoveries on the molecular and cellular mechanisms governing mammary branching morphogenesis in near future.

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Figure legends

Figure1. Distribution of progenitor cells driving branching morphogenesis. A) The salivary

epithelium is specified into distal and proximal progenitors that regulate the expansion of the tip and stalk domains of the gland, respectively. B) Mammary epithelium is specified into basal and luminal progenitors, whose coordinated behaviors are likely required for branching. C) In the pubertal mammary gland, progenitors have been proposed to reside in terminal end buds (TEBs) whereas the ducts contain their quiescent progeny. Quiescent stem cells have been shown to contribute to side branching during pregnancy. Whether side branching contributes to pubertal morphogenesis, is controversial in the field.

Figure2. Cell behaviors regulating branch elongation and branch point generation. A) In the mammary gland, Fgf signaling activates Erk and Akt pathways to promote cell migration driving tip advancement, whereas cell proliferation provides material for growth. Survival of epithelial cells in the basal layer is regulated by Wnt signals produced by mesenchymal macrophages (blue). Remodeling of the stromal collagen fibers by MT1-MMP/Mmp14 of stromal fibroblasts (red) is required for branching. Cell rearrangements are thought to regulate assortment of cells between equipotent tips during tip bifurcation. However, the signals and cell behaviors that drive mammary TEB bifurcation are currently unknown. Canonical Wnt signaling has been implicated for budding of side branches from the mammary duct, whereas maintenance of the ductal architecture is associated with non-canonical Wnt signaling. Side branches are generated by CD61+ progenitors, whose local differentiation is controlled by the transcription factor Id2. **B)** In the salivary gland, cell movement in the salivary tip is compartmentalized into faster migrating outer-bud cells and slower migrating inner bud cells. The outer-bud cells move laterally along the BM, remodeling it by creating microperforations, which allow the epithelium to expand. The pressure for epithelial expansion is likely generated by cell proliferation in the tip, although contribution of inner bud cell motility is also possible. Clefts are initiated by localized assembly of fibronectin (FN) by bud epithelial cells into a wedge-like structure that progressively splits the tip. FN also induces Btdb7, which regulates cell-cell detachment through repression of E-cadherin and motility at the cleft site to aid cleft progression.

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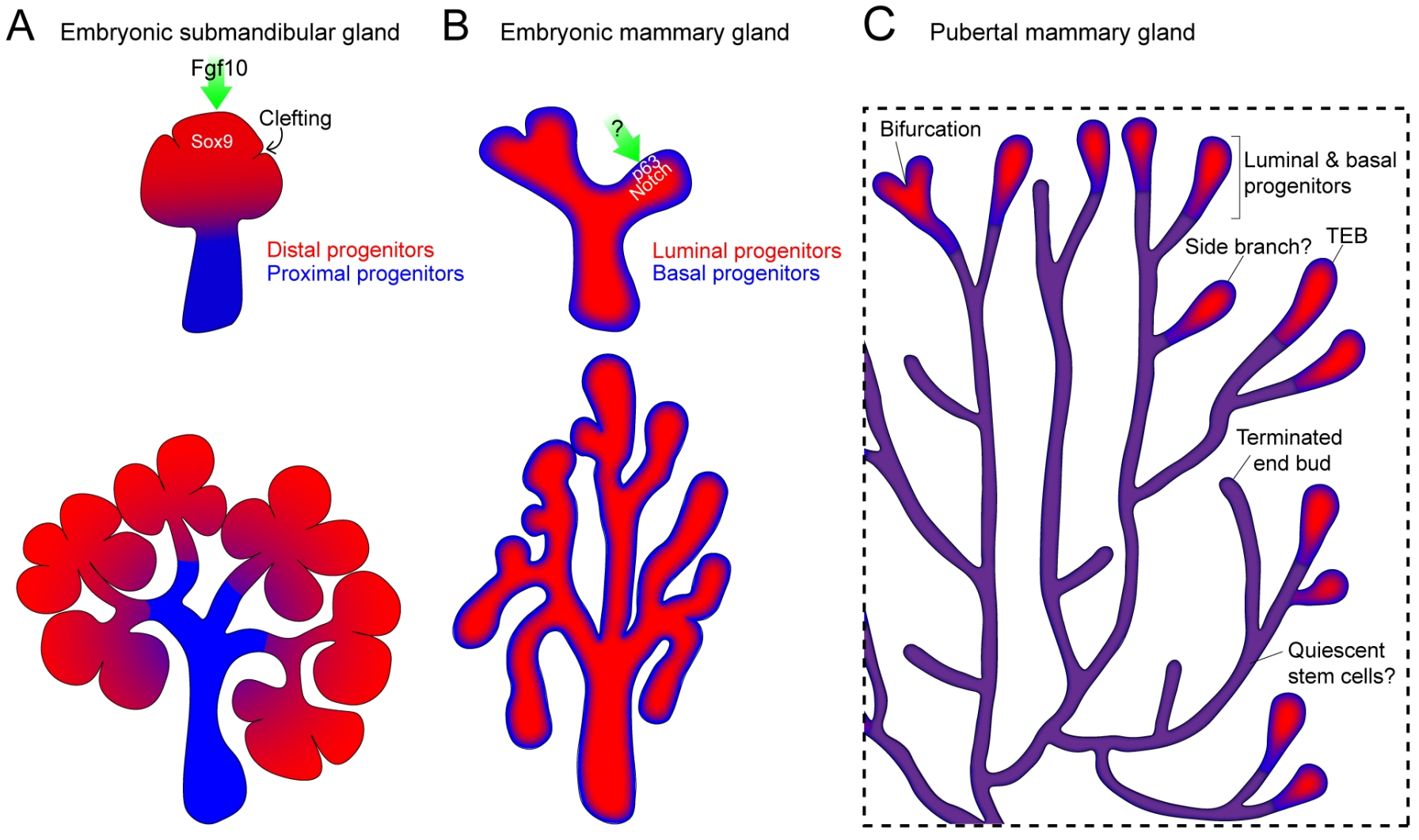
A
Embryonic submandibular gland
Fgf10
Clefting
Sox9
Distal progenitors
Proximal progenitors
B
Embryonic mammary gland
?
p63
Notch
Luminal progenitors
Basal progenitors
C
Pubertal mammary gland
Bifurcation
Luminal & basal progenitors
Side branch?
TEB
Terminated end bud
Quiescent stem cells?

Figure 1.

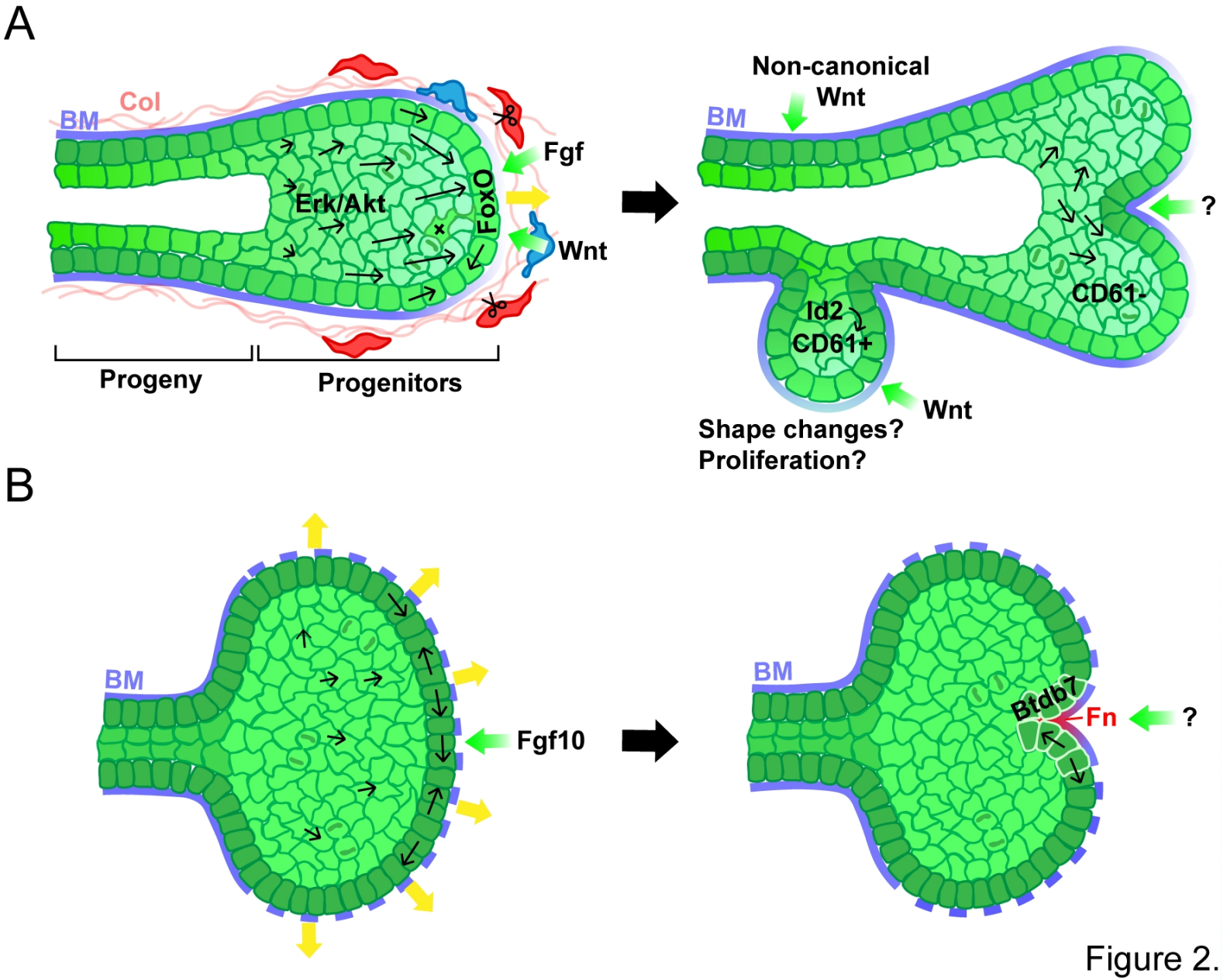
A
Col
BM
Erk/Akt
FoxO
Fgf
Wnt
Progeny
Progenitors
Non-canonical
Wnt
BM
?
CD61-
Id2
CD61+
Wnt
Shape changes?
Proliferation?
B
BM
Fgf10
BM
Btdb7
Fn
?

Figure 2.